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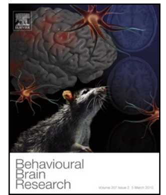

Research report

Effect of the bone marrow cell transplantation on elevated plus-maze performance in hippocampal-injured mice

Luís Bruno da Cruz e Alves-de-Moraes [a], João Tadeu Ribeiro-Paes [b], Beatriz Monteiro Longo [c], Enéas Galdini Ferrazoli [c], Telma Gonçalves Carneiro Spera de Andrade [b,*]

[a] *Post-Graduation in Biotechnology, USP/IPT/I. Butantan, Avenida Prof. Lineu Prestes 2415, Cidade Universitária, 05508-900, São Paulo, SP, Brazil*
[b] *UNESP – Univ Estadual Paulista, FCL, Department of Biological Science, Avenida Dom Antonio 2100, 19806-900, Assis, SP, Brazil*
[c] *Department of Physiology, ECB, UNIFESP, Rua Botucatu 862, 04023-062, São Paulo, SP, Brazil*

HIGHLIGHTS

- Electrolytic lesion in mice dorsal hippocampus induced behavioral disinhibition.
- Cell transplantation provided a discreet relief in the injury-induced anxiolysis.
- Re-exposures to the plus-maze had an anxiogenic effect on normal and treated mice.
- Transplantation-derived cells failed to graft in the injured hippocampus.
- Marrow cells possibly had a stimulating influence on neural self-repair mechanisms.

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ABSTRACT

Several reports have shown that the hippocampus plays an important role in different aspects of the emotional control. There is evidence that lesions in this structure cause behavioral disinhibition, with reduction of reactions expressing fear and anxiety. Thus, to portray the aptitude of cell therapy to abrogate injuries of hippocampal tissue, we examined the behavioral effects of bone marrow mononuclear cells (BMMCs) transplantation on C57BL/6 mice that had the hippocampus damaged by electrolytic lesion. For this purpose, mice received, seven days after bilateral electrolytic lesion in the dorsal hippocampus, culture medium or BMMCs expressing the enhanced green fluorescent protein (EGFP) transgene. One week after transplantation, animals were tested in the elevated plus-maze (EPM). On the whole, three assessment sessions in the EPM were carried out, with seven days separating each trial. Thirty-five days after the induction of injury, mice were sacrificed and their brains removed for immunohistochemistry. The behavioral evaluation showed that the hippocampal lesion caused disinhibition, an effect which was slightly lessened, from the second EPM test, in transplanted subjects. On the other hand, immunohistochemical data revealed an insignificant presence of $EGFP^{+}$ cells inside the brains of injured mice. In view of such scenario, we hypothesized that the subtle rehabilitation of the altered behavior might be a result from a paracrine effect from the transplanted cells. This might have been caused by the release of bioactive factors capable of boosting endogenous recuperative mechanisms for a partial regaining of the hippocampal functions.

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1. Introduction

The hippocampal structure of an adult mammalian is characterized by a large variety of neurons, precisely interconnected and working in a highly organized manner [1]. By function, hippocampus is involved with the most essential higher brain activities, playing a critical role in processing systems necessary to provide, for instance, continuity in time, personal history and awareness [2,3]. It is also well established that the hippocampus is crucial in certain aspects of the emotional regulation. There are lines of evidence showing, for example, that hippocampal lesions cause behavioral disinhibition, leading to a reduction of reactions that express fear and anxiety [4]. In fact, that area takes part in a group of interconnected structures, the so-called septo-hippocampal system, claimed as the neural substrate of the behavioral inhibition and which operates in synergy to control the

* Corresponding author. Tel.: +55 18 3302 5858.
E-mail address: raica@assis.unesp.br (T.G.C.S. de Andrade).

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expression of behaviors linked to anxiogenic and aversive stressors [4]. More specifically, it works in response to stimuli associated with novelty, punishment and non-reward to trigger outputs that enhance the weight of the emotionally negative information and give rise to reactions that contribute to the adaptation and survival, including behavioral inhibition and increased levels of attention and alertness [4]. In view of that, hippocampal lesions, while eliminating the output signal that favors the tendency of avoidance in defensive situations, interfere in the inhibition processing. Thus, they lead to an increased behavior of approaching to the stimulus source [4].

In a health care perspective, failures of the hippocampus functioning have been associated to a severe impairment of the normal mental functioning. Many times its neural circuits are overpowered and put in prejudice by complex morphofunctional abnormalities associated with neuronal atrophy and/or a severe and progressive loss of nerve cells. This causes neurobiological consequences frequently present in the neurological diseases associated to the aging [5]. In particular, the hippocampus appears to be critically vulnerable to psychopathologies and neurodegenerative conditions such as ischemia [6], epilepsy [7], schizophrenia [8] and Alzheimer's disease [9]. Depending on the type and extent of the damage, affected individuals may undergo substantial cognitive deficits and serious emotional and behavioral disturbances, manifestations that inflict a significant decline on their life quality [5].

In this way, the different processes of neurodegeneration and injury of the hippocampus put in evidence the necessity for the development of new strategies to replace destroyed cells in that structure. In the last few years, an extensive set of reports has suggested that bone marrow-derived cells, because of the ability to migrate to the brain and develop morphological aspects of nervous tissue mature cells, would be potential candidates to a cell therapy approach. Actually, several independent groups have obtained evidence that, under specific conditions, marrow cells can assume phenotypic characteristics and express antigens of neural progenitors, neurons and glial cells both in vitro [10,11] and in vivo [12,13]. Additionally, a number of studies, employing different experimental approaches, have consistently documented therapeutic benefits – especially by the induction of functional recovery – following the administration of cells from bone marrow in animal models of central nervous system disease. For example, marrow cell transplantation have achieved promising results in models of acute injury such as ischemic brain damage [14–16], traumatic brain injury [17,18] and spinal cord lesion [19,20]. Findings by other teams point out that the therapeutic potential of bone marrow cells can also be of use in chronic and neurodegenerative ailments including multiple sclerosis [21–23], amyotrophic lateral sclerosis [24,25], epilepsy [26,27], Parkinson's disease [28,29] and Alzheimer's disease [30,31].

Considering this background, the present study was designed to assess the competence of bone marrow mononuclear cells (BMMCs) transplantation as an element of recovery in mice that had the hippocampus damaged by electrolytic lesion. The central hypothesis of the study was based on the expectation that those cells, once transplanted, could migrate to the site of injury to promote a tissue and/or functional recovery. The consequence of that procedure would induce, in the lesioned mice, a behavioral profile similar to that of the uninjured animals in the elevated plus-maze (EPM).

2. Experimental procedures

2.1. Animals

Animals were wild type C57BL/6 and transgenic EGFP (enhanced green fluorescent protein)-expressing C57BL/6 male mice, aged 8–12 weeks at the beginning of the experiments. They were housed under standard laboratory conditions, having free access to food and water. Room temperature was kept at $21 \pm 1\,^{\circ}$C and lights were on from 07:00 h to 19:00 h. All procedures were conducted in conformity with the Brazilian Society of Neuroscience and Behavior Guidelines for Care and Use of Laboratory Animals, which are in compliance with international laws and policies, and were approved by the Ethic Committee on Research from the Faculty of Sciences and Letters of Assis (FCLA) – State University of São Paulo (UNESP) (Protocol number 1520/2008).

2.2. Stereotaxic surgery

C57BL/6 mice were anaesthetized with tribromoethanol (300 mg/kg, i.p.; Sigma–Aldrich, USA) associated with subcutaneous local anesthesia (3% lidocaine; Dentsply Pharmaceutical, Brazil) in the incision line on the head and fastened to a stereotaxic instrument (David Kopf, USA) equipped with an adapter for mouse (Insight, Brazil). The following coordinates were used: 2.0 mm posterior to bregma, ±1.6 mm lateral to the midline and 2.2 mm ventral to the skull surface [32]. An unipolar stainless steel electrode, 0.12 mm in diameter, insulated with enamel, except for 0.5 mm from the tip, was inserted bilaterally into holes (one on each parietal bone) drilled in the skull, at an angle of 0° with the vertical plane. To perform the injury, an electronic lesion producing apparatus (3500 Lesion Producing Device, Ugo Basile, Italy) was connected to the electrode. A current of 3 mA was applied for 10 s and soon after the electrode was removed. Soon after the lesions, the holes in the skull were closed with acrylic cement, and the skin sutured with cotton thread. Control animals were submitted to the same surgical procedure, but no electric current was applied through the electrode (Fig. 1).

2.3. Cell transplantation

Cell transplantation occurred seven days after the surgical procedure. Fresh BMMCs were obtained from transgenic mice expressing constitutively (with the exception of erythrocytes and hair) an enhanced GFP cDNA under control of the chicken β-actin promoter and cytomegalovirus enhancer [33], and transplanted in recipient animals with the same genetic background (C57BL/6) but not carriers of the fluorescent protein reporter gene. Briefly, total bone marrow cells were aseptically harvested by flushing femora and tibiae with Dulbecco's Modified Eagle's Medium (DMEM; LGC Biotecnologia, Brazil) using a syringe with a 26 ga needle. The samples were pooled, homogenized, and centrifuged at $400 \times g$ for 10 min. The cell pellet was resuspended with DMEM and fractionated on a density gradient generated by centrifugation at $400 \times g$ for 30 min over a Ficoll-Paque solution (LGC Biotecnologia). The mononuclear fraction over the Ficoll-Paque layer was collected and washed by further centrifugation ($400 \times g$, 10 min) with DMEM. The concentration of the recovered cells was verified in a Neubauer-counting chamber, and the number of viable cells was determined by Trypan Blue exclusion. Each animal of the treated groups was inoculated via the retro-orbital venous plexus with 1.75×10^6 viable BMMCs diluted in 250 μl of DMEM, while non-treated mice underwent a similar procedure, but just being injected with the vehicle. Groups were named as it follows: sham mice injected with DMEM (Sham + Medium, $n = 15$), sham mice transplanted with BMMCs (Sham + BMMC, $n = 15$), lesioned mice injected with DMEM (Lesioned + Medium, $n = 16$) and, finally, lesioned mice transplanted with BMMCs (Lesioned + BMMC, $n = 15$). An additional cohort of control non-operated animals was included to provide baseline behavioral data and intact brain samples for histology, receiving DMEM (Intact + Medium, $n = 12$) or BMMCs (Intact + BMMC, $n = 12$) as described above.

2.4. Behavioral assessment

Three sessions of behavioral assessment were conducted for each mouse. Trials were performed at 7 (D7), 14 (D14) and 21 (D21) days after the cell transplantation, employing the elevated plus-maze model [34] under environmental conditions similar to the holding facilities ones. It was used a wooden maze tailored for mice [35], comprising two open (30 cm × 5 cm) and two enclosed arms (30 cm × 5 cm × 14 cm) which extended from a common central platform (5 cm × 5 cm). Open arms were bounded at their perimeter by an edge (0.2 cm in height and 0.4 cm wide), thus encouraging the exploration and preventing the fall of the mice. The apparatus was raised 39 cm above the floor. All sessions were conducted between 12:00 h and 16:00 h. On the testing, each subject was transported individually in a holding cage (28 cm × 17 cm × 13 cm) to the experimental room and positioned on the maze central platform facing an enclosed arm. Test sessions lasted 5 min and, in all of them, the experimenter stayed outside the experimental room. All trials were video recorded and scored later, through the software EthoLog (version 2.2.5) [36]. For the former measures, the data were expressed as percentage of entries (with the four paws) into, and time spent on the open arms in relation to the total number of entries and time, respectively, in both open and enclosed arms. The total number of entries into the enclosed arms was also recorded.

2.5. Immunohistochemistry

Thirty-five days after the hippocampal injury induction, the animals were sacrificed in order to carry out the immunohistochemical analysis. For the tissue preparation, mice were intraperitoneally anesthetized with thionembutal

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