

Preventive effects of bone marrow-derived mesenchymal stem cell transplantation in a D-galactose-induced brain aging in rats

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Background: Aging is a complex process accompanied by numerous morphological, functional, and metabolic impairments in the brain, and a critical risk factor involved in the increasing incidence of neurodegenerative diseases. Few studies have evaluated the efficacy of different sources of mesenchymal stem cells (MSCs) in ameliorating the early morphological and functional alterations in the aging brain. This study, for the first time, evaluated the potential efficacy of intravenous injection of bone marrow-derived mesenchymal stem cells (BMMSCs) in a D-galactose-induced rat model of brain aging.

Materials and methods: BMMSCs (1×10^6) were intravenously injected into brain aging model rats once every 2 weeks for 8 weeks.

Results: The transplanted cells survived and migrated to the brain, and differentiated into astrocytes and neurons, including choline acetyltransferase neurons. BMMSC transplantation improved locomotor activity and cognitive functions, restored cholinergic system function, protected atrophic cholinergic neurons in the basal forebrain, induced antioxidative effects and restored neurotrophic factors, and modulated hippocampal synaptic plasticity by upregulating PSD95 and Egr1 expression.

Conclusions: Our findings demonstrated the efficacy of BMMSC injection in an aging rat model and suggest that these cells may be developed into an effective cell therapy for the aging brain. (Folia Morphol 2022; 81, 3: 632–649)

Key words: bone marrow-mesenchymal stem cells, D-galactose, rat, brain

INTRODUCTION

Aging is a progressive, complex process accompanied with morphological, functional, and metabolic alterations in the brain, and a critical risk factor in-

involved in the escalating prevalence of neurodegenerative, age-related diseases, such as Alzheimer's disease (AD) and Parkinson's disease (PD) [19, 34, 63, 71, 94]. A distinction can be made between the gradual

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decline in the structural and functional brain status in the non-diseased stage (primary or normal aging) and the progressive structural and functional loss resulting from age-related diseases (secondary aging) [43].

Cognitive functions controlled by the hippocampus and prefrontal cortex are greatly affected by normal aging. Both brain regions experience cellular and synaptic changes that are related to a deterioration in cognitive activities [8, 11, 35, 52, 53]. The brain cells the most vulnerable to the deleterious consequences of aging are cholinergic neurons. During normal aging, the cholinergic system of the basal forebrain undergoes moderate neurodegenerative alterations, while in AD, it shows severe deteriorations. Aging and AD are associated with a progressive degeneration of the cholinergic neurons, characterised by a decline in choline acetyltransferase (ChAT) activity, followed by a reduction in acetylcholine (ACh) release [28, 31, 33, 46, 48, 49, 72, 82]. The levels of neurotrophic factors, such as brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF), are also remarkably decreased in aging and AD, which can be linked to cognitive impairments [15, 50, 83]. In addition, it is well-established that aging is associated with declines in neurotransmitter and receptor levels, reduced synapse numbers, and increased oxidative stress, leading to the marked neurodegenerative status associated with age-related diseases [29, 38, 43, 47, 75, 94].

The structural and functional impairments associated with aging are accelerated in the presence of age-related diseases; hence, therapies that ameliorate primary and/or secondary aging are a principal target in aging research [42, 43]. Stem cell therapy has proven its efficacy in both AD and PD in pre-clinical and clinical studies. Among the different types of stem cells, mesenchymal stem cells (MSCs) are the most promising because they can differentiate toward the neuronal fate, release neurotrophic factors, and enhance endogenous brain repair. In addition, they have immunomodulatory, neuroprotective, angiogenic, and chemotactic properties [4, 10]. In rodent AD models, MSC transplantation has been demonstrated to down-regulate A β deposits, enhance neurogenesis and neuronal differentiation, and alleviate spatial learning and memory deficits. Further, MSCs have anti-inflammatory and immunomodulatory effects [25, 41, 54, 56, 58–60, 68, 85, 90, 92, 95]. Based on these and other studies, in 2015, the Food and Drug Administration (FDA) approved the first phase 2A clinical trial of MSCs for AD treatment, and sim-

ilar trials were designed in Europe and Asia [36]. Recently, the FDA approved a phase 1/2 trial of autologous, adipose-derived MSCs for the treatment of AD (NCT04228666).

Most studies evaluating the efficacy of stem cells have been conducted in preclinical animal models or in patients with AD and PD, in which structural and functional brain capacities are extensively deteriorated. It could be postulated that early intervention to encounter the neuropathological alterations during primary aging would prevent or at least slow down the pathological processes leading to secondary aging, and hence reduce the incidence of age-related diseases [3, 42, 43, 64, 75, 94]. Notably, studies investigating the administration of human (h)MSCs derived from bone marrow or adipose tissue in mouse models of hind limb ischaemia have reported controversial outcomes regarding which type of MSCs is the most effective [13, 40]. Proof for the superiority of specific MSCs source for the treatment of neurodegenerative disorders is lacking. Since the potential differences between MSCs isolated from different sources may result in diverse clinical effects, studies to decide the most efficacious MSC types for each clinical condition are needed [84].

Few studies have evaluated the efficacy of MSC transplantation in animal models of aging [17, 39, 61, 74, 91]. Therefore, we sought to assess, for the first time, the potential beneficial effect of systemic transplantation of bone marrow (BM)-derived MSCs (BMMSCs) on the cortex, hippocampus, and forebrain in a D-galactose-induced rat model of brain aging in order to evaluate their potential as a preventive approach for age-related neurodegeneration.

MATERIALS AND METHODS

Animals

Thirty male Sprague Dawley rats (8 weeks old, 180–200 g) were purchased from the Theodor Bilharz Research Institute, Imbaba, Egypt and were kept in the animal house of the Faculty of Medicine, Menoufia University, Egypt. The rats were housed in standard polycarbonate cages with 2 rats in cage under standard laboratory conditions ($22 \pm 5^\circ\text{C}$, $60 \pm 5\%$ humidity, and a 12-h/12-h light/dark cycle). Standard laboratory chow and tap water were available *ad libitum*. All experimental procedures involving animals were approved by Institutional Review Board of Princess Nourah bint Abdulrahman University, KSA [IRB# 18-0165] and Institutional Review Board

of Menoufia University, Faculty of Medicine, Egypt [IRB# 191219ANAT] and were conducted in accordance with the guidelines on the ethical use of animals in the European Community Council Directive 2010/63/EU.

BMMSC isolation and culture

Bone marrow-derived MSCs were obtained from 6- to 8-week-old male Sprague Dawley rats as previously reported [65]. Briefly, BM plugs were collected from the femurs and tibias of the rats using a 23-gauge needle and centrifuged for 5 min at room temperature (RT) at 1,800 rpm. The pelleted cells were then resuspended in Dulbecco's modified Eagle's medium (DMEM, Gibco, Carlsbad, CA, USA) containing 10% foetal bovine serum (FBS) (Gibco) and 1% penicillin–streptomycin (Gibco) and seeded at a density of 1×10^6 cells/cm in 25 cm² cell culture flasks. The cells were incubated at 37°C in a humidified atmosphere with 5% CO₂. A complete medium change was performed every 3–4 days to remove non-adherent hematopoietic cells. When the confluence of the cells reached 70%, they were harvested for 2–5 min using 0.25% Trypsin–EDTA (Sigma-Aldrich, St. Louis, MO, USA), then neutralised with complete medium and centrifuged at $500 \times g$ for 5 min. Cell pellets were resuspended in complete medium. The viability of the cells was assessed by adding equal volumes of the cell suspension and 0.4% Trypan blue (Gibco), and loading 10 μ L of the stained suspension into each chamber of a haemocytometer. Viable and dead cells were calculated within 5 min of sample preparation. Cells with greater than 90% viability were subcultured at a 1:3 (passage 1). Cells were used at passage 4.

Flow cytometry

Cells were resuspended in staining buffer (2% FBS/PBS) and surface-stained with FITC-conjugated mouse anti-rat CD44 (BioLegend, UK), FITCH-conjugated mouse anti-rat CD90 (BD Pharmingen, USA), or PE-conjugated rabbit anti-rat CD34 (Abcam, UK) at 4°C for 30 min. Isotype-matched antibodies served as controls. The cells were analysed using an EPICS XL flow cytometer (Beckman Coulter).

Experimental design

The rats were randomly assigned to three groups: control, D-galactose (D-gal)-treated, and D-gal + BMMSCs-treated ($n = 10$ in each group). The sample size was calculated using the G Power software. Rats

in the D-gal- and D-gal + BMMSCs-treated groups received a subcutaneous injection of D-gal (300 mg/kg, Sigma-Aldrich, St. Louis, MO, USA) every day for 8 weeks. Rats in the D-gal + BMMSCs group were intravenously administered 1×10^6 BMMSCs labelled with the membrane-bound fluorescent marker PKH26 (Sigma-Aldrich) once every 2 weeks.

Behavioural tests

All animals were acclimatised 1 week following arrival to behavioural testing. Tests were conducted 1 week after the last transplantation. Test sessions were conducted between 2 PM and 5 PM. Two observers were present throughout each session and were blind to experimental condition.

Open-field test

The open-field test allows simultaneous evaluations of exploration, locomotion, and anxiety. A box of 1 m \times 1 m and 50 cm in height was made of wood. The floor of the box was divided into equally areas. Each rat was positioned in the centre of the open field arena and the rearing frequency, number of crossing the lines (with both forepaws), and number and duration of central square entries (with both forepaws) were recorded using a video camera installed 2.5 m above the box for 5 min. The box was placed in a noiseless room with controlled illumination.

Y-maze test

The Y-maze task was used to evaluate spatial working memory. A Y-maze with three equal-sized wooden arms (60 cm, 12 cm, 25 cm) was designed. Each rat was placed in the centre of the maze and permitted to explore the three arms for 8 min. A correct choice was scored when any three successive choices of three different arms were entered. An alternation score was obtained as the total number of alternations divided by the total number of choices minus 2.

Measurement of body weight and the brain index

The general condition of the rats, including behavioural activity and glossiness and colour of the hair coat, was observed daily. Body weights were assessed weekly. At the end the experiment, the rats were anaesthetised through intraperitoneal injection of ketamine (90 mg/kg) and xylazine (15 mg/kg) and decapitated. Brains were immediately harvested from all rats and weighed. Brain indices were calculated in the following manner: brain tissue weight (mg)/final body weight (g).

Table 1. List of primers used in quantitative reverse-transcription polymerase chain reaction

Gene name	Gene accession	Primer sequence forward/reverse 5'→3'
ChT1	NM_053521	CAAGACCAAGGAGGAAGCAG GCAAACATGGAACCTTGCTGA
ChAT	XM_224626	TGAACGCCTGCCTCCATTGCGC CTGCTGA GTGCCATCTCGGCCACCACG AACTGCA
VACht	NM_031663	GCCACATCGTTCACCTCTCTTG CGGTTTCATCAAGCAACACATC
M1AChR	NM_080773	CCTACAGCTGGAAGGAAGAA GCCTGTGCTTCAAGATCTAC
nAChR α 5	NM_017078	TGGAACACCTGAGCGACAAG CGTGACAGTGCCGTTGTACC
nAChR β 2	NM_019297	CGGGAAGCAGTGGATGGCGTA GTCTCCCTCACACTCTGGTCATCA
Egr1	NM_012551	AAGACACCCCCCATGAAC CTCATCCGAGCGAGAAAAGC
BDNF	NM_012842	TGTCCGAGGTGGTAGTACTTCATC CATGCAACCGAAGTATGAAATAACC
VEGF	AF062644	GAGGAAAGGAAAGGGTCAAAA CACAGTGAACGCTCCAGGATT
NGF	XM_227525	TGC ATA GCG TAA TGT CCA TGT TG CTG TGT CAA GGG AAT GCT GAA
BETA-ACTIN	NM_031144	ATTGGCACCACACTTTCTACA TCACGCACGATTCCCTCTCAG

Abbreviations — see text.

Assessment of oxidative stress and antioxidants indices

A spectrophotometer was used to determine the levels of malondialdehyde (MDA) and glutathione (GSH) in brain tissue. To assess the extent of lipid peroxidation, rats' cortices and hippocampi (100 mg) were homogenised in 1 mL of phosphate buffer solution (PBS; pH 7.0) and the MDA concentration was measured [87]. The homogenates were centrifuged after being mixed with trichloroacetic acid (TCA; 20%) at 5000 rpm for 15 min. The supernatants were treated with a 5% thiobarbituric acid (TBA) solution before being boiled in a water bath for 10 min. The absorbance at 532 nm was determined, and the MDA concentration was estimated using the standard curve. The results were given in nanomoles (nmol) per milligram (mg) of protein.

Ellman's method [22] was used to assess GSH levels. A solution of dithiobis nitrobenzoate (DTNB) was added to cortices and hippocampi tissue homogenate and incubated for 1 h. At 412 nm, the absorbance was measured. The standard curve was used to measure the GSH concentration. The findings were expressed in micromoles (mmol) per mg of protein.

Quantitative reverse-transcription polymerase chain reaction (RT-qPCR)

Total RNA was extracted from homogenised cortices and hippocampi of rats of each group using RNeasy Purification Reagent (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. RNA purity was assessed with a spectrophotometer; the wavelength absorption ratio (260/280 nm) was between 1.8 and 2.0 for all preparations. The RNA was reverse transcribed into cDNA using Superscript II (Gibco Life Technologies, Grand Island, NY, USA). qPCRs were run and analysed in a StepOneTM instrument with software version 3.1 (Applied Biosystems, Foster City, CA, USA). The reaction mixtures contained SYBR Green Master Mix (Applied Biosystems), a gene-specific primer pair (listed in Table 1), cDNA, and nuclease-free water. The cycling conditions were: 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. The ABI Prism sequence detection system software was used to analyse the data and quantification was achieved using the Sequence Detection Software v1.7 (PE Biosystems, Foster City, CA). Relative target gene expression was calculated using the comparative cycle threshold method [45]. All values were normalised to β -actin mRNA.

Immunohistochemical analysis

For immunohistochemical staining, brains were fixed in 10% formalin and embedded in paraffin wax. Five-micrometre sections were deparaffinised, rehydrated, rinsed with PBS, and blocked in 0.1% H₂O₂ for 30 min to block endogenous peroxidase activity. The sections were incubated in 10% normal goat serum (blocking solution) at RT for 1 h, then incubated with rabbit anti-polysynaptic density protein 95 (PSD95) primary antibody (1:500, Abcam, Cat. #ab18258) at RT for 1 h. After rinsing with PBS, the sections were incubated with a biotinylated goat anti-rabbit secondary antibody (1:200, Vector Labs, BA-1000, Peterborough, UK) at RT for 20 min. The sections were treated with the enzyme conjugate streptavidin–horseradish peroxidase solution for 10 min. Secondary antibody binding was detected using 3,3-diaminobenzoic acid dissolved in PBS with H₂O₂ (0.03%) added immediately before use. The sections were washed with PBS, counterstained with two drops (100 µL) of haematoxylin, and rinsed in distilled water until they turned blue. Finally, the slides were dehydrated in an ascending graded ethanol series (70%, 95%, and 100%) for 5 min per concentration, cleared in xylene, mounted with Histomount, and covered with a coverslip.

For immunofluorescence staining, brains were dissected and fixed at 4°C for 24 h, then cryoprotected in 30% sucrose at 4°C. Serial sections (40 µm) were cut by a cryostat and stored at –20°C until use. The sections were incubated in 10% blocking solution (10% normal goat serum in 0.3% Triton X-100 in PBS) at RT for 1 h, then incubated at 4°C overnight in the primary antibodies rabbit anti-Neun (1:1000, Abcam, Cat. #ab177487), rabbit anti-GFAP (1:1000, Abcam, Cat. #ab7260), or rabbit anti-ChAT (1:1000, Abcam, Cat. #ab1778850). The sections were then rinsed in PBS and a secondary antibody was applied (1:500, Alexa-488, Cat. #A-11034, Molecular Probes) at RT for 1 h. Finally, the sections were rinsed in PBS and mounted in Fluoroshield Mounting Medium with DAPI (Abcam, Cat. #ab104139).

Quantitative histological assessments

Five non-overlapping images per section were randomly captured from the cerebral cortex, whereas the entire basal forebrain and dentate gyrus area were analysed for each brain section for each marker. Immunohistochemical images were captured using a Leica DML B2/11888111 microscope equipped

with a Leica DFC450 camera, using the Leica C PLAN 4×/0.10 or 10×/0.22 objectives. Immunofluorescence images were captured using a Leica DM5500 B/11888817/12 microscope equipped with a Leica DFC450C camera, using the Leica HI PLAN 10×/0.25 objective. For each image, the region of interest was the field of view at a magnification of 10×. From at least three sections/rat, immunopositive cells were counted using the ImageJ software (National Institutes of Health, Bethesda, Maryland, US) by a manual approach using the plugin/cell counter tool [70] and then averaged per field for each rat. Calculated numbers for 10 animals/experimental group were considered for comparison and statistical analyses.

Statistical analysis

The data are expressed as the mean ± standard error of mean (SEM). Normal distributions were evaluated using the D'Argostino and Pearson normality test, and data were analysed using one-way or two-way analysis of variance (ANOVA) followed by a post hoc Bonferroni test. *P* < 0.05 was considered statistically significant. Statistical analyses were performed using GraphPad Prism 5.03 (GraphPad Software, San Diego, California, USA).

RESULTS

Characterisation of BMMSCs

After 10 days of culture, MSCs derived from the BM of Sprague-Dawley rats were spindle-shaped fibroblast-like cells. Cells from passage 4 were evaluated by flow cytometry for the expression of the markers CD90, CD44 (mesenchymal cell marker), CD34 (hematopoietic lineage marker). More than 90% of the cells were CD90+ and CD44+, whereas less than 10% were CD34+ (Fig. 1). These results indicated that the cells were mostly non-hematopoietic MSCs.

BMMSC transplantation improves the physical characteristics, body weight, and brain indices

Rats in the D-gal group exhibited signs of general aging in terms of physical appearance, i.e., reduced activity and rough, dull, yellow hair coat with hair loss, while rats in the transplanted group exhibited normal activity and smooth, glossy, brightly coloured hair coat, suggesting that BMMSC treatment had beneficial effects on D-gal-induced aging. In the current study, the body weights of the rats in the control, D-gal, and transplanted groups were not

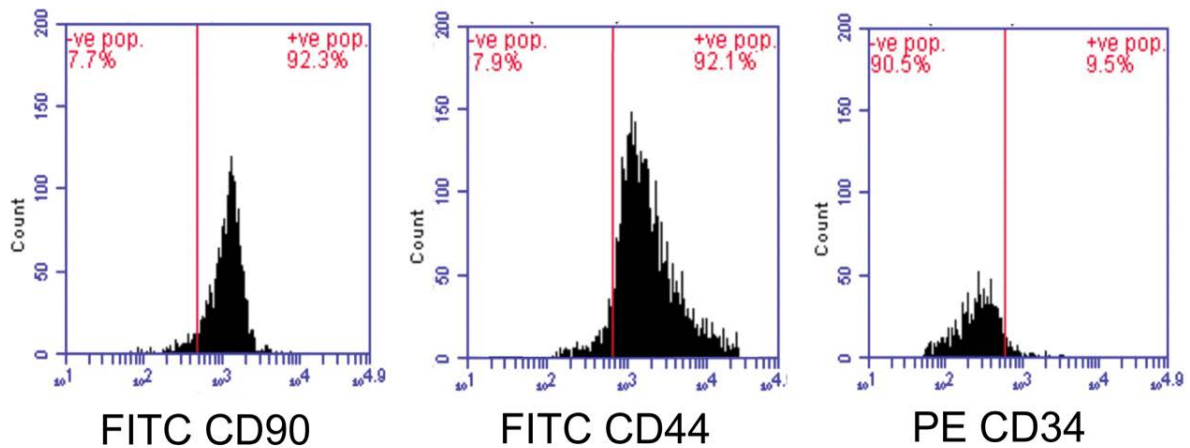


Figure 1. Characterisation of the bone marrow-derived mesenchymal stem cells (BMSCs) population. The cell-surface phenotype of the BMSCs was assessed by flow cytometry using antibodies against CD90, CD44, and CD34. In total, 92.3%, and 92.1% of the cells expressed CD90 and CD44, respectively, whereas only 9.5% expressed CD34.

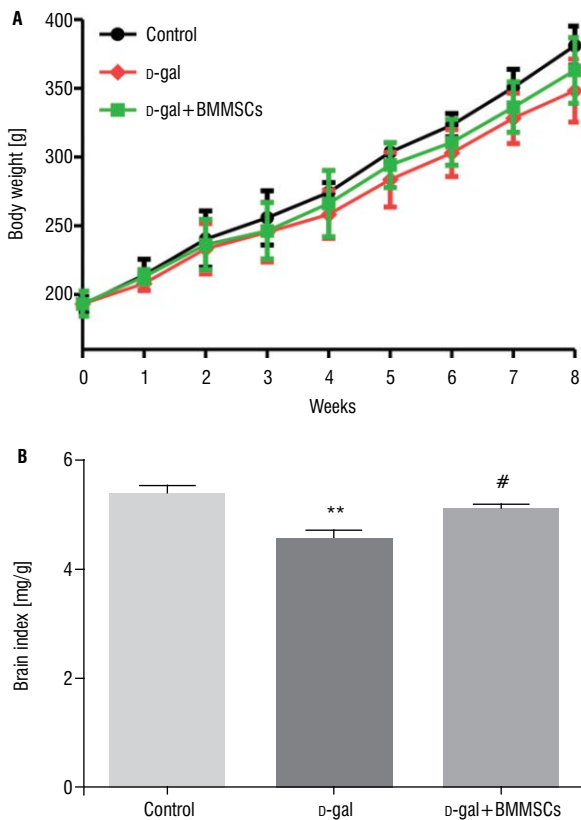


Figure 2. Body weight (A) and brain index (B) were evaluated in the control, aged (D-gal), and transplanted (D-gal + BMSCs) rats; ** $P < 0.001$ vs. control rats; # $P < 0.01$ vs. aged rats. Data are expressed as means \pm standard error of mean; $N = 10$ /group; BMSCs — bone marrow-derived mesenchymal stem cells.

significantly different. However, the brain index was significantly decreased in D-gal-treated rats compared with that in the control rats, whereas BMSC

treatment dramatically improved the brain index as compared with that in the aged rats (Fig. 2), indicating that transplanted cells prevented D-gal-induced brain atrophy.

BMSCs improve spatial working memory, exploratory behaviour, and locomotion, and reduce anxiety in D-gal aging rats

Seven days after the last BMSC injection, the reaction to a novel environment was evaluated using the open-field test. Aged rats showed significantly decreased exploratory behaviour and locomotion as indicated by significant declines in the rearing frequency and number of line crossings when compared to those in the control group (Fig. 3A, B). This was significantly improved by BMSC injection as indicated by the significant increases in the rearing frequency and number of line crossings when compared to the values in the aging group (Fig. 3A, B). Parameters reflecting anxiety increased with aging. In the D-gal group, the frequency and duration of central square entries were significantly reduced as compared to those in the control group (Fig. 3C, D). BMSC treatment ameliorated anxious behaviour and significantly increased the frequency and duration of central square entries as compared to those in the aged group (Fig. 3C, D).

The alternation score in the Y-maze task was dramatically decreased in the aged group when compared with the control group, which reflects impaired spatial working memory (Fig. 3E). BMSC treatment significantly improved the spatial working memory

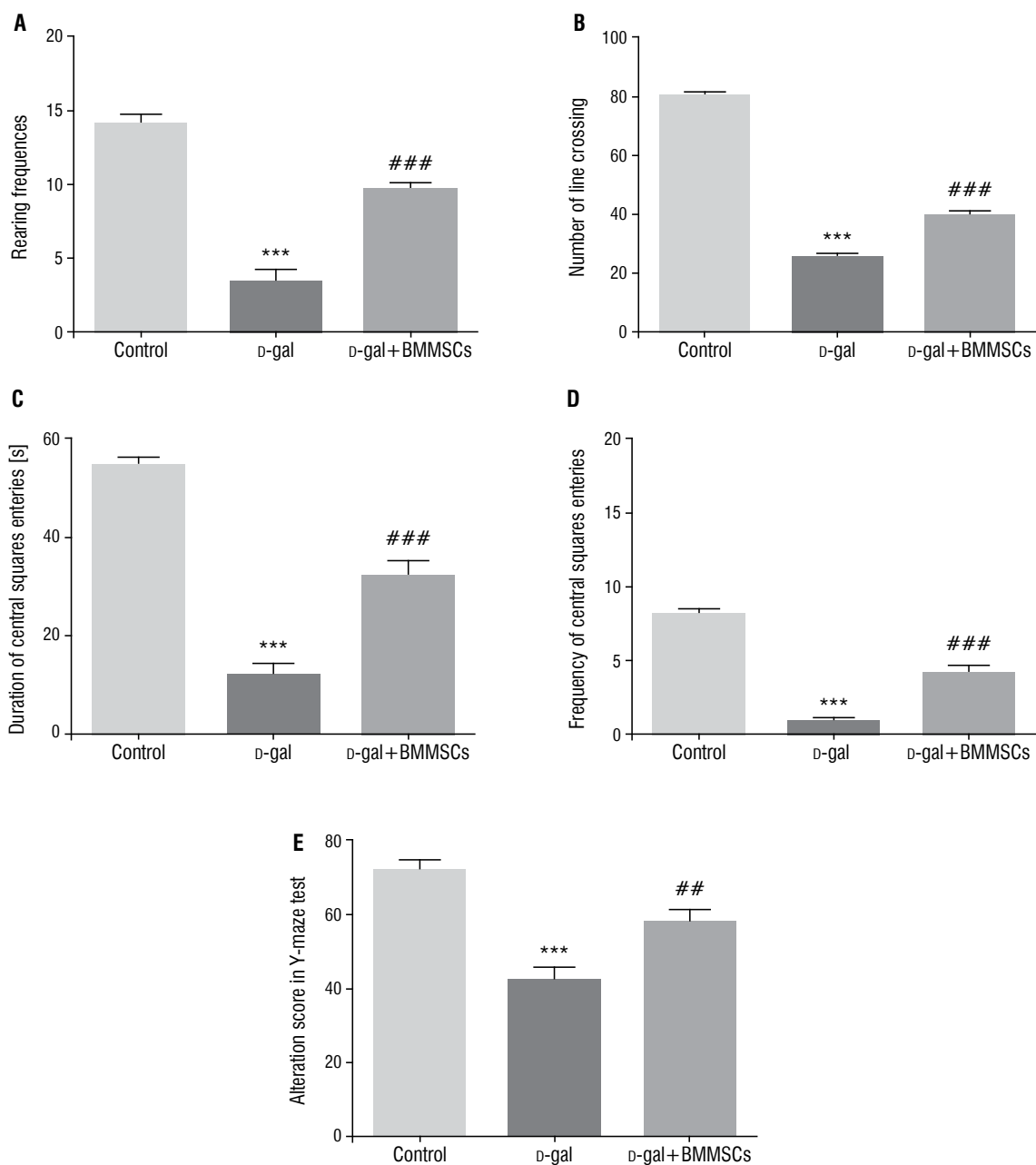


Figure 3. A–E. Locomotion, exploratory behaviour, spatial working memory, and anxiety were evaluated in control, aged (d-gal), and transplanted (d-gal + BMMSCs) rats. Locomotion and explanatory behaviour (horizontal locomotion and vertical rearing) and anxious behaviour (frequency and duration of central squares entries) were assessed for 5 min in an open field test, and spontaneous alternations between the arms of a Y-maze was assessed for 8 min; *** $P < 0.001$ vs. control rats; ## $P < 0.01$ and ### $P < 0.001$ vs. aged rats. Data are expressed as means \pm standard error of mean; $N = 10$ /group; BMMSCs — bone marrow-derived mesenchymal stem cells.

as indicated by an increased alternation score when compared to that of the aged group (Fig. 3E).

BMMSCs restore cholinergic system function

The functional integrity of the cholinergic system in the cerebral cortex and hippocampus is largely affected during aging [18, 20, 46]. The correlation between cholinergic system hypofunction and cog-

nitive deficits has led to the formulation of the cholinergic hypothesis of cognitive impairments in aging and AD [46]. Gene expression of the main functional elements of cholinergic neurons was assessed in the different groups. Gene expression of ChAT for acetylcholine synthesis; high-affinity choline transporter 1 (ChT1) and the vesicular ACh transporter (VAChT) for ACh transport into the vesicles; and the cholinergic

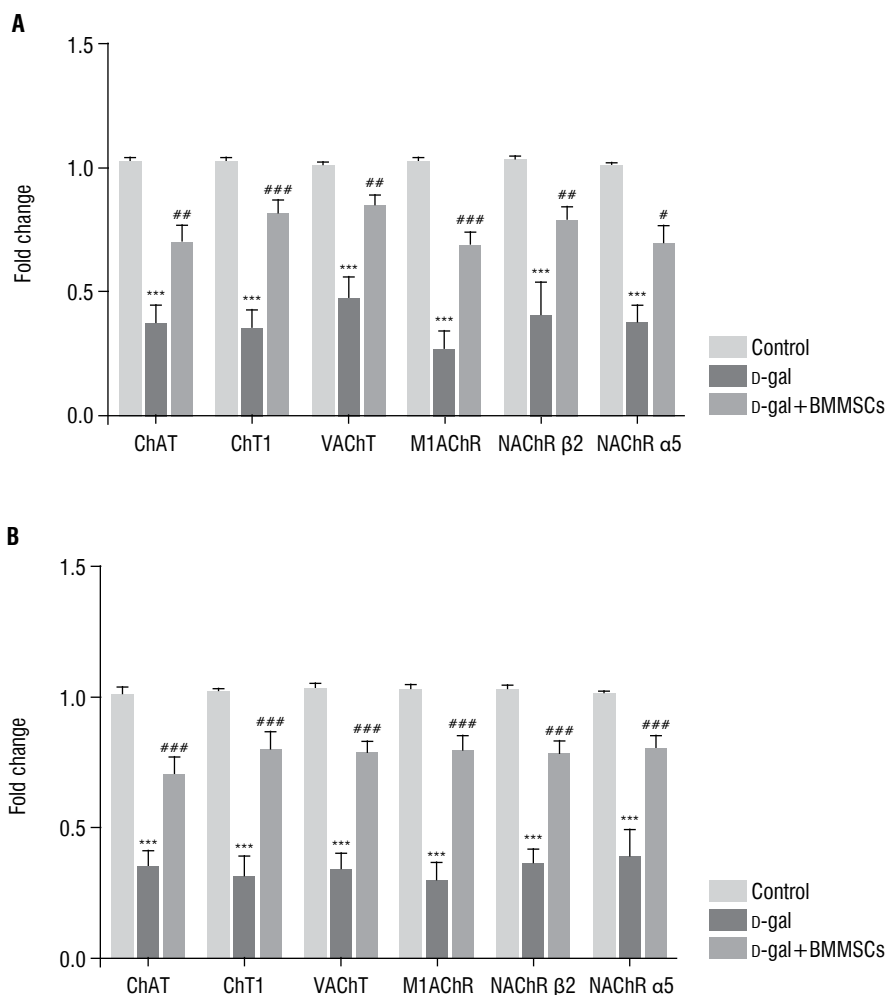


Figure 4. Gene expression of cholinergic nervous system markers in the cortex (**A**) and hippocampus (**B**) in control, aged (D-gal), and transplanted (D-gal + BMMSCs) rats as measured by quantitative reverse-transcription polymerase chain reaction; *** $P < 0.001$ vs. control rats; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. aged rats. Data are expressed as means \pm standard error of mean; $N = 10$ /group; BMMSCs — bone marrow-derived mesenchymal stem cells.

muscarinic (m1AChR) and nicotinic ACh receptors (nAChR $\alpha 5$ and nAChR $\beta 2$) for synaptic signalling were decreased in both the cortex (Fig. 4A) and the hippocampus (Fig. 4B) of aged as compared with control rats. These changes in gene cholinergic nerve marker expression were markedly prevented in the D-gal + BMMSCs group in both the cortex and the hippocampus (Fig. 4A, B).

Transplanted BMMSCs survive and migrate to the brain, and differentiate into neurons and astrocytes

To assess the potential mechanisms by which BMMSCs improved motor and cognitive deficits and restored cholinergic system alterations, we first assessed whether the systemically administered BMMSCs homed to and survived in the brains of the transplanted

rats. PKH-labelled transplanted BMMSCs were found in brain sections of the transplanted group and were detected extensively in all examined brain regions. To characterise the fate of the migrated cells, the cells were recognised by PKH labelling, while their differentiation status was confirmed based on the expression of the neuronal marker Neun or the astrocyte marker glial fibrillary acidic protein (GFAP). In the cerebral cortices and hippocampi of transplanted rats, approximately 10% and 30% of the PKH-labelled cells co-expressed Neun (Fig. 5), and GFAP (Fig. 6), respectively, whereas a few transplanted cells expressed ChAT.

BMMSC transplantation protects cholinergic neurons in the basal forebrain

The cholinergic system involves neurons found mainly in the basal forebrain and their long axons

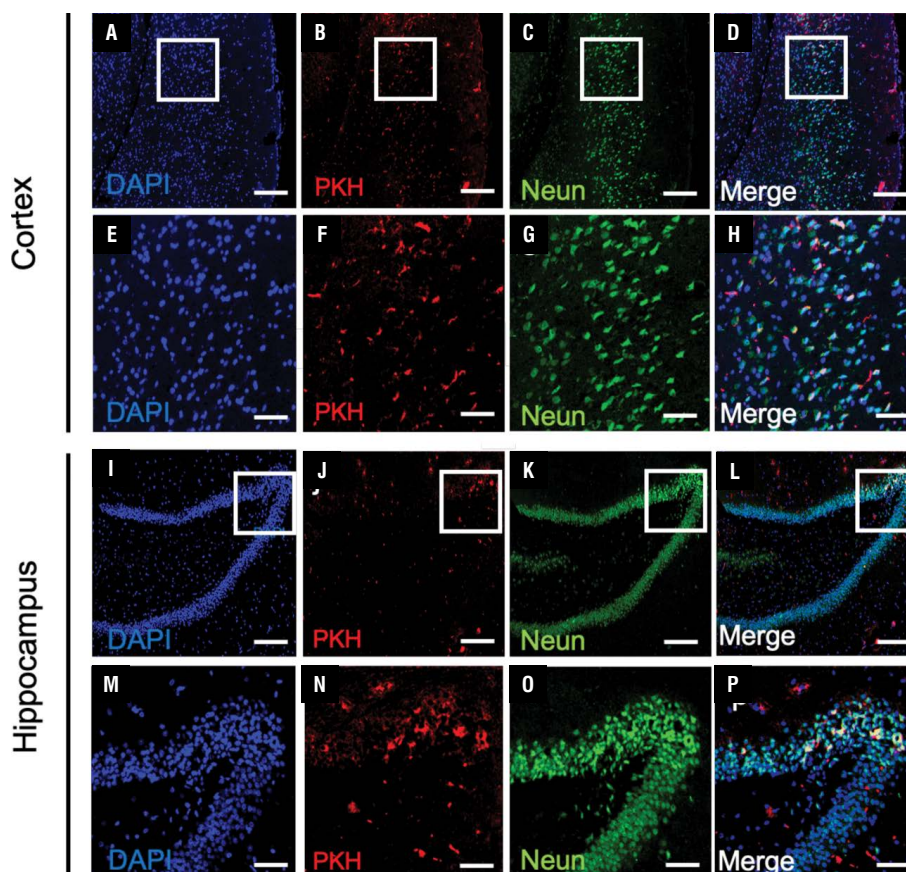


Figure 5. Survival and differentiation of transplanted bone marrow-derived mesenchymal stem cells (BMMSCs) into neurons in transplanted (D-gal + BMMSCs) rats. A number of PKH-labelled BMMSCs (red) (**B, J**) co-expressed Neun (green) in the cortex (**C, D**) and in the hippocampus (**K, L**). The boxed areas in **A–D** and **I–L** are magnified in **E–H** and **M–P**, respectively. PKH-labelled cells (red) (**B, F, J, N**), Neun-positive cells (green) (**C, G, K, O**), DAPI-stained nuclei (blue) (**A, E, I, M**), and merged images (**D, H, L, P**). Scale bar = 500 μm (**A–D, I–L**) and 100 μm (**E–H, M–P**).

that projects diffusely to the cerebral cortex and the hippocampus. To determine whether BMMSC transplantation rescued atrophic cholinergic neurons in the basal forebrain in aged rats, ChAT-positive neurons were analysed. In D-gal-treated rats, ChAT-positive cells in the basal forebrain were markedly declined (Fig. 7A, B, D), whereas in the transplanted group, they were markedly increased (Fig. 7B–D). In the transplanted rats, some PKH-labelled cells in the basal forebrain co-expressed ChAT (Fig. 7C'–C''').

BMMSCs exert antioxidative effects and restore neurotrophic factors

One of the most well accepted theories for the mechanistic cause of brain ageing is the free-radical hypothesis of aging [26]. D-gal-treated rats had higher levels of MDA, an index of lipid peroxidation, in their cerebral cortex and hippocampus than control rats (Fig. 8A, C). In addition, the GSH levels in these brain regions were significantly (Fig. 8B, D) reduced in aged compared to control rats. In D-gal + BMMSCs-treated

rats, MDA levels in the cerebral cortices and hippocampi were significantly reduced (Fig. 8A, C), whereas the GSH levels were increased (Fig. 8B, D) when compared with the levels in D-gal-treated rats.

Neurotrophic factors, such as BDNF, NGF, and vascular endothelial growth factor (VEGF) play important role in cholinergic innervation and ChAT activity in the cortex and hippocampus, and their decline has been linked to impaired cognitive function in aged animals [23, 79]. The expression of *BDNF*, *NGF*, and *VEGF* in the cortex and hippocampus were remarkably decreased in aged rats (Fig. 9) when compared with the expression in control rats, and these declines were suppressed in both brain regions (Fig. 9) after BMMSC transplantation.

BMMSCs modulate hippocampal synaptic plasticity by upregulating the PSD95 expression and triggering Egr1 expression

The PSD95 protein is a component of the postsynaptic complex and plays a crucial role in synaptic plas-

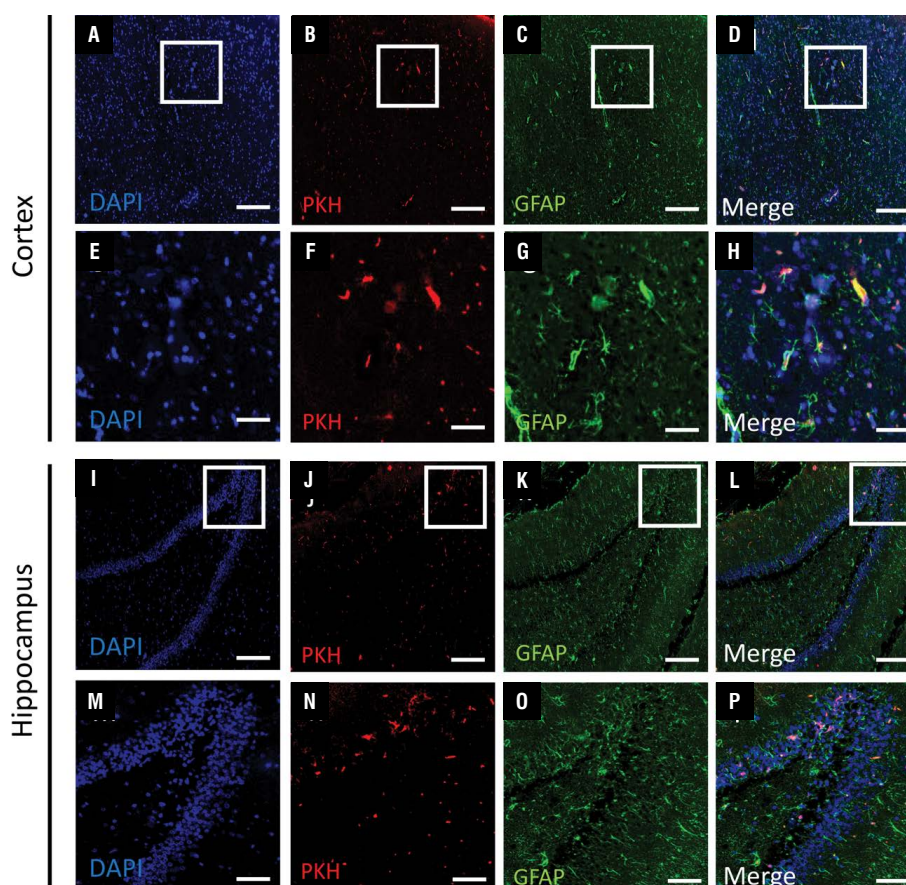


Figure 6. Survival and differentiation of transplanted bone marrow-derived mesenchymal stem cells (BMMSCs) into astrocytes in the transplanted (D-gal + BMMSCs) group. A number of PKH-labelled BMMSCs (red) (B, J) co-expressed GFAP (green) in the cortex (C, D) and in the hippocampus (K, L). The boxed areas in A–D and I–L are magnified in E–H and M–P, respectively. PKH-labelled cells (red) (B, F, J, N), GFAP-positive cells (green) (C, G, K, O), DAPI-stained nuclei (blue) (A, E, I, M) and merged images (D, H, L, P). Scale bar = 500 μ m (A–D, I–L) and 100 μ m (E–H, M–P).

ticity [16]. The numbers of PSD95-positive neurons in the hippocampus were markedly decreased in aged rats (Fig. 10A, B), whereas they were significantly increased in the transplanted group (Fig. 10A, B).

Reduced transcription of early growth response protein 1 (*Egr1*), an immediate early gene, in the hippocampus has been related to age-related memory deficits [21, 62, 93]. *Egr1* expression in the hippocampus was reduced in aged rats when compared with control rats (Fig. 10C), but was significantly restored in BMMSCs + D-gal rats (Fig. 10C).

DISCUSSION

In the central nervous system, aging is associated with altered structure and connectivity, which leads to a decline in normal function. The decreases in neuron numbers and brain function during aging may be a determinant factor in the morphological and functional changes observed in neurodegenerative diseases [19, 34, 63, 71, 94]. These changes

are accompanied by the deterioration of motor coordination and cognition in normal aging, which is worsened in age-associated neurodegenerative disorders such as AD [8, 11, 35, 52, 53]. Thus, therapeutic strategies to ameliorate primary (normal) aging are a major goal in aging research [3, 42, 43, 64, 75, 94]. The replenishment of lost/malfunctioning cells by stem cell therapy has become the focus of recent research. Studies have demonstrated the therapeutic potential of different types of MSCs such as adipose-, umbilical cord (UC)-, and amniotic-derived MSCs in rodent aging models [17, 39, 61, 74, 91]. Given the lack of proof for the superiority of specific source of MSCs in alleviating structural and functional alterations in different parts of the aging brain as well as for the most effective injection route [84], this study aimed to evaluate, for the first time, the potential beneficial outcome of intravenous transplantation of BMMSCs on the brain in D-gal aging rats.

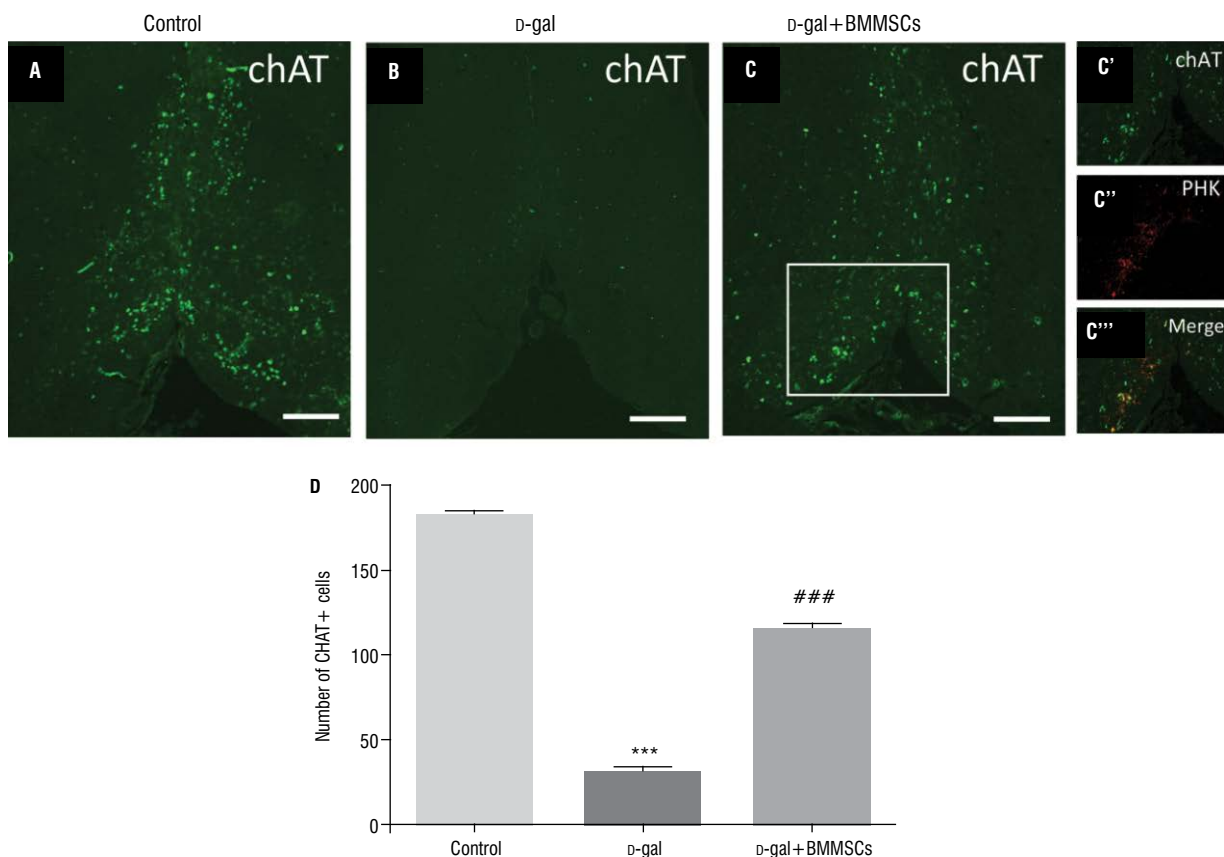


Figure 7. Number of cholinergic neurons in the basal forebrain in control, aged (D-gal), and transplanted (D-gal + BMMSCs) rats. A number of PKH-labelled BMMSCs (C', red) co-expressed ChAT (C'', green). The boxed areas in panel C are magnified in panel C'. PKH-labelled cells (red) (C'), ChAT-positive cells (green) (A–C and C'), and merged images (C'''). Scale bar = 500 μm A–C; ***P < 0.001 vs. control rats; ###P < 0.001 vs. aged rats. Data are expressed as means \pm standard error of mean; N = 10/group; BMMSCs — bone marrow-derived mesenchymal stem cells.

Aging is featured by a gradual decline in locomotion and cognition. With aging, various aspects of learning and memory progressively decline. In our study, BMMSCs improved exploratory behaviour, locomotion, and anxiety in D-gal + BMMSCs rats. Our results are consistent with previous findings. Intra-cerebroventricular human BMMSC transplantation in aged rats improved spatial memory accuracy capacity in locating goal sector in a Barnes maze [91]. Spatial learning and memory functions in D-gal aging mice enhanced after repeated intraperitoneal injections of hUCMSCs [17]. Repeated intravenous transplantations of human amniotic membrane (AM)-derived MSCs (AMMSCs) or adipose tissue (AD)-derived MSCs (ADMSCs) in 10-month-old male F344 rats improved motor coordination and cognitive function as indicated by improved rotarod, passive avoidance, and Morris water-maze performance [39]. Similarly, single or repeated intravenous or intra-cerebroventricular transplantation of ADMSCs increased

physical activity and enhanced learning and memory performance in aged mice [61].

The strong involvement of the cholinergic circuits in age-related brain functional deterioration has led to the development of cholinergic hypothesis of geriatric cognitive impairments [9, 30, 72, 77, 81]. In normal aged brains, loss of cholinergic neurons, depletion of ACh, reduced ChAT activity, and declines in the densities of muscarinic and nicotinic ACh receptors have been reported [9, 18, 28, 49, 72, 77, 82]. Based on positron emission tomography, Albin et al. [2] reported region-specific declines in VAcHT binding sites in the cortex and striatum in the aged brain. In our study, the gene expression of the cholinergic factors ChAT, ChT1, VAcHT, m1AChR, and nAChRs $\alpha 5$ and $\beta 2$ was increased in transplanted compared to aged rats. Therefore, it is plausible to conclude that the detected improvements in cognitive and motor activities might be due to improvements in the cholinergic system.

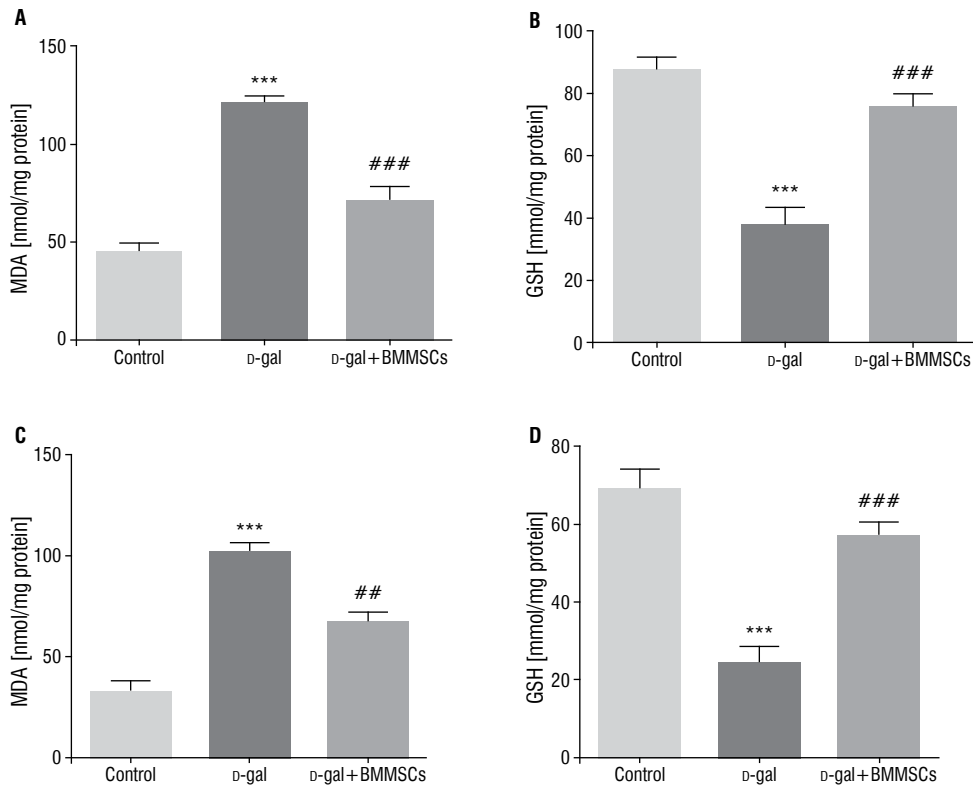


Figure 8. A–D. Status of malondialdehyde (MDA) and glutathione (GSH) in the cortex (**A, B**) and the hippocampus (**C, D**) of control, aged (D-gal), and transplanted (D-gal + BMMSCs) rats. *** $P < 0.001$ vs. control rats; ## $P < 0.01$ and ### $P < 0.001$ vs. aged rats. Data are expressed as means \pm standard error of mean; $N = 10$ /group; BMMSCs — bone marrow-derived mesenchymal stem cells.

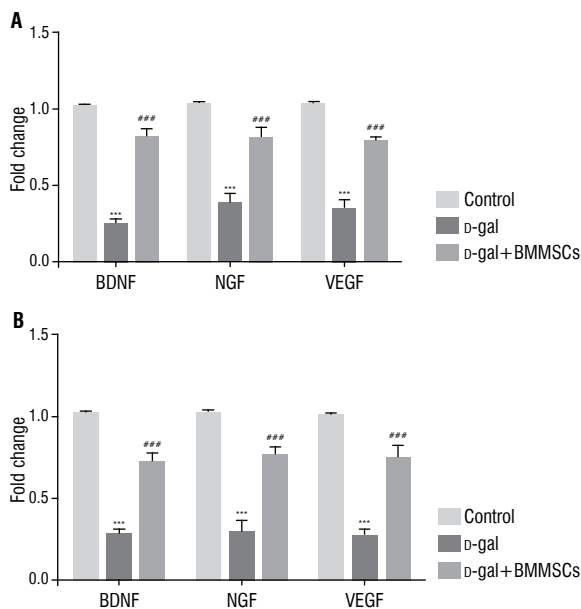


Figure 9. Gene expression of *BDNF*, *NGF*, and *VEGF* in the cortex (**A**) and the hippocampus (**B**) of control, aged (D-gal), and transplanted (D-gal + BMMSCs) rats as measured by quantitative reverse-transcription polymerase chain reaction. *** $P < 0.001$ vs. control rats; ### $P < 0.001$ vs. aged rats. Data are expressed as means \pm standard error of mean; $N = 10$ /group; BMMSCs — bone marrow-derived mesenchymal stem cells.

To reveal the mechanism underlying the behavioural and cholinergic functional improvements observed after BMMSC injection, we evaluated the survival, migration, and differentiation potential of the transplanted BMMSCs in D-gal aging rats. Repeated intravenous BMMSC transplantation was accompanied with a massive migration of the cells into all brain regions examined. These results were in line with previous findings. Interestingly, in both animals and humans, aging has been linked with increased blood-brain barrier permeability, which might be triggered by several aging-mediated events, such as increased oxidative stress and enhanced microglial activation [66, 76]. Demonstrating the therapeutic efficacy of a relatively non-invasive approach such as the intravenous route is of clinical relevance as invasive implantation techniques such as intracranial transplantation may damage intact brain tissues, increasing the burden of aging-related cell loss. Approximately 10% of the migrated cells differentiated into neurons and 30% differentiated into astrocytes. In addition, only few transplanted cells differentiated into ChAT+ cells. Substantial evidence suggests

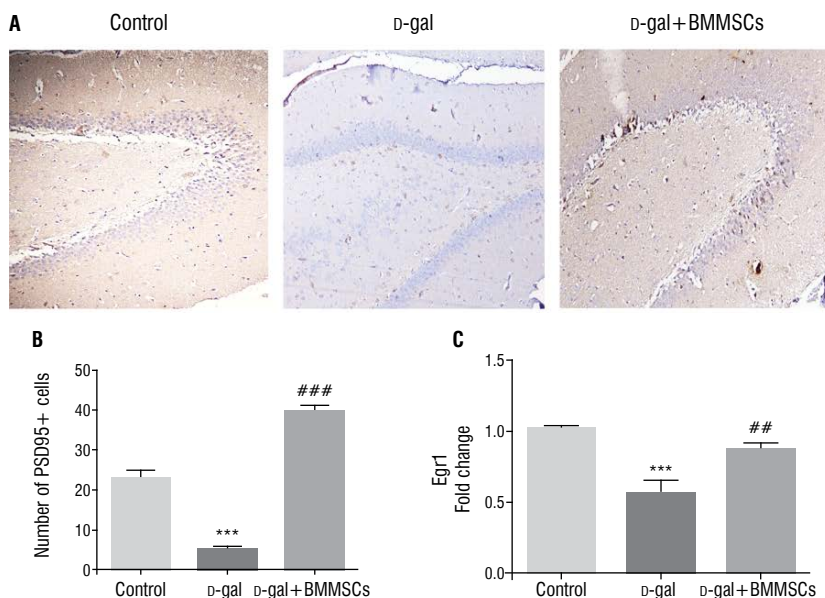


Figure 10. PSD95 expression as indicated by immunohistochemical staining (**A, B**) and *Egr1* gene expression as indicated by quantitative reverse-transcription polymerase chain reaction (**C**) in the hippocampus of control, aged (D-gal), and transplanted (D-gal + BMMSCs) rats. *** $P < 0.001$ vs. control rats; ## $P < 0.001$ and ### $P < 0.001$ vs. aged rats. Data are expressed as means \pm standard error of mean; $N = 10$ /group; BMMSCs — bone marrow-derived mesenchymal stem cells.

that BMMSCs can differentiate into neurons, and in particular, ChAT neurons [1, 5, 6, 12, 32, 55, 67, 78, 80, 88, 89]. The capabilities of intravenously injected MSCs from different sources to migrate and differentiate into the neuronal fate have been previously reported. In 10-month-old male F344 rats, repeated intravenously transplanted hAMMSC and hADMSCs homed to the cerebral cortex and hippocampus and differentiated into neurons, some of which co-expressed ChAT, and, in part, into astrocytes [39]. Similar results have been reported after repeated intravenous transplantation of hADMSCs [61]. However, these studies reported a higher potential of the transplanted cells to differentiate into neuronal phenotypes; in particular, ChAT-positive cells, than we observed. Differences in aging animal models, tissue source and species of the MSCs, and intravenous dose frequency may account for this discrepancy.

Previous studies have shown that the differentiation of transplanted cells into neurons and their subsequent projection to the target anatomical region is challenging, and that the beneficial effects observed in the transplanted brains could be mainly explained by transplanted stem cell-mediated protection rather than replacement. The cholinergic system involves neurons found mainly in the basal forebrain and their long axons that project diffusely to the cerebral cortex and the hippocampus. We observed limited differentiation

of the transplanted cells into the neuronal fate in the cortex and hippocampus. Interestingly, we observed a significant increase in the number of endogenous ChAT-positive cells in the basal forebrain in transplanted rats when compared with aged rats, which cannot be explained by the limited number of transplanted cells that colocalised with ChAT immunostaining in the basal forebrains of these rats. These results indicated an endogenous regeneration of the host cholinergic system in the basal forebrain in transplanted rats. Therefore, we next sought to assess potential bystander-like mechanisms mediated by the transplanted BMMSCs that would contribute to the endogenous regeneration of the host cholinergic system and lead to the observed beneficial effects on functional and cognitive activities.

Cholinergic neurons in the basal forebrain depend for their survival and function on neurotrophic factors, such as BDNF and NGF, which are retrogradely transported from basal forebrain targets. Aging is accompanied by alterations in the neurotrophic signalling pathways, which have crucial roles in the cholinergic and cognitive deficits found in aging and AD [14, 15, 23, 50, 51, 79]. In cultured embryonic rat basal forebrain and cortical neurons in microfluidic chambers used as models of normal aging, significant declines in BDNF and proNGF transport have been observed, suggesting the susceptibility of aged forebrain cholinergic neurons to age-induced transport

malfunction [73]. BDNF-knockout mice possessed reduced numbers of cholinergic cells in the medial septum, which was associated with reduced ChAT activity and NGF expression in the hippocampus [27]. We observed decreased *BDNF* and *NGF* expressions in both the hippocampus and cortex in D-gal aging rats, which is in line with findings reported in several previous studies [24, 37, 44, 57]. These decreases were remarkably restored in BMMSC-transplanted rats. A substantial body of evidence suggests the neurotrophic and paracrine potential of stem cell transplantation, which is accomplished by increased concentrations of various neurotrophic factors such as BDNF, NGF, and glial cell-derived neurotrophic factor [7]. BDNF and NGF levels in F344 rat brains markedly increased after transplantation of hAMMSC and hADMSCs [56], and in aged mice after transplantation of ADMSCs [61]. In addition, hBMMSCs upregulate the BDNF level, rescuing cultured rodent cortical neurons from degeneration by trophic factor absence or oxidative stress [86] and ameliorating spinal cord injury *in vivo* by increasing both BDNF and NGF levels [69]. Hence, the observed restoration of cholinergic functions is most likely due to the secretion of neurotrophic factors by the migrated cells, which were then taken up by projected axonal terminals and retrogradely transported into cholinergic neuron bodies, preventing their loss.

To explore the pathways involved in the observed improvement of cognitive deficits in BMMSCs + D-gal rats, we investigated factors involved in synaptic activity and plasticity. Reduced transcription of the immediate early gene *Egr1* in the hippocampus has been related to age-related memory deficits [21, 62, 93]. Our results showed that *Egr1* expression was reduced in the hippocampi of aged rats when compared with control rats. The restoration of competent spatial memory in the water maze test correlated with *Egr1* expression in the hippocampi of aged mice [93]. In the present study, BMMSC transplantation triggered *Egr1* expression in the hippocampus in aging rats. Cao et al. [17] reported that hUCMSCs stimulated the intracellular MAPK-ERK signalling and subsequently enhanced several effectors, including *Egr1* and PSD95. These effectors play important roles in neuron morphology, synaptic plasticity, and cognitive integrity in aged brain. Therefore, it is likely that the enhancement of *Egr1* expression by BMMSCs in transplanted rats have contributed to the countering of the age-related cognitive decline.

Limitations of the study

Our study had some limitations. First, neuronal differentiation of the transplanted cells was limited. Enhancing the neuronal differentiation of the transplanted cells would further improve their therapeutic capacity; therefore, optimising strategies to further improve the neuronal differentiation potential of the transplanted cells are needed. Second, the effect of transplanted cells on neurogenesis was not assessed. Third, the transplanted BM population did not represent pure MSCs. According to phenotypic profiling, 9.5% of the BMMSCs utilised in this study expressed CD34. This cell population may have contributed to the beneficial effects observed in this study. Finally, the optimisation of the cell-delivery protocol, including the number of transplanted cells, frequency of injections, and route of delivery requires further investigation.

CONCLUSIONS

The current study demonstrated that intravenous transplantation of BMMSCs prevented cognitive and physical deficits in a D-gal aging rat model by restoring cholinergic system function, protecting atrophic cholinergic neurons in the basal forebrain, inducing antioxidative effects and restoring neurotrophic factors, and modulating hippocampal synaptic plasticity by triggering PSD95 and *Egr1* expression. Our results provide evidence of the usability of systemic transplantation of BMMSCs as a potential therapeutic approach for the prevention of neurodegenerative changes associated with aging. However, prior to commencing clinical trials, further studies are needed to enhance the differentiation of these cells into the neuronal fate *in vivo* and to untangle the molecular mechanisms underlying the beneficial effects of BMMSC-secreted factors.

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