ORIGINAL ARTICLE

Intravenous infusion of bone marrow mononuclear cells promotes functional recovery and improves impaired cognitive function via inhibition of Rho guanine nucleotide triphosphatases and inflammatory signals in a model of chronic epilepsy

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Abstract

Temporal lobe epilepsy is the most common form of intractable epilepsy in adults. More than 30% of individuals with epilepsy have persistent seizures and have drug-resistant epilepsy. Based on our previous findings, treatment with bone marrow mononuclear cells (BMMC) could interfere with early and chronic phase epilepsy in rats and in clinical settings. In this pilocarpine-induced epilepsy model, animals were randomly assigned to two groups: control (Con) and epileptic pre-treatment (Ep-pre-t). The latter had status epilepticus (SE) induced through pilocarpine intraperitoneal injection. Later, seizure frequency was assessed using a video-monitoring system. Ep-pre-t was further divided into epileptic treated with saline (Ep-Veh) and epileptic treated with BMMC (Ep-BMMC) after an intravenous treatment with BMMC was done on day 22 after SE. Analysis of neurobehavioral parameters revealed that Ep-BMMC had significantly lower frequency of spontaneous recurrent seizures (SRS) in comparison to Ep-pre-t and Ep-Veh groups. Hippocampus-dependent spatial and non-spatial learning and memory were markedly impaired in epileptic rats, a deficit that was robustly recovered by treatment with BMMC. Moreover, long-term potentiation-induced synaptic remodeling present in epileptic rats was restored by BMMC. In addition, BMMC was able to reduce abnormal mossy fiber sprouting in the dentate gyrus. Molecular analysis in hippocampal tissue revealed that BMMC treatment down-regulates the release of inflammatory cytokine tumor necrosis factor-α (TNF-α) and Allograft inflammatory factor-1 (AIF-1) as well as the Rho subfamily of small GTPases [Ras homolog gene family member A (RhoA) and Ras-related C3 botulinum toxin substrate 1 (Rac)]. Collectively, delayed BMMC treatment showed positive effects when intravenously infused into chronic epileptic rats.

Keywords Chronic epilepsy · Bone marrow mononuclear cells · Memory · Exploratory behavior · Mossy fiber sprouting · Long-term potentiation · Inflammatory responses

Introduction

Epilepsy, which is characterized by recurrent seizures, is a common neurological disorder, affecting approximately 50 million people of all ages around the world (Organization 2019). Temporal lobe epilepsy (TLE) is the most common form of intractable epilepsy in adults (Ramey et al. 2013), and the most frequent type of drug-resistant epilepsy (Bartolini and Sander 2019). Animal models have been necessary for the study of epilepsies, particularly the pilocarpine rat model of epilepsy in which many features of TLE are reported to be reproduced (Curia et al. 2008). Pilocarpine-induced status epilepticus (SE) leads to cell loss in several

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hippocampal and extrahippocampal structures and subsequent delayed spontaneous recurrent seizures (SRS) (Leite et al. 1990; Turski et al. 1983). Systemic administration of pilocarpine, with consequent excessive activity of hippocampal neuronal networks, leads to neuronal loss in the dentate hilus and in the CA1 and CA3 sub-regions (Costa-Ferro et al. 2012; Covolan and Mello 2000; Tai et al. 2018), as well as aberrant sprouting of granule cell axons (mossy fibers) into the molecular layer of the dentate gyrus (Cavarsan et al. 2018; Mello et al. 1993; Queiroz and Mello 2007; Sutula et al. 1989; Tauck and Nadler 1985).

Long-term potentiation (LTP) is an activity-induced long-lasting increase in excitatory synaptic strength that is thought to represent a cellular correlate of learning and memory (Bliss and Collingridge 1993; Bliss and Lomo 1973; Malenka and Nicoll 1999). Morphological plasticity of dendritic spines also contributes to the formation of new synapses during LTP induction (Matsuzaki et al. 2004; Park et al. 2006; Toni et al. 1999). Nevertheless, LTP in hippocampal slices of epileptic animals are impaired days to weeks after SE onset (Costa-Ferro et al. 2010; Suarez et al. 2012; Zhou et al. 2011).

Interestingly, some small molecules of the Rho family play several important roles in cellular function including cytoskeletal rearrangement, cell movement, involvement in the regulation and timing of cell division and epilepsy (Choi et al. 2017; Hall 2012; Hodge and Ridley 2016; Liu et al. 2016; Sit and Manser 2011; Xiao-Lan Ai 2018). Particularly, RhoA, which participates in many normal and pathophysiological processes, may be involved in neuronal apoptosis signaling (Hu and Selzer 2017), dendritic remodeling, synapse formation, long-term spatial memory (Dash et al. 2004) and plasticity in the central nervous system (CNS) (Hall 1998). Rac1 (ras-related C3 botulinum toxin substrate 1) plays an important role in synaptic plasticity by regulating α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) expression, cytoskeletal rearrangement and gene expression (Hall 1998; Heasman and Ridley 2008; Ridley 2001; Takai et al. 2001; Wiens et al. 2005) and may contribute to the pathophysiology of epilepsy (Li et al. 2016; Sharma et al. 2009).

Studies have reported increased levels of interleukins 1β (IL-1β) and 6 (IL-6) and tumor necrosis factor alpha (TNF-α) associated with reactive gliosis in various time-frames after seizures (Aronica et al. 2012; Boison et al. 2013; Costa-Ferro et al. 2014; Leal et al. 2014; Vezzani et al. 2013). Allograft inflammatory factor-1 (AIF-1) is a cytokine expressed by the microglial cells in areas of glial reaction in the brain (Zhao et al. 2013). The gene, situated in the major histocompatibility class III genomic region (Harney et al. 2008), is surrounded by surface glycoprotein genes and complement cascade proteins genes as well as TNF-α, TNF-β, and NF-κB genes (Iris et al. 1993). Recent studies have demonstrated that AIF-1 modulates inflammatory processes in various pathological (Autieri 1996; Schluesener et al. 1998).

Bone marrow-derived mononuclear cells (BMMC) are a heterogeneous group of cells with unilobulated nuclei that can be extracted from animal and human bone marrow. Intravenous injections of BMMC has been demonstrated to be beneficial for multiple conditions (Agadi and Shetty 2015; Arnous et al. 2012; Behbahan et al. 2013; Kocher et al. 2001; Vahidy et al. 2016). Recent research has suggested that BMMC treatment has host-protective paracrine proprieties, which might account for its observed effects in various diseases (Arnous et al. 2012; Guerra et al. 2018; Kocher et al. 2001). In previous studies, the effects of BMMC treatment on the pilocarpine model of epilepsy were assessed during the acute epileptogenic (Costa-Ferro et al. 2010; Leal et al. 2014) and chronic periods (Costa-Ferro et al. 2012; Venturin et al. 2011; Zanirati et al. 2015), as well as in clinical investigations (DaCosta et al. 2018). Recently, an anti-inflammatory effect of BMMC treatment was demonstrated in the unpredictable chronic stressors experimental model (do Prado-Lima et al. 2019). Systemic administration of BMMC in this epilepsy model promotes functional recovery, reduce SRS, attenuates astrocyte morphological changes and neuronal loss, increase expression of genes encoding anti-inflammatory cytokines and normalize expression of genes encoding proinflammatory cytokines, when transplanted 22 days post-SE (Costa-Ferro et al. 2012). Additionally, BMMC administered 10 months post-SE is efficacious for reducing SRS frequency by 65%, with associated diminished astrocyte hypertrophy, improved neurogenesis, and enhanced expression of anti-inflammatory cytokine genes in the hippocampus (Costa-Ferro et al. 2012). An increase in protein levels and mRNA expression of brain derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF), nerve growth factor (NGF) and vascular endothelial growth factor (VEGF) and a reduction in TGF-β1 levels in the hippocampus of BMMC-treated epileptic animals have also been found (Zanirati et al. 2015).

In this study, BMMC treatment was used to investigate the mechanisms associated with improved functional outcome observed in this pilocarpine-induced epilepsy rat model. Evaluation of behavioral outcomes, spatial and nonspatial memory, LTP and mossy fiber sprouting (MFS) in the hippocampus was performed to study the structural and functional changes induced by BMMC infusion. Additionally, this study was also focused on the characterization of the neuroprotective spectrum of BMMC on the level of inflammatory mediators such as TNF-α, AIF-1 and Rho family RhoA and Rac-GTPases in epileptic hippocampi. Consequently, potential mechanisms underlying neuroprotective effects of BMMC were elucidated for this chronic epilepsy rat model.

Materials and methods

Animals

In the current study, adult male Wistar rats (60 days old, 280–300 g) were housed 4 per cage and kept with free access to food and water under a 12-h light/dark cycle (lights on at 7:00 a.m.). All of the experimental procedures were performed in accordance with guidelines of the USA National Institutes of Health Guide for the Care and Use of Laboratory and were approved by the Animal Care and Use Committees of the Pontifical Catholic University of Rio Grande do Sul (CEUA 7839-PUCRS). The rats were randomly divided into two groups: control animals treated with saline, (Control, $n = 25$) and pilocarpine-induced epileptic rats called (Ep-pre-t, $n = 45$). The latter was further divided into epileptic rats injected with saline (Ep-Veh, $n = 20$) and epileptic rats injected with BMMC (Ep-BMMC, $n = 20$). Additionally, $n = 5$ animals from Ep-pre-t were used for histological analysis (see below, title 2.5). A timeline of the drug administrations, treatments, tests and groups of animals is shown in Table 1.

Pilocarpine model

Initially, seventy rats were submitted to the pilocarpine model of epilepsy (Turski et al. 1983). On experiment day 1, the rats were subjected to an intraperitoneal injection of 1 mg/kg methyl-scopolamine (Sigma Aldrich, St. Louis, MO, USA) followed 30 min later by an injection of (280 mg/kg ip) pilocarpine (Sigma-Aldrich, St. Louis, MO, USA). Control animals received all drugs and treatments, except they were given saline instead of pilocarpine. Following pilocarpine injection, animals were observed for seizure scoring according to Racine (1972). Racine's scale has been frequently used to score seizure intensity induced by various chemical convulsants, such as pilocarpine. All

behavioral manifestations during seizures could be classified (Cavalheiro et al. 1987; Leite et al. 2002; Turski et al. 1984). Stage 1: immobility, eye closure, ear and vibrissae twitching, salivation, sniffing, facial clonus; Stage 2: head nodding and mastication associated with more severe facial clonus; Stage 3: clonus of one forelimb; Stage 4: bilateral clonus accompanied by rearing; Stage 5: rearing and falling accompanied by generalized tonic–clonic seizures. SE induction was defined as Racine Stage 5, followed by several discontinuous convulsive seizures (Racine 1972). SE was terminated after 90 min by the administration of diazepam (10 mg/kg, intraperitoneal) (Costa-Ferro et al. 2014). Animals were hydrated by subcutaneous injection of 0.9% sterile saline, twice a day, for 3 days after the SE. Control group underwent identical treatment but received injections of comparable volume of saline in lieu of pilocarpine and did not exhibit any seizures.

Cavalheiro et al. demonstrated that the mean time of delayed SRS onset in rats treated with this pilocarpine protocol is 14.8 days (Cavalheiro et al. 1991). Surviving animals (45 out of 70) were continuously monitored 24 h per day from days 15 to 29 and 82 to 89 for detection of spontaneous recurrent seizures (SRS), using a video-monitoring system (Sv3204, Vmis, MG, Brasil). SRS frequency was recorded. All subjects presenting criteria for SE and subsequent SRS during video-monitoring days 15–21 and this period was called Ep-pre-t group. On day 22, $n = 5$ random Ep-pre-t animals were killed for histological analysis of mossy fiber sprouting. The remaining Ep-pre-t animals were randomly assigned to experimental groups Ep-Veh ($n = 20$) and Ep-BMMC ($n = 20$) and subjected to treatment protocols with BMMC or saline.

Bone marrow mononuclear cells

Bone marrow cells were harvested from enhanced green fluorescent protein (EGFP) transgenic mice. The animals were killed with 200 µl of 8% ketamine hydrochloride (Cristália, Brazil) and 2% chlorpromazine (União

Table 1 Timeline of experimental procedures

Day 1	Pilocarpine-induced SE	
Days 15–21	Video-monitoring of SRS	Ep-pre-t group ($n = 40$)
Day 22	Neo-Timm staining	Ep-pre-t and control groups ($n = 5$)
	BMMC or saline administration	Ep-BMMC and Ep-Veh groups ($n = 20$)
Days 22–29	Video-monitoring of SRS	Ep-BMMC and Ep-Veh groups ($n = 20$)
Days 82–89	Video-monitoring of SRS	Ep-BMMC and Ep-Veh groups ($n = 20$)
Day 92	Neo-Timm staining	Ep-Veh, Ep-BMMC and control groups ($n = 5$)
	Object recognition task	Ep-Veh, Ep-BMMC and control groups ($n = 10$)
Day 100	Inhibitory avoidance task	Ep-Veh, Ep-BMMC and control groups ($n = 10$)
Day 104	Morris water maze task	Ep-Veh, Ep-BMMC and control groups ($n = 10$)
Days 112–122	Long-term potentiation	Ep-Veh, Ep-BMMC and control groups ($n = 5$)
Day 112	qRT-PCR	Ep-Veh, Ep-BMMC and control groups ($n = 5$)

Química, Brazil), which was followed by dissection. Fresh bone marrow was extracted from the humerus, femurs and tibiae by flushing with phosphate-buffered saline (PBS). After centrifugation, the cell pellet was resuspended with RPMI-1640 cell culture growth 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 $400 \times g$ for 30 min at room temperature. The mononuclear fraction over the Ficoll–Paque layer was collected and washed twice with PBS. BMMCs were prepared for transplantation in saline at a concentration of 1×10^7 cells in 100 μL total volume. Cells or saline were administered via tail vein injection 22 days after SE, according to Costa-Ferro et al. (2012).

Behavioral characterization

On days 92, 100 and 110 after SE-onset, experimental groups were compared using a battery of behavioral tests.

Object recognition

To assess recognition-associated memory and learning, object recognition task (OR) was used on day 92 after SE onset. See Fig. 2. The experimental apparatus used was an open-field arena ($60 \times 40 \times 50$ cm) placed in a dimly illuminated room (Furini et al. 2014). The objects to be discriminated were made of glass and varied in shape and texture and were chosen based on previous observations that demonstrated a lack of preferential exploration for one object over the other. Objects were secured to the floor of the arena with Velcro tape. The open-field arena and the objects were thoroughly cleaned with 70% ethanol after each animal to ensure the absence of olfactory cues. Exploration was defined as sniffing or touching the stimulus object with the nose and/or forepaws. Sitting on or moving around the objects was not considered exploratory behavior. Before test phase, animals were habituated to the experimental apparatus by allowing them to freely explore it for 20 min per day for consecutive 4 days. In the training day, two different objects (named A and B) were placed in the apparatus; in the first moment animals could explore them freely for 5 min. The test consisted on replacing one of the objects with a novel object (either C or D) and reintroducing the subject into the apparatus for additional 5 min period of free exploration. To reduce the likelihood of carryover testing effects, two cohorts of animals per group were randomly chosen; respectively, the first and the second cohorts were tested 180 min and 24 h after the first moment and named short-term memory (STM) and long-term memory (LTM).

Inhibitory avoidance task

On day 100 after SE onset, animals were assessed with the inhibitory avoidance task (IA) to measure aversive memory learning and retention. See Fig. 3. The apparatus consists of a 50-cm × 25-cm × 25-cm plexiglass box with a 5-cm-high, 7 –cm-wide and 25-cm-long formica platform on one end of a series of 0.3-cm caliber bronze bars spaced 1.0 cm apart that made up the floor of the box. Animals are placed on the platform facing the rear left corner of the training box. During IA training, rats are carefully placed on the elevated platform and observed. When they step down the platform placing their four paws on the grid they receive a single scrambled foot shock with 0.7 mA for 2 s (Katche et al. 2010). Training retention was tested in two cohorts 180 min (STM) and 24 h (LTM) after training by measuring subject latency to step down from the platform (300 s maximum). The difference between step-down latency in the training and in the test session was used as a measure of aversive memory learning and retention (de Vargas et al. 2017; Pereira et al. 2005).

Morris water maze

On day 104 after SE-onset, the Morris water maze (MWM) was used to test spatial memory learning and retention. See Fig. 4. The MWM is a black circular pool (200 cm in diameter) conceptually divided into four equal imaginary quadrants for the purpose of data collection. The water temperature was 21–23 °C. Two centimeters beneath the surface of the water and hidden from the rat's view was a black circular platform (12 cm in diameter). The MWM was set in a well-lit white room with several posters and other distal visual stimuli hanging on the walls to provide spatial clues. The training trials consisted of introducing the subject into the pool in a random starting location, allowing free swimming for up to 60 s, followed by 30 s sitting on the platform. Rats that did not find the platform within 60 s were guided to the platform by the experimenter. Different starting locations were used for each trial. The swimming path of the rats was recorded using a video camera mounted above the center of the pool and analyzed using a video tracking path and analysis system. The MWM training was carried out during five consecutive days. In each training day, the rats were submitted to eight consecutive training trials, while the hidden platform was kept in a constant position. The escape time, time in the target quadrant and frequency crossing the target quadrant were recorded as previously described (Venturin et al. 2011). Spatial memory learning and retention were evaluated on a test trial carried out 24 h after the last training session in the absence of the escape platform.

Hippocampal long-term potentiation

After behavioral characterization, we examined the effects of BMMC treatment on synaptic plasticity by measuring LTP through ex vivo electrophysiological recording in the CA1 region of hippocampal slices (Fig. 5).

Anesthetized rats were decapitated, their brains were rapidly removed and 400-µm coronal slices containing the dorsal hippocampus were prepared using a vibratome. Slices were consecutively incubated in saturated artificial cerebrospinal fluid (ACSF) containing 125 mmol NaCl, 2.8 mmol KCl, 1.0 mmol NaH2PO4, 26 mmol NaHCO3, 10 mmol glucose, 2.0 mmol CaCl2, 1.5 mmol MgSO4, per liter. ACSF was previously gasified with a 95% O_2 and 5% CO_2 mixture to obtain a pH value of 7.3–7.4. Individual slices were transferred into a submerge-type recording chamber and continuously perfused with (95% O_2, 5% CO_2) gas-saturated ACSF at the rate of 2.0 mL/min at 30 °C. Field excitatory postsynaptic potentials (fEPSP) were triggered by electrical stimulation of the Schaffer collaterals with constant-current pulses of 0.2 ms duration delivered every 20 s (0.05 Hz) using a differential alternating current stimulator (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). Field EPSPs were recorded extracellularly on the pyramidal layer of the CA1 hippocampal region using an Axoclamp 2-B amplifier (Axon Instruments, Foster City, CA, USA). Field EPSPs were amplified and low-pass filtered at 600 Hz (Cyber Amp 320, Axon Instruments), digitized (Digidata 1322A, Axon Instruments) and recorded on a computer using the Axoscope 9.2 software (Axon Instruments). The amplitude of fEPSPs was measured using the Clampfit 9.2 software (Axon Instruments). At the beginning of each recording, an input–output (I/O) curve for the fEPSP amplitude relative to the stimulus intensity was recorded using 50 µA stepwise increases (ranging from 50–250 µA) until saturation of the fEPSP amplitude. Current intensity was then adjusted to evoke baseline fEPSP amplitude ranging from 50 to 60% of the maximal fEPSP amplitude obtained by the I/O curve. Baseline responses obtained to 0.05 Hz paired-pulse stimuli (0.2 ms) were recorded for 20–30 min before the induction of long-term potentiation (LTP). After reaching stable baseline fEPSP recordings, LTP was induced using a high-frequency stimulation (HFS) protocol consisting of four trains of 1 s duration delivered at 100 Hz (pulse duration 0.2 ms), with an intertrain interval of 20 s (de Vargas et al. 2017; Wearick-Silva et al. 2019).Field EPSPs were monitored for 60 min after tetanic stimuli. Statistical significance of differences between means was calculated with appropriate statistical tests, as indicated below and in figure legends. Statistical significance of differences between means was calculated with appropriate statistical tests, as indicated below and in figure legends.

Histological analysis

Rats that were killed at different times following the SE. Rats were killed at three different time points: on day 21 after SE induction for random animals in the Ep-pre-t ($n=5$) and control ($n=5$) groups, day 92 for Ep-BMMC ($n=5$) and Ep-Veh ($n=5$) and control ($n=5$) groups. Once deep anesthesia was achieved with halothane, rats were transcardially perfused with Millonig's buffer followed by glutaraldehyde 3% and 200 ml of sodium sulfide fix 0.1% in Millonig's buffer. Brains were then removed, immersed in 30% sucrose overnight for cryoprotection and quickly frozen in isopentane previously cooled in liquid nitrogen (-70 °C) until use. Tissue blocks for cryostat sectioning at serial 30 µm thick were then processed for neo-Timm stain (Mello et al. 1993). The staining was assessed for the entire septotemporal axis of the hippocampal complex (the region between 1.7 and 4.6 mm to the bregma (Herman and Watson 1987). The intensity of sprouting in the inner molecular layer (IML) was evaluated by a subjective graduation score, suggested by Tauck and Nadler (1985). A semi-quantitative scale was used to evaluate the degree of axonal sprouting. The score criteria were 0: no granules; 1: occasional discrete granule bundles; 2: occasional-to-moderate granules; 3: prominent granules; 4: a prominent near continuous granule band; and 5: a continuous or nearly continuous dense granule band (Tauck and Nadler 1985). Next, each section was analyzed in ImageJ software (four to six bilateral sections per slide) using the densitometric analysis to evaluate the mossy fiber sprouting by measuring the optical density of the IML in the representative sections. Digital images were taken using a light microscope (Nikon 80i) and were captured and digitized using the Nikon ACT-1 v.2 system.

Quantitative reverse-transcription polymerase chain reaction

Day 112 after SE induction, the brains of the animals submitted to the different experimental conditions were removed and the hippocampus of both hemispheres dissected out and was snap frozen in liquid nitrogen and stored at -80 °C for quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis (five rats per experimental group). RNA extraction was performed using SV-Total RNA kit (Promega, Madison, Wisconsin, EUA) according to the manufacturer's instructions. Quantitation of total RNA was assessed using NanoDrop (Thermo Fisher Scientific, Massachusetts, EUA). Synthesis of cDNA was performed using SuperScript VILO MasterMix (Thermo Fisher Scientific, Massachusetts, EUA) according to the manufacturer's instructions and quantified using NanoDrop (Thermo Fisher Scientific, Massachusetts, EUA). The qRT-PCR analysis was performed using equipment StepOne Plus (Thermo Fisher

Scientific, Massachusetts, EUA). Each sample was amplified from initial 20 ng of cDNA. Primers used in the study are shown in Table 2. For subsequent data analysis related to gene expression, the 2-ΔΔCt relative quantification method was used with reference to the endogenous control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene.

Statistical analyses

GraphPad Prism (Version 8.0; GraphPad Software, San Diego, CA, USA) was used for statistical analysis. Group data for SRS were compared using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Object exploration time in OR task was converted to percent of total exploration time and so a one-sample t test was used to compare the percent of total time exploration spent in each object with a theoretical mean (50%) and the statistical significance was determined using two-way ANOVA followed by Bonferroni t test (de Vargas et al. 2017). IA data were analyzed using two-way ANOVA followed by the Dunnett's multiple comparisons test (de Vargas et al. 2017). MWM data were analyzed using a two-way ANOVA followed by the Bonferroni post hoc test (Venturin et al. 2011). Data from LTP, MFS and qRT-PCR were compared using one-way ANOVA followed by Tukey's post hoc test (de Vargas et al. 2017; Malheiros et al. 2012; Wearick-Silva et al. 2019). Values are presented as means ± standard deviation (S.D.). Statistical significance was defined as p (or corresponding variable) < 0.05 for all tests.

Results

BMMC decreases SRS frequency

All animals that developed SE subsequently developed spontaneous recurrent seizures, which was evident from the occurrence of repeated stage 5 seizures during the observation period from days 15 to 21 (Table 1). Statistical analysis comparing groups (Ep-pre-t, Ep-Veh and Ep-BMMC) revealed a significant difference between their interaction

$[F_{(2, 82)} = 126.4, P < 0.0001]$. BMMC treated rats had significantly decreased frequency of SRS than saline treated ones (Ep-BMMC: 4.45 ± 0.56 vs. Ep-Veh: 20.35 ± 0.75 seizures/days, $P < 0.0001$, Tukey's post hoc test, $n = 20$/group, Fig. 1).

BMMC improves object recognition

In the OR, in training day, all the rats from different groups explored the two new objects for a similar percent of total exploration time (~ 50% for A and B; training (Tr), $P > 0.05$, t test, Fig. 2b). Animals from the control group explored significantly more than 50% of total exploration time the new object (B) ($t_{(27.83)} = 9$, $P < 0.0001$, t test, Fig. 2b, test, control). Ep-Veh rats presented deficits in
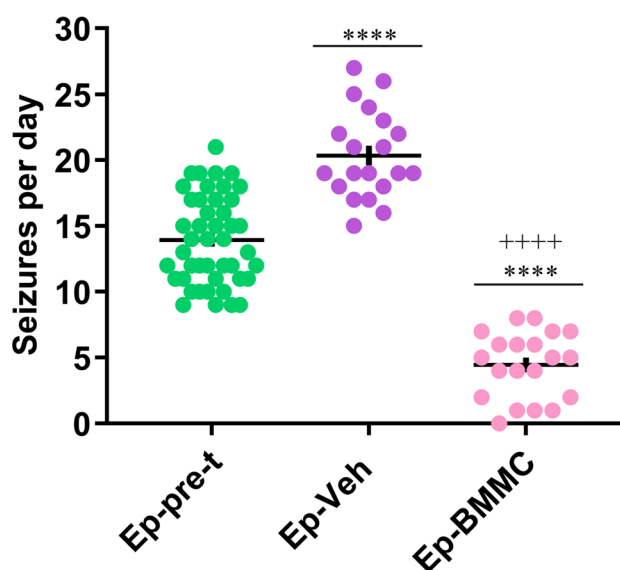

Fig. 1 BMMC decreases SRS in the chronic epilepsy phase. **a** Seizure frequency observed for epileptic animals prior to (Ep-pre-t) and after cell saline (Ep-Veh) or BMMC (Ep-BMMC) injection. Data are presented as the mean ± S.D., n: 40, Ep-pre-t; $n = 20$, Ep-Veh and $n = 20$, Ep-BMMC. ****$P < 0.0001$, Ep-Veh vs. Ep-pre-t; ++++$P < 0.0001$, Ep-BMMC vs. Ep-pre-t and ****$P < 0.0001$ EP-BMMC vs. Ep-Veh in Tukey's test after one-way ANOVA

Table 2 The primer sequences for RT-qPCR analysis

Gene	Forward primer	Reverse primer
GAPDH	TGCCACTCAGAAGACTGTGGATG	GCCTGCTTCACCACCTTCTGAT
TNF-α	AGAACAGCAACTCCAGAACACCCT	ATCTCGGATCATGCTTTCCGTGCT
RHOA	AAGTCGGGGGTGCCTCAT	AATAATCATGGTTGGCTTCTAAAT
RAC-1	CAATGCGTTCCCTGGAGAGTACA	ACGTCTGTTTGCGGGTAGGAGAG
AIF-1	GCCTCATCGTCATCTCCCCA	AGGAAGTGCTTGTTGATCCCA

RT-qPCR, reverse-transcription quantitative polymerase chain reaction; *GAPDH* glyceraldehyde-3-phosphate dehydrogenase; *TNF-α* tumor necrosis factor-α; *RHOA* Ras homolog gene family member A; *RAC-1* Ras-related C3 botulinum toxin substrate 1; *AIF-1* allograft inflammatory factor-1
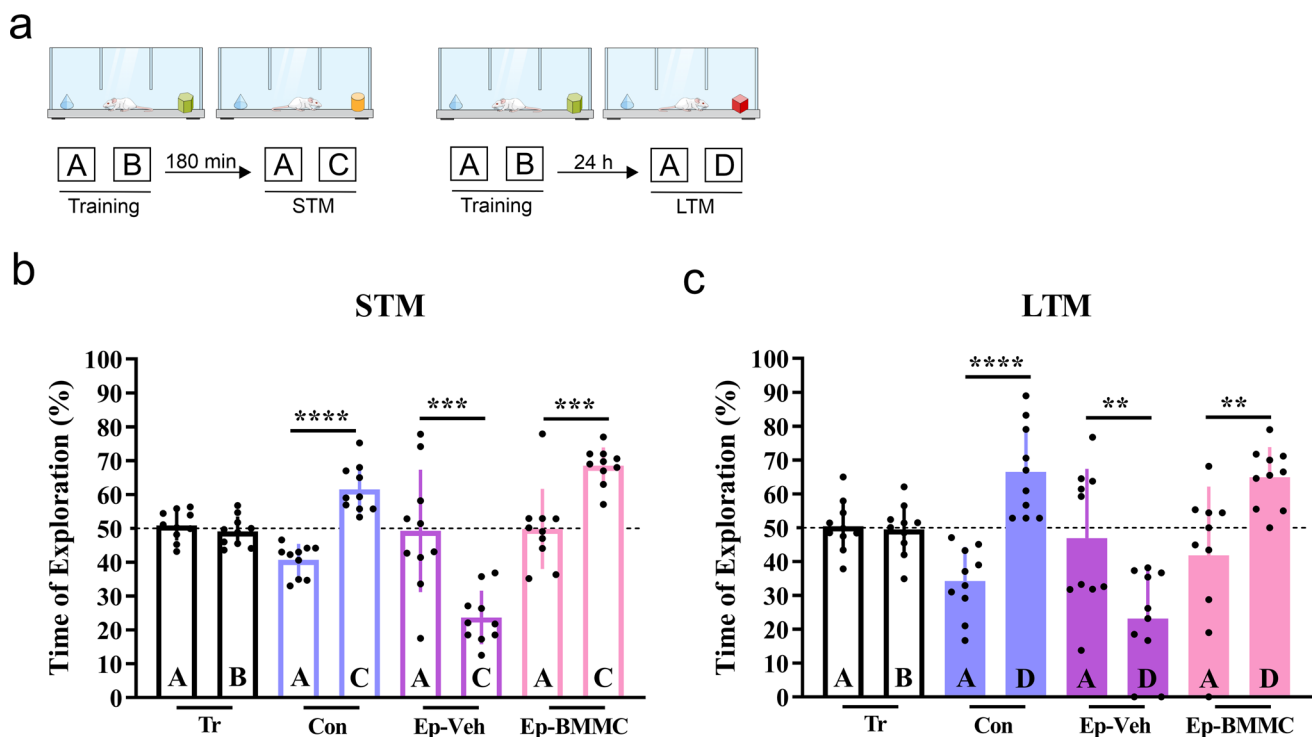
Fig. 2 BMMC improved the consolidation of object recognition in the epileptic animals. **a** For behavioral experiment, rats were divided into three groups: control, Ep-Veh and Ep-BMMC. All animals were habituated and trained in OR task. OR test sessions were made 180 min or 24 h after training. **b** On test session, animals were exposed to a familiar (**a**) and a novel object (**c**) for 5 min to evaluate short-term memory (STM). **c** 24 h after training, animals were exposed to a familiar (**a**) and a novel object (**d**) for 5 min to evaluate long-term memory retention (LTM). Data (mean ± SD) are presented as percentage of total exploration time. **P < 0.001 and ***P = 0.0004, ****P < 0.0001 in one-sample Student's t test with theoretical mea n = 50. These effects were further supported by two-way ANOVA analysis; n = 10 per group. *Cont*, control group

STM, as they spent less time exploring the novel object (C) than the familiar object (A) ($t_{(9.468)} = 9$, $P = 0.0007$, t test, Fig. 2b, test, Ep-Veh]. Further analysis indicated that time exploring the novel object (C) was higher than time exploring the familiar object (A) in Ep-BMMC rats ($t_{(39.91)} = 9$, $P = 0.0003$, Fig. 2b, Ep-BMMC, t test). The interaction between the groups was confirmed by two-way ANOVA [$F_{(2, 54)} = 329.1$, $P < 0.0001$].

In the LTM testing session, the animals from the control group showed exploratory preference for the novel object (C) [$t_{(15.54)} = 9$, $P < 0.0001$, t test, Fig. 2c, test, control]. Ep-Veh rats presented deficits in long-term OR memory, as they spent less time exploring the novel object (C) [$t_{(5.04)} = 9$, $P = 0.0081$, Fig. 2c, t test, test, Ep-Veh]. The results demonstrate that the BMMC group spent significantly more time exploring the novel object (C) during the test [$t_{(22.92)} = 9$, $P = 0.0041$, Fig. 2c, t test, test, Ep-BMMC], demonstrating preserved memory for the familiar object. These effects were further supported by two-way ANOVA analysis, which confirmed that preference for the novel object was different among treatments [$F_{(2, 54)} = 19.12$, $P < 0.0001$].

BMMC recovered inhibitory avoidance memory performance of epileptic animals

Figure 3 shows the effects of BMMC on memory consolidation in step-down latency during testing in the inhibitory avoidance task. We observed a significant difference in the IA performance among experimental groups in the test session measured 180 min ($F_{(2, 18)} = 38.43$, $P < 0.0001$, two-way ANOVA) and 24 h later ($F_{(2, 18)} = 50.52$, $P < 0.0001$, two-way ANOVA). The step-down latency was significantly lower in the Ep-Veh group compared to Ep-BMMC group (STM: Ep-Veh vs. Ep-BMMC: $P < 0.0001$ Dunnett's test after two-way ANOVA) and (LTM: Ep-Veh vs. Ep-BMMC: $P < 0.0001$; Dunnett's test after two-way ANOVA).

BMMC reversed spatial memory impairment of epileptic animals

In MWM test, as show in Fig. 4, animals in control and Ep-BMMC groups easily learned to find the invisible hidden platform. A progressive reduction of average escape latency during the training period of five consecutive days and the
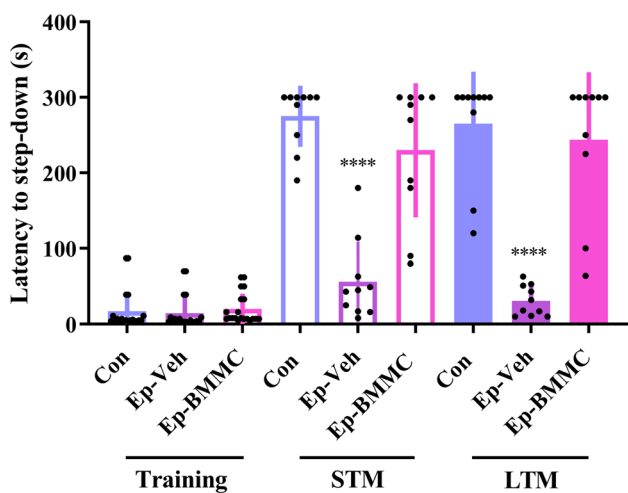
Fig. 3 BMMC recovered inhibitory avoidance memory performance of epileptic animals. IA latencies on 180 min (STM) and 24 h (LTM) were significantly declined in the Ep-Veh animals but not in the Ep-BMMC (Dunnett's test after two-way ANOVA ****$P < 0.0001$ vs. Ep-Veh ($n = 10$ per group)

interaction between them [$F_{(8, 120)} = 5.591$, $P < 0.0001$, two-way ANOVA, $n = 10$ per group] was observed. The escape latency was decreased for Ep-BMMC group compared to Ep-Veh group already in the 4th and 5th training days ($P < 0.001$ for both, Bonferroni's post hoc test, Fig. 4b) and mainly in the test day ($P < 0.0001$, Bonferroni's test after two-way ANOVA, $n = 10$/group, Fig. 4c). Memory was assessed by removing the platform and measuring elapsing time in the correct quadrant of maze. During testing phase Ep-BMMC had significantly lower latency to the first entrance into target quadrant [$F_{(2, 18)} = 33.13$, $P = 0.0003$, two-way ANOVA, $n = 10$ per group, Fig. 4d] and higher frequency crossing the target quadrant [$F_{(2, 18)} = 24.46$, $P < 0.0001$, two-way ANOVA, $n = 10$ per group, Fig. 4e] of the platform than the Ep-Veh rats that received vehicle.

BMMC rescues synaptic plasticity in epileptic animals' neurons

Since we aimed to investigate functional plasticity in the hippocampal CA1 area in control and chronically epileptic animals, three experimental groups were assigned (Fig. 5). Slices from EP-BMMC rats exhibited significantly more
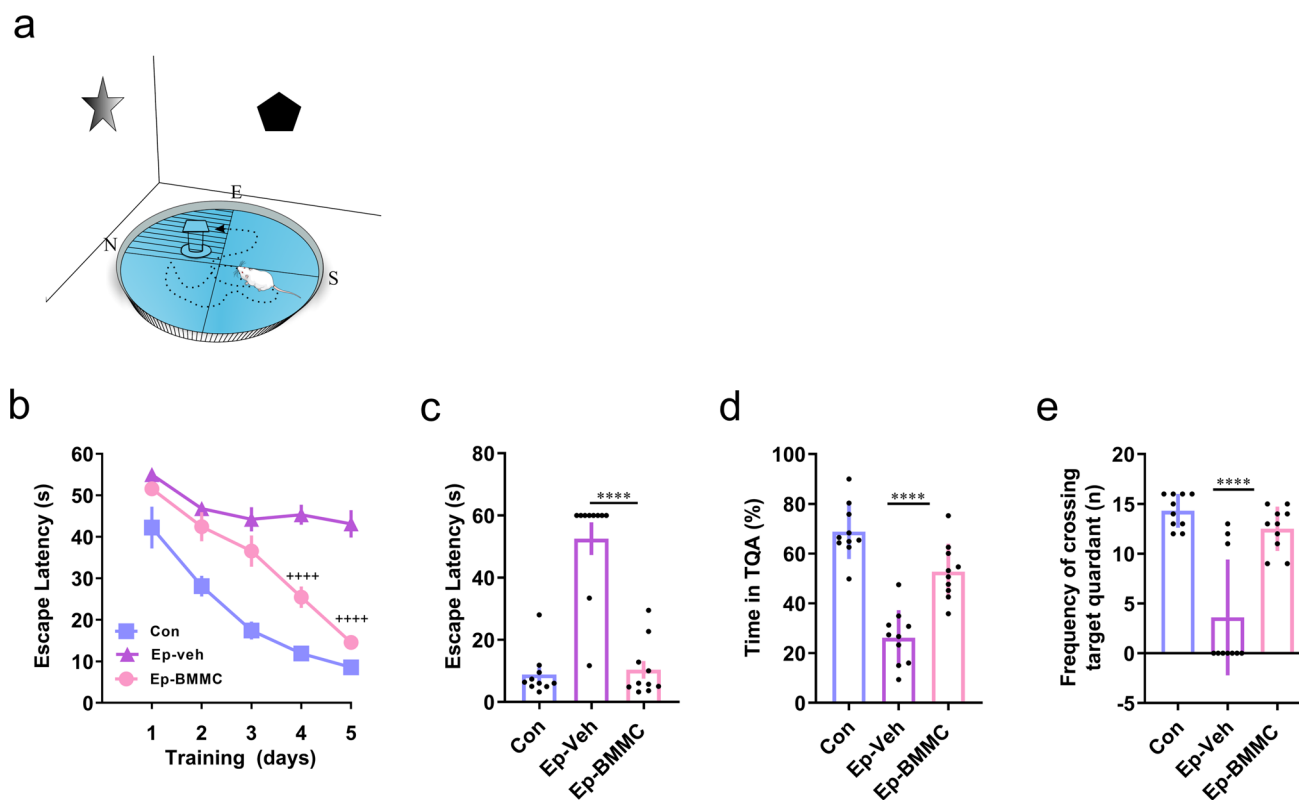

Fig. 4 BMMC improves the memory in epileptic animals in the Water maze test. **a** Photomicrograph showing the experiment, **b** Mean escape latency during the 5 days of training in the MWM, **c** Mean escape latency in the test, **d** percentage of time spent in the target quadrant, and **e** Mean frequency crossing in the target quadrant. Data are presented as the mean ± SD ($n = 10$ per group). ****$P < 0.0001$ Ep.Veh vs. Ep-BMMC. Bonferroni's post hoc test after two-way ANOVA)
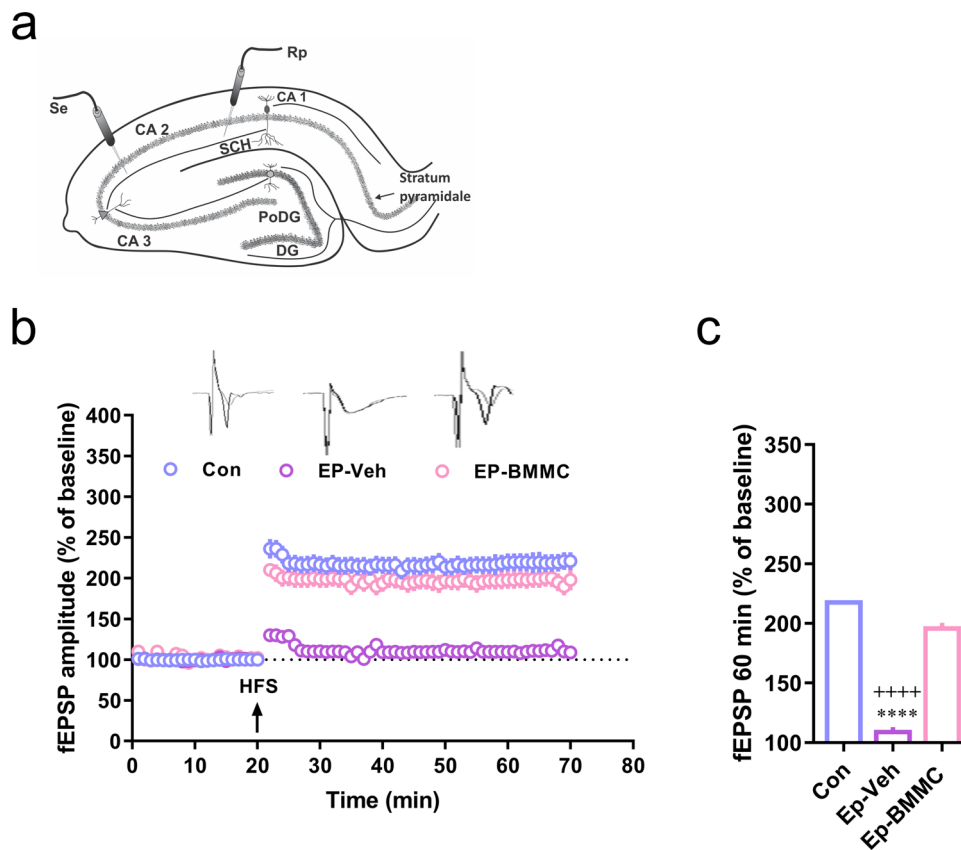
Fig. 5 BMMC rescues synaptic plasticity in slices of epileptic animals. **a** Schematic diagram showing the configurations for hippocampal slices experiments. In the configuration shown, an extracellular recording electrode is placed among apical dendrites of CA1 pyramidal cells, and stimulating electrodes are positioned in the Schaffer collaterals. **b** The fEPSP amplitude and insets are superimposed representative traces of the averaged fEPSPs before (−20 to −15 min; gray line) and after (50–60 min; dark line) in Control, Ep-Veh and Ep-BMMC groups, respectively. **c** Normalized amplitudes of fEP-SPs after 50–60 min to HFS showed that hippocampal LTP was significantly suppressed in EP-Veh group when compared Ep-BMMC (****$P < 0.001$, Tukey's test after one-way ANOVA) and controls (++++$P < 0.0001$, Tukey's test after one-way ANOVA). Data are presented as mean ± SD ($n = 8$–12 slices/group). *Con*, Control group; *CA1, CA2 and CA3*, cornus ammonis; *GD*, dentate gyrus; *HFS*, high-frequency stimulation; *PoDG* polymorph layer dentate gyrus; *Sch*, Schaffer collaterals; *Se*, stimulating; *Rp*, recording

LTP (197.6 ± 0.86% of baseline, $n = 12$ slices from 5 animals) than slices from EP-Veh animals (110.7 ± 0.74%, $n = 10$ slices from 5 animals (Fig. 5b). A one-way ANOVA comparing the different treatment groups revealed a significant difference among them ($F_{(2, 204)} = 68.2$, $P < 0.0001$, Fig. 5c) with a post hoc Tuckey comparison revealing the highest significant difference between the Ep-Veh and Ep-BMMC groups ($P < 0.0001$) and Ep-Veh and control groups ($P < 0.0001$, control = 8 slices from 5 animals).

Mossy fiber sprouting was reduced in BMMC epileptic animals

After pilocarpine administrated, we recorded the Timm's score after 21 days after SE induction (Ep-pre-t group) and 92 days after SE (or 70 days after BMMC injection) in the Ep-BMMC group in to evaluate the severity of MFS. The Timm's score was significantly increased after SE induction. One-way ANOVA [$F_{(2, 12)} = 62.28$] revealing the highest significant difference between the groups ($P < 0.0001$; Fig. 6a and c; $n = 5$ animals per group. MFS score was graded (1.5–2.0 in Ep-pre-t group and was graded (2.5–3.0) in Ep-Veh group (Ep-pre-t vs. Ep-Veh, $P = 0.0011$, Tukey's test) indicating the occurrence of aberrant mossy fiber sprouting (MFS) in the hippocampus in this animals group. BMMC treatment significantly reduced Timm's score (graded zero to 1.0) in Ep-BMMC group ($P < 0.0001$, Ep-BMMC vs. Ep-Veh, Tukey's test).

As is seen in (Fig. 6b), the densitometry analysis revealed significant higher sprouting in the IML of the dentate gyrus (DG) in the epileptic groups. The density of sprouted axons in Ep-pre-t group was (68.46 ± 8.05, $n = 9$ slices from 5 animals) and in Ep-Veh was (84.38 ± 5.92,
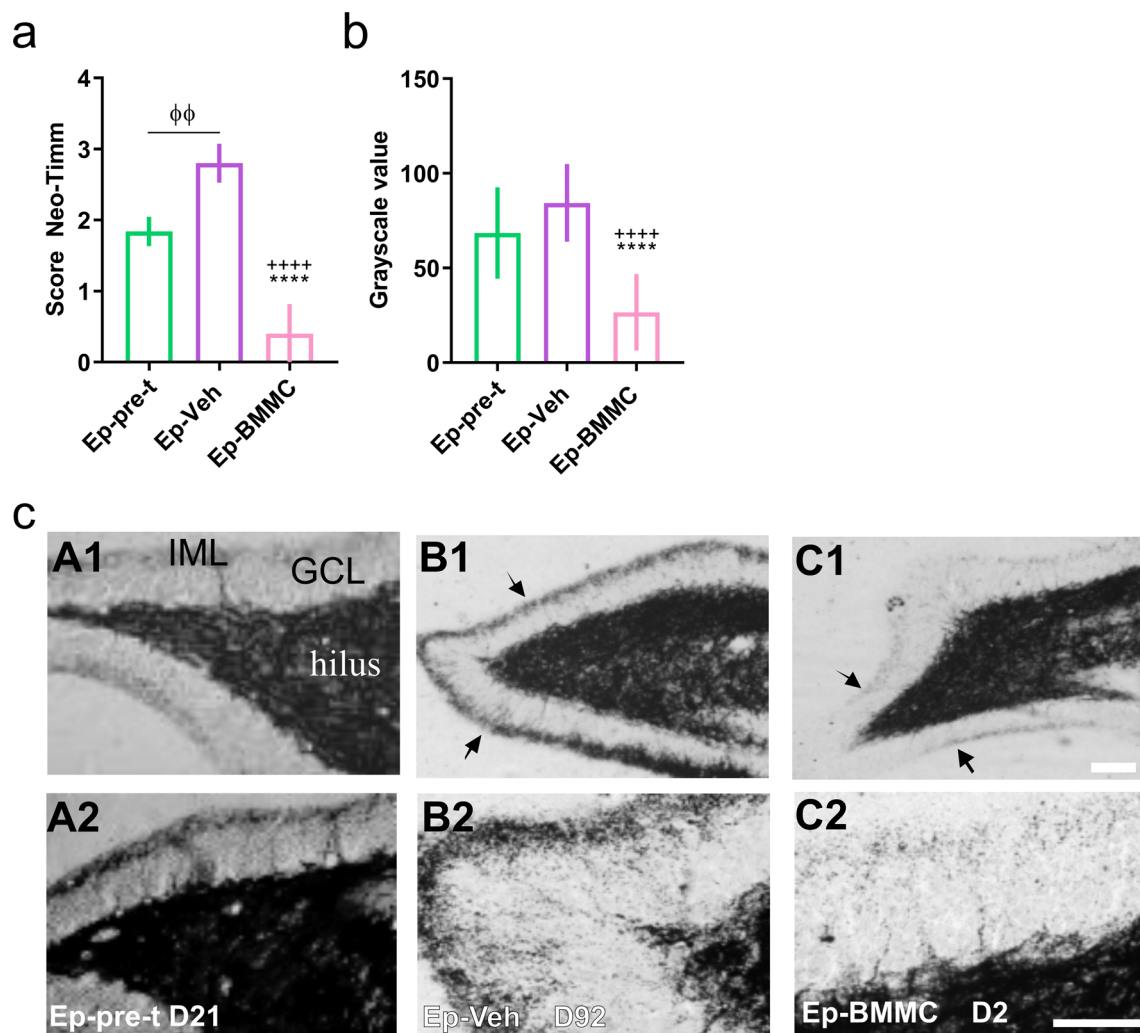
a

b

c

Fig. 6 BMMC transplantation reduces mossy fiber sprouting in the epileptic animals. **a** Graphic representation of neo-Timm score of dentate gyrus subfield from rats were perfused 21 (Ep-pre-t) and 92 (Ep-saline and Ep-BMMC) days after pilocarpine-induced SE. Semi-quantitative analysis demonstrating a significant increase in Timm score $\Phi\Phi P < 0.001$, Ep-Veh vs. Ep-pre-t; $****P < 0.0001$ Ep-Veh vs. Ep-BMMC, Tukey's test after one-way ANOVA ($n = 5$ per group). **b** Graphic representation of neo-Timm densitometry in the IML from representative slices of the Ep-pre-t, Ep-Veh vs. Ep-BMMC ($****P < 0.0001$) rats, Tukey's test after one-way ANOVA. **c** neo-Timm staining (A1, A2) Ep-pre-t, (B1, B2) in Ep-veh animals and Ep-BMMC (C1, C2). Arrows (B1) indicate mossy fiber sprouting extending beyond its usual limits (i.e., beyond the inner molecular layer) and (C1) staining for mossy fibers, was decreased in the inner molecular layer of Ep-BMMC animals. Scale bars: 50 μm, ($n = 5$ per group). *IML*, inner molecular layer; *GCL*, granule cell layer

$n = 9$ slices from 5 animals, P > 0.05, Tukey's test). The BMMC administration was able to reduce the amount of axonal sprouting. The density of sprouted axons was (26.62 ± 5.59, $n = 13$ slices from 5 animals) in Ep-BMMC group and was (84.38 ± 5.92, $n = 9$ slices from 5 animals) in Ep-Veh group ($P < 0.0001$, Ep-BMMC vs. Ep-Veh, Tukey's test). Comparing different groups using one-way ANOVA showed a significant difference among them [$F_{(2, 28)} = 23.02$, $P < 0.0001$]. Rats that did not experience SE did not show mossy fiber sprouting (data dot shown). In both evaluating methods, similar results were obtained regardless of which measurement system was used.

BMMC treatment inhibited the expression of TNF-α, AIF-1, RhoA, and RAC in epileptic animals

The effect of the BMMC injected in the epileptic chronic period on TNF-α, AIF-1, RhoA, and RAC mRNA expression in the hippocampus is shown in Fig. 7. Statistical analyses were carried out with one-way ANOVA followed by Tukey's test showed that animals injected with BMMC the expression of TNF-α was significantly decreased compared Ep-Veh group ($P < 0.008$) and no differences were found between control group ($P > 0.05$, Fig. 7a). The mRNA expression levels of AIF-1 were significantly decreased in the Ep-BMMC group compared to the Ep-Veh ($P < 0.0001$),
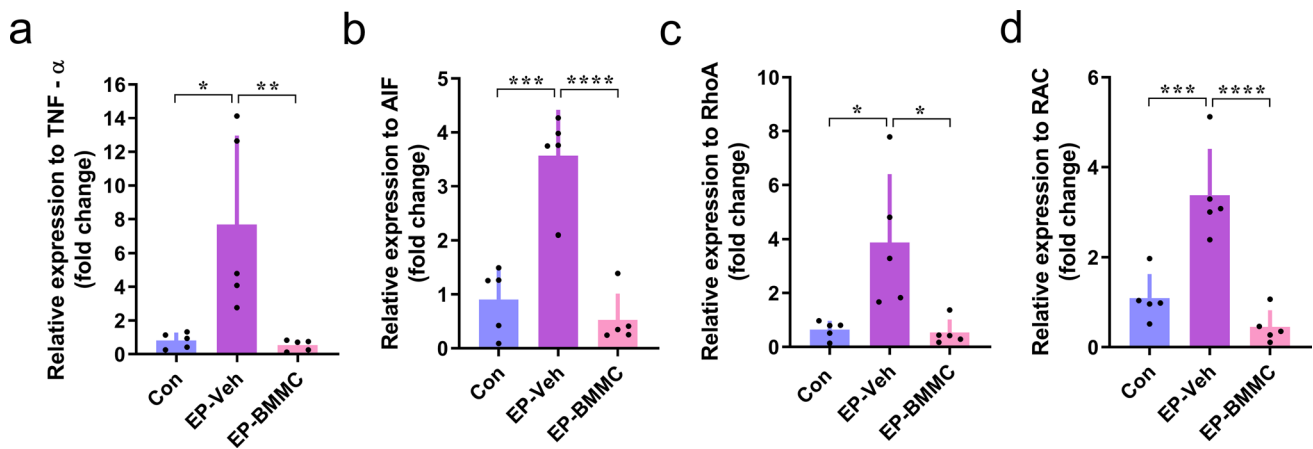
Fig. 7 BMMC transplantation inhibited the expressions of TNF-α, AIF-1, RhoA, and RAC at the mRNA level in the epileptic animals. **a** Comparison between the levels of TNF-α, **b** AIF, **c** RhoA and **d** Rac in the hippocampus of animals in control, Ep-Veh and EP-BMMC groups. *$P < 0.01$, **$P < 0.001$, ***$P = 0.0007$, ****$P < 0.0001$ in Tukey's test after one-way ANOVA indicates a significant statistical difference between the groups. The values are presented as mean ± SD, $n = 5$ per group. *Con*, Control group

while there were no significant differences between the control group ($P > 0.05$, 7B]. BMMC treatment significantly inhibited the expression levels of RhoA ($P < 0.0108$, Tukey's test after one-way ANOVA, Fig. 7c) and Rac (Ep-BMMC, $P < 0.0001$, Tukey's test after one-way ANOVA, Fig. 7d) compared to the Ep-veh group. The mRNA expression levels of RhoA and Rac were no significantly different between the Ep-BMMC and control group ($P > 0.05$).

Discussion

In this study, the effect of intravenous infusion of BMMC was evaluated on seizures' behavioral signs, memory deficit, LTP induction and mossy fiber sprouting in the pilocarpine-induced chronic epilepsy model. In addition, genes pertaining to inflammatory response, such as TNF-α and AIF-1, and to neuronal plasticity, such as RhoA and Rac-GTPase, were evaluated. Our data demonstrate that seizures' memory impairments are significantly attenuated even in delayed BMMC treatment. Functional analysis using electrophysiological recordings on hippocampal slices revealed that LTP amplitude can be restored by BMMC treatment. Moreover, MFS has decreased in animals treated with BMMC. Furthermore, expression of TNF-α, AIF-1 and RhoA and Rac1–GTPase are modulated in the hippocampus by BMMC treatment.

Accordingly, we have previously reported that BMMC exhibited anti-epileptogenic effects and prevented the development of epilepsy in pilocarpine rat model when treated after SE (Costa-Ferro et al. 2014; Leal et al. 2014) and in already epileptic rats (Costa-Ferro et al. 2012; Venturin et al. 2011; Zanirati et al. 2015). The present study corroborates

our previous data indicating that administration of BMMC caused a significant reduction in the frequency and severity of seizures.

Similar to the clinical disease, experimental seizures in rodents result in long-term epilepsy and cognitive dysfunctions, especially related to memory (Jensen 2011; Lenck-Santini and Holmes 2008; Lynch et al. 2000; Mameniskiene et al. 2006). Seizures early in life can cause life-long cognitive impairments in object recognition memory, inhibitory avoidance and spatial memory, as supported by earlier studies (de Oliveira et al. 2008; Mazumder et al. 2017; Pearson et al. 2014; Smolensky et al. 2019; Venturin et al. 2011). Based on the present results, spatial memory impairments in TLE animals were improved BMMC corroborating earlier report (Venturin et al. 2011). Moreover, our finding demonstrated for the first time that BMMC-treated rats had memory scores in inhibitory avoidance and object recognition tasks similar to that of healthy controls for both short-term and long-term memory. Collectively, the performance of epileptic rats in the three behavioral tests suggests that BMMC promoted an effective modulation to rescue memory deficits. Accordingly, as suggested by clinical studies, cognitive improvement was also observed in epileptic patients (DaCosta et al. 2018; Milczarek et al. 2018).

Previous studies have found strong correlation between behavior associated with memory retrieval and CA1-LTP, a cellular correlate of memory (Lynch 2004; Morris et al. 1986; Moser et al. 1998; Tim et al. 2006). Also, the hippocampal region in animal models of epilepsy exhibits structural abnormalities, including sprouting of mossy fibers (Mello et al. 1993), enhanced neurogenesis (Parent et al. 1997) and sclerosis, characterized by selective neuronal loss (Costa-Ferro et al. 2012; Mello et al. 1993). These events

may alter physiological properties of hippocampal networks. Electrophysiological studies have shown that LTP could be altered by seizures (Costa-Ferro et al. 2010; Muller et al. 2013; Zhou et al. 2007), thus affecting spatial learning in animals (Reid and Stewart 1997; Venturin et al. 2011).

In this study, our results clearly indicate that the epileptic condition reduces significantly the ability to generate LTP in the affected slices. In contrast, we found that BMMC-treated epileptic rats exhibited LTP at Schaffer collateral-CA1 synapses similar to controls.

Mainly based on transgenic mice and optogenetics studies, the DG has been involved in cognitive processes and several behavioral functions (Anacker and Hen 2017; Scharfman 2016). However, the continuous reorganization and high level of plasticity of the DG that are crucial for neuroplasticity may also predispose to epileptogenic processes. MFS, rather pro- or anti-epileptogenic phenomena, is the best-studied form of axonal plasticity in epilepsy, which has been observed in many patients with TLE (Buckmaster 2014; Sutula et al. 1989) and in animal models of epilepsy (Mello and Longo 2009; Mello et al. 1993). MFS in the hippocampus of pilocarpine-treated rats results from the initial insult in TLE and gives rise to later spontaneous motor seizures (Hester and Danzer 2013), although other hypotheses have emerged trying to explain the participation of MFS in epilepsy processes, either positive or negatively (Mello and Longo 2009). Because MSF in the inner molecular layer of DG was reported to reach a plateau by 100 days after SE in the pilocarpine TLE model, we measured the abnormal integrations at days 22 and 92 after SE onset days after SE. We found that hippocampal MFS was present in epileptic rats and that BMMC effectively reduced aberrant sprouting onto the molecular layer. These results are consistent with previous report that showed a suppression of MFS in the epileptic rats infused with bone marrow mesenchymal stem cells, one day after the SE induction, in lithium-pilocarpine model (Fukumura et al. 2018).

Repetitive seizures lead to release of inflammatory cytokines (Vezzani et al. 2013). The role of TNF-α and AIF-1 in apoptotic, inflammatory and immune responses in epilepsy has been investigated extensively. TNF-α was upregulated in pilocarpine-induced epilepsy in rats, during early epileptogenesis and late SRS phase (Costa-Ferro et al. 2012; Leal et al. 2014; Ravizza et al. 2005; Xiao-Lan Ai 2018; Zhao et al. 2013), and also in resected hippocampal tissue from TLE patients (de Vries et al. 2016). In our study, a significant decrease of mRNA levels for these two genes was obtained with BMMC treatment. These data of TNF-α are consistent with our previous studies that showed decreased in the level of TNF-α by BMMC treatment shortly after pilocarpine administration in different brain regions and serum (Costa-Ferro et al. 2012; Leal

et al. 2014). Concomitantly, we found that the RhoA and Rac-GTPases analysis showed that treatment with BMMC decreased mRNA levels in epileptic hippocampal tissue. The downregulation of these transcripts after BMMC treatment suggests the involvement of these genes in the aberrant sprouting of mossy fiber that was observed at specific temporal intervals. In fact, it was demonstrated by Sharma and colleagues that RhoA and Rac-GTPases are up-regulated in different time points and directly correlates with aberrant mossy fiber generation in a rat kainic acid treatment experimental model (Sharma et al. 2009). In this context, several studies have shown that normal activity of RhoA are required for LTP (Kim et al. 2014; Murakoshi et al. 2011), and Rac1 is required for the structural enlargement of spines that accompanies (Saneyoshi et al. 2019). Here, hippocampal LTP data from epileptic rats treated with BMMC demonstrated an increase in LTP in CA1 area with the same pattern as non-epileptic LTP rats.

In this study, data revealed that genes pertaining to neuronal plasticity RhoA, Rac and inflammation and immune-response pathways AIF-1 and TNF-alpha were modulated by BMMC treatment, which occurred in parallel with decreased seizure threshold and inhibition of hippocampal mossy fiber sprouting. Among these, the anti-inflammatory effects of BMMC treatment were also accompanied by induction of LTP and improvement in cognitive and memory impairments in chronic epileptic rats. In conclusion, we suggest that inhibition of AIF-1, TNF-alpha and Rho GTPase by BMMC may contribute to the control of epilepsy.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All experimental procedures were performed in accordance with guidelines of the USA National Institutes of Health Guide for the Care and Use of Laboratory Animals (Council 2011) and were approved by the Animal Care and Use Committees of the Pontifical Catholic University of Rio Grande do Sul (CEUA 7839-PUCRS).

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