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Lercanidipine boosts the efficacy of mesenchymal stem cell therapy in 3-NP-induced Huntington's disease model rats via modulation of the calcium/calcineurin/NFATc4 and Wnt/β-catenin signalling pathways

Eman M. Elbaz[a], Hebatullah S. Helmy[a], Ayman E. El-Sahar[b], Muhammed A. Saad[b,c], Rabab H. Sayed[b,*]

[a] Department of Biochemistry, Faculty of Pharmacy, Cairo University, Cairo, Egypt
[b] Department of Pharmacology and Toxicology, Faculty of Pharmacy, Cairo University, Cairo, Egypt
[c] School of Pharmacy, Newgiza University, Cairo, Egypt

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ABSTRACT

3-Nitropropionic acid (3-NP) induces a spectrum of Huntington's disease (HD)-like neuropathologies in the rat striatum. The present study aimed to demonstrate the neuroprotective effect of lercanidipine (LER) in rats with 3-NP-induced neurotoxicity, address the possible additional protective effect of combined treatment with bone marrow-derived mesenchymal stem cells (BM-MSCs) and LER, and investigate the possible involvement of the Ca^{2+}/calcineurin (CaN)/nuclear factor of activated T cells c4 (NFATc4) and Wnt/β-catenin signalling pathways. Rats were injected with 3-NP (10 mg/kg/day, i.p.) for two weeks and were divided into four subgroups; the first served as the control HD group, the second received a daily dose of LER (0.5 mg/kg, i.p.), the third received a single injection of BM-MSCs (1 x 106/rat, i.v.) and the last received a combination of both BM-MSCs and LER. The combined therapy improved motor and behaviour performance. Meanwhile, this treatment led to a marked reduction in striatal cytosolic Ca^{2+}, CaN, tumour necrosis factor-alpha, and NFATc4 expression and the Bax/Bcl2 ratio. Combined therapy also increased striatal brain-derived neurotrophic factor, FOXP3, Wnt, and β-catenin protein expression. Furthermore, haematoxylin-eosin and Nissl staining revealed an amelioration of striatum tissue injury with the combined treatment. In conclusion, the current study provides evidence for a neuroprotective effect of LER and/or BM-MSCs in 3-NP-induced neurotoxicity in rats. Interestingly, combined LER/BM-MSC therapy was superior to cell therapy alone in inhibiting 3-NP-induced neurological insults via modulation of the Ca^{2+}/CaN/NFATc4 and Wnt/β-catenin signalling pathways. LER/BM-MSC combined therapy may represent a feasible approach for improving the beneficial effects of stem cell therapy in HD.

1. Introduction

Huntington's disease (HD) is a genetic neurodegenerative disease caused by the expansion of CAG triplicate repeats in the huntingtin (Htt) gene, which leads to the accumulation of polyglutamine-expanded Htt protein within intranuclear inclusion bodies or neurites (Nagahara and Tuszynski, 2011). HD is initiated by the death of neurons in the striatum and is characterized by abnormal movements (chorea, dyskinesia), psychiatric disturbances, and progressive dementia (Lindvall and Kokaia, 2006). Among the experimental models of HD, the 3-nitropropionic acid (3-NP) model has been widely used in HD research. 3-NP is a plant and fungal neurotoxin known to mediate preferential neurodegeneration similar to that found in HD (Brouillet et al., 2005; Ludolph et al., 1991).

A key pathological feature of neurodegenerative diseases is the accumulation of misfolded aggregated proteins in the brain, leading to neuronal dysfunction and disease (Quist et al., 2005). Misfolded proteins damage cells by inducing endoplasmic reticulum stress and calcium (Ca^{2+}) concentration changes (Mukherjee and Soto, 2011). Ca^{2+} dyshomeostasis induces calcineurin (CaN), a key phosphatase in the brain, that may trigger synaptic dysfunction and neuronal death (Wu et al., 2010). Upon activation by CaN, the nuclear factor of activated T cells (NFAT) translocates to the nucleus and guides the expression of target genes involved in neuroinflammation and synaptic development and plasticity (Schwartz et al., 2009). Mounting evidence suggests that aberrant Ca^{2+}/CaN/NFAT signalling significantly contributes to the

* Corresponding author. Postal address: Faculty of Pharmacy, Kasr El Aini St., Cairo, Postal code: 11562, Egypt.
E-mail address: rabab.sayed@pharma.cu.edu.eg (R.H. Sayed).

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pathologic and clinical features of neurodegenerative disorders (Abdul et al., 2010; Sompol and Norris, 2018). Thus, Ca^{2+}/CaN/NFAT signalling is likely to serve as a potential therapeutic target for neurodegenerative disorders.

Wnts are a large family of proteins that participate in a signalling cascade that regulates organogenesis and stem cell proliferation (Inestrosa and Varela-nallar, 2014). Wnt signalling plays an essential role in the development and function of the neuronal network (Ciani and Salinas, 2005). The canonical Wnt signalling pathway is activated by the binding of the ligand to its receptor, leading to the liberation of β-catenin (Angers and Moon, 2009). As a consequence, β-catenin accumulates in the cytoplasm and translocates to the nucleus where it regulates Wnt target gene expression (Inestrosa et al., 2012). Dysfunctional Wnt signalling is associated with neuron pathology and synapse degeneration (Silva-Alvarez et al., 2013). Synapse loss is an early feature of neurodegenerative diseases and correlates with the severity of the disease (Purro et al., 2014). Therefore, protecting synapses early in the disease through modulating Wnt/β-catenin signalling could provide an effective therapeutic approach for the treatment of these diseases.

Mesenchymal stem cells (MSCs) are multipotent cells that have been shown to be effective in the treatment of preclinical animal models of neurological diseases such as experimental autoimmune encephalomyelitis, multiple sclerosis, and HD (Colpo et al., 2019; Uccelli et al., 2011). Intravenously injected MSCs are primarily trapped in the lungs where they can secrete various factors for a systemic response. Over time, MSCs migrate from the lung to various tissues, including the brain Kean, 2013; Kim, 2015. MSCs have been reported to secrete neurotrophic growth factors, including brain-derived neurotrophic factor (BDNF) (Teixeira et al., 2017). MSCs modulate inflammatory and apoptotic responses (Foraker et al., 2011; Li et al., 2015) and migrate to injury sites (Prockop and Oh, 2012). Bone marrow-derived MSCs (BM-MSCs) are, to date, the most commonly used stem cells for the treatment of haematopoietic diseases, and thus, their isolation and application are well studies (Gögel et al., 2011). Furthermore, BM-MSCs provide superior therapeutic effects over other stem cell types because of their low immunogenicity, making them the stem cell type with the greatest foundation for successful clinical use (Tao et al., 2015).

Lercanidipine (LER) is a vasoselective dihydropyridine calcium channel blocker (CCB) used for the treatment of hypertension (Wirtz and Herzig, 2004). Because LER is an L-type CCB and L-type calcium channels are considered the primary source of Ca^{2+} that activates CaN/NFAT signalling, LER has been speculated to inhibit this signalling. Likewise, LER possesses high lipophilicity, long duration of action and minimal adverse effects (Selvaraj et al., 2015). LER has been found to be neuroprotective in a focal cerebral ischaemic-reperfusion injury model via its antioxidant, anti-inflammatory and anti-apoptotic properties (Gupta et al., 2017).

Taken together, these observations suggest that a treatment that modulates both the Ca^{2+}/CaN/NFAT and Wnt/β-catenin signalling pathways may have therapeutic potential in mitigating HD. Therefore, the aim of the current study was to demonstrate the neuroprotective effect of LER in rats with 3-NP-induced neurotoxicity, address the possible additional protective effect of combined treatment with BM-MSCs and LER, and investigate the possible role of the Ca^{2+}/CaN/NFAT and Wnt/β-catenin signalling pathways.

2. Materials and methods

2.1. Animals

Adult male Wistar rats weighing 200–250 g were purchased from Theodor Bilharz Research Institute (Giza, Egypt). Animals were housed under controlled environmental conditions: constant temperature of 25 ± 2 °C, 60 ± 10% humidity, ventilation with 10–20 changes/h, and a 12/12-h light/dark cycle. Rats were allowed free access to a standard chow diet and water. The protocols used in this study complied with The Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 2011) and were approved by the Ethics Committee for Animal Experimentation at Faculty of Pharmacy, Cairo University (Permission Number: 2249). All efforts were exerted to minimize animal suffering and to reduce the number of animals used.

2.2. Isolation, culture and labelling of MSCs

Once male Wistar rats (150–200 g) were euthanized with thiopental (Sigma-Aldrich Co., USA) overdose, whole marrow from the femurs and tibias was flushed in Mesencult basal medium supplemented with MSC stimulatory enhancements (Stem Cell Technologies, Vancouver, Canada), 100 U/mL penicillin, and 100 mg/mL streptomycin (Biowest, France). Cells were seeded at a density of 3×10^7 nucleated cells per millilitre at 37 °C in a 5% humidified CO_2 incubator (Sanyo, Osaka, Japan) for 72 h. Detached cells were removed on the third day, and the adherent population was cultured for more than 4–10 days to reach the maximum number of fibroblast colony-forming units prior to initial passage. Adherent cells were trypsinized (using 0.1% trypsin/0.1% ethylene diamine tetra acetic acid) and subcultured at a density of 5×10^3 cells/cm. All cultures were used between passages 2 and 5 (Liu et al., 2009). MSCs in culture were characterized by their flexible adhesiveness and spindle-shaped fibroblast-like appearance. Flow cytometric immunophenotyping was used to identify and characterize BM-MSCs ($CD34^+$, $CD90^+$ and CD105 +) using a flow cytometer (Beckman Coulter, EPICS-XL, USA). MSCs harvested during the 4th passage were stained with red fluorochrome PKH26 (Sigma–Aldrich Co., USA), which labels cells with both biological and proliferating activities preserved and is convenient for in vitro and in vivo cell labelling (Alhadlaq and Mao, 2004). Flow cytometric analysis depicted that 96.8% and 96.1% cultured cells were positive for CD105 and CD90, respectively. In contrast, CD34 was expressed in only 1.05% of total cells (Fig. 1A, B and C).

2.3. Drugs and chemicals

3-NP purchased from Sigma-Aldrich Co. (St Louis, MO, USA) was dissolved in normal saline (0.9% w/v) in a volume of 0.5 mL 100 g^{-1} animal body weight for intraperitoneal (i.p.) injection. Lercanidipine

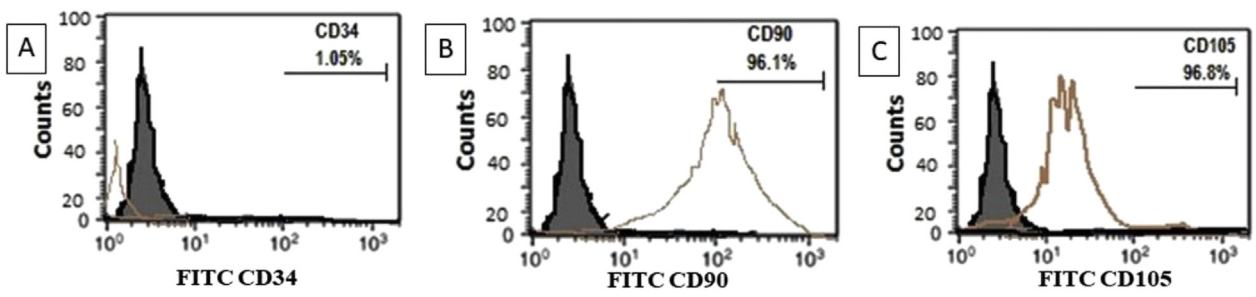

Fig. 1. Flow cytometric immunophenotyping for the identification and characterization of BM-MSCs (A–C).

hydrochloride was provided as a gift from Pharmacare Egypt (Cairo, Egypt) and was prepared in 20% ethanol, 20% dimethyl sulfoxide (DMSO), and 60% normal saline. All other chemicals were of the highest purity and analytical grade.

2.4. Experimental design

Sixty rats were randomly divided into five groups (12 animals each). Rats in group 1 (the control group) received i.p. injection of ethanol:DMSO:saline at a ratio of 20:20:60 for 14 days and served as a normal control. Rats in group 2 (the 3-NP group) received 3-NP (10 mg/kg/day, i.p.) for 14 days and served as the HD control group (Shalaby et al., 2018). Rats in group 3 (the BM-MSC group) received 3-NP (10 mg/kg/day, i.p.) for 14 days and a single intravenous injection of BM-MSCs (1×10^6/rat) into the tail vein 1 h before the first 3-NP injection (Mohamed et al., 2015). Rats in group 4 (the LER group) received 3-NP (10 mg/kg/day, i.p.) and LER (0.5 mg/kg/day, i.p.) (Gupta et al., 2017) 1 h before 3-NP injection for 14 days. Rats in group 5 (the BM-MSC + LER group) received 3-NP (10 mg/kg/day, i.p.) and a combined therapy of BM-MSCs as in group 3 and LER as in group 4. The dose of LER was selected on the basis of the previous study by Gupta et al. (2017) who reported the neuroprotective activity of LER against stroke in rats and was confirmed by our pilot study results (data not shown).

2.5. Behavioural and motor assessments

Twenty-four hours after the last 3-NP injection, animals were subjected to behavioural tests with a minimum of 30 min between each test. All testing was conducted during the animal's light cycle.

2.5.1. Open field test

An open field was performed to examine the spontaneous motor activity of rats. The apparatus was made of a square wooden box 80 × 80 × 40 cm in size with a red painted wall. The floor was divided with white lines into a 4 × 4 grid of 16 equal squares. Each rat was gently placed in the central area of the open field, and the locomotor behaviour was video recorded for 3 min. The test was performed in a quiet room under white light, and the apparatus was cleaned with 20% ethyl alcohol after each rat was tested. The ambulation frequency (the number of squares traversed by the rat), rearing frequency (the frequency of standing on its hind legs) and latency time (time passed with lack of movement during testing) were recorded for each animal (Moreira et al., 2010).

2.5.2. Rotarod test

Motor coordination was examined using the rotarod test. In brief, the rotarod apparatus consisted of a metallic rotating rod (120 cm in length, 3 cm in diameter and 30 cm in height) operating at a constant speed of 25 rpm. The rod was partitioned into four sections by discs 24 cm in diameter. Prior to the test, rats were adapted on the rotarod in training sessions. Each rat performed 3 separate trials with a 5-min gap, and the average latency to fall was recorded as the time on rotarod (Jones and Roberts, 1968).

2.5.3. String test for grip strength

A rat grip strength metre (Model 47200, Ugo Basile, Comerio, Italy) was used to assess forelimb grip strength. Animals were gently placed over a base plate in front of a triangle bar. When the rat grasped the bar, the animal was gently pulled steadily by their tail away from the triangle bar. The maximum pulling force (in grams) was recorded when the animal lost its grip on the grasping bar. The mean of three values for each rat was recorded (Massicotte et al., 2015).

2.6. Tissue processing

At the end of behavioural tests, rats were weighed and sacrificed by cervical dislocation under light anaesthesia, and their brains were rapidly dissected, washed with ice-cold saline and dried. Afterward, each of the groups was divided into two subgroups. In the first set (n = 3), brains were fixed in 10% (v/v) formalin for 24 h for histopathological staining. In the other set (n = 9), the striatal tissues were excised from each brain on an ice-cold glass plate and used for biochemical investigations.

2.7. Biochemical parameters

One striatum was homogenized in ice-cold saline to prepare 10% homogenates for the determination of cytosolic Ca^{2+} concentration, tumour necrosis factor-alpha (TNF-α), BDNF, forkhead box P3 (FOXP3), and CaN expression using Enzyme-linked immunosorbent assay (ELISA). The other striatum was used for Western blot analyses. The protein contents of the tissue homogenates were determined using the Bradford method (1976).

2.7.1. Cytosolic Ca^{2+} concentration

An aliquot of the 10% homogenate was ultracentrifuged at 105,000 × *g* for 15 min at 4 °C. A cytosolic fraction was used to assay cytosolic Ca^{2+} concentration using a Unicam 939 atomic absorption spectrophotometer (Cambridge, UK) (Subramanian, 1995).

2.7.2. ELISA

Brain TNF-α, BDNF, FOXP3 and CaN levels were estimated using rat ELISA kits purchased from Elabscience Biotechnology Co., Ltd. (Wuhan, China), Abnova Corporation (Jhongli, Taiwan), R&D Systems Inc. (Minneapolis, USA) and Cusabio Life Science (Wuhan, Hubei, China), respectively. The procedures were performed according to the manufacturer's instructions. The results are expressed as pg/mg protein for TNF-α, BDNF and FOXP3 and ng/mg protein for CaN.

2.7.3. Western blot analysis of Bax, Bcl2, Wnt and β-catenin protein expression

After total protein was extracted from the striatal tissue, equal amounts of protein (20–30 μg of total protein) were separated by SDS-PAGE (10% acrylamide gel) using a Bio-Rad Mini-Protein II system. The protein was transferred to polyvinylidene difluoride membranes (Pierce, Rockford, IL, USA) with a Bio-Rad Trans-Blot system. Subsequently, the membranes were washed with phosphate-buffered saline (PBS) and blocked for an hour at room temperature with 5% (w/v) skim milk powder in PBS. The manufacturer's guidelines were followed for the primary antibody reactions. After the membranes were blocked, the blots were developed using antibodies against Wnt and β-catenin obtained from R&D Systems Inc. (Minneapolis, USA) as well as Bax, Bcl2, and β-actin obtained from Thermo Fisher Scientific Inc. (Rockford, IL, USA). After the membranes were washed, peroxidase-labelled secondary antibodies were added, and the membranes were incubated at 37 °C for an hour. The band intensity was analysed using the ChemiDoc™ imaging system with Image Lab™ software version 5.1 (Bio-Rad Laboratories Inc., Hercules, CA, USA). The results are expressed as arbitrary units after normalization to β-actin protein expression.

2.8. Histological examinations

2.8.1. Assessment of striatal damage and Nissl staining

The fixed brain tissues were serially sectioned (5-μm thickness) and embedded in paraffin. The striatal sections were then stained with haematoxylin and eosin (H&E) and examined under a light microscope. Morphological changes, including necrosis, neurophagia, congestion of blood vessels and cellular oedema, were defined in five different fields

for each section. Changes were semi-quantitatively evaluated using the following scoring system: 0 = no changes, 1 = mild changes, 2 = moderate changes and 3 = severe changes. A total score for striatal injury was recorded for each rat with a maximum score of 12 (Woodruff et al., 2006). Nissl staining was performed to demonstrate degenerated and intact neurons in the striatum, as outlined by Bancroft and Stevens (Bancroft and Stevens, 1990).

2.8.2. Glial fibrillary acidic protein (GFAP) immunostaining

Immunohistochemical staining of GFAP was performed using a mouse monoclonal antibody (Thermo Fisher Scientific Inc., Rockford, IL, USA). All procedures were performed according to the manufacturer's instructions. The percentage of GFAP immunoreactivity in five random non-overlapping fields per tissue section was measured using a Full HD microscopic camera operated by the Leica application module for tissue section analysis (Leica Microsystems GmbH, Wetzlar, Germany).

2.8.3. Estimation of the number of labelled BM-MSCs

Other brains were excised, snap frozen in liquid nitrogen and cut into 5-μm-thick sections. PKH26, a red fluorochrome, was used to label BM-MSCs. The homing of injected BM-MSCs in the brain tissue was detected by a fluorescence microscope (Leica DM 5500 B, Germany) and estimated by manually counting the number of PKH26-stained cells in 5 random fields for each frozen section (5 sections for each brain) (Ali et al., 2017).

2.9. Statistical analysis

All data were tested for normality using the Shapiro test. The data are presented as the mean ± S.D. Data were analysed using one-way ANOVA followed by the Tukey-Kramer multiple comparison test, except for the histopathological scores, which were analysed using Kruskal–Wallis ANOVA followed by Dunn's multiple comparison test and expressed as a median and range. Unpaired Student's *t*-test was used to analyse the number of labelled BM-MSCs in the striatum. GraphPad Prism software (version 7.04; GraphPad Software, Inc., San Diego, CA, USA) was used to perform the statistical analysis and present the data. The level of significance was fixed at $p < 0.05$ for all statistical tests.

3. Results

3.1. Effect of LER, BM-MSCs and their combination on 3-NP-induced changes in body weight and mortality rate

3-NP intoxication caused a significant reduction in body weight together with an increase in mortality rate compared to control treatment. In contrast, treatment with either BM-MSCs or LER significantly hindered body weight loss and decreased the mortality rate compared to 3-NP treatment alone. However, co-treatment with LER and BM-MSCs improved the abovementioned parameters (Fig. 2, Table 1).

3.2. Effect of LER, BM-MSCs and their combination on 3-NP-induced behavioural and motor abnormalities

3-NP-intoxicated animals showed behavioural and motor abnormalities, as evidenced by the open field (ambulation frequency, rearing frequency, and latency time), rotarod, and grip strength tests. In the open field test, the 3-NP group exhibited a significant reduction in ambulation and rearing frequencies, together with a striking elevation in the latency time, compared to the control group. Moreover, 3-NP administration decreased grip strength (rotarod and grip strength metre). Treatment with LER, BM-MSCs or their combination significantly ameliorated the aforementioned behavioural and motor changes in the open field and grip strength tests. Interestingly, only the combined therapy succeeded in prolonging the time on the rotarod compared to 3-NP alone (Table 1).

3.3. Effect of LER, BM-MSCs and their combination on 3-NP-induced striatal inflammation

3-NP induced a state of inflammation as revealed by a significant elevation in the TNF-α level together with a marked decrease in the FOXP3 level compared to control treatment. Conversely, treatment with either LER or BM-MSCs significantly attenuated the elevation in TNF-α levels and the reduction in FOXP3 levels. However, combined therapy caused a more pronounced anti-inflammatory effect (Fig. 3).

3.4. Effect of LER, BM-MSCs and their combination on the 3-NP-induced alteration in the Ca^{2+}/CaN/NFATc4 and Wnt/β-catenin signalling pathways

Compared to the control animals, the 3-NP-treated animals exhibited increased striatal cytosolic Ca^{2+} concentration, CaN level, and NFATc4 expression. In addition, 3-NP down-regulated expression of Wnt and its downstream effector β-catenin. However, treatment with either LER or BM-MSCs significantly decreased cytosolic Ca^{2+} concentration, CaN level, and NFATc4 expression and up-regulated Wnt and β-catenin expression. Furthermore, combined therapy further ameliorated the changes in NFATc4, Wnt, and β-catenin expression levels as compared to monotherapy with BM-MSCs (Fig. 4). Notably, LER significantly decreased cytosolic Ca^{2+} concentration as compared with BM-MSCs group value.

3.5. Effect of LER, BM-MSCs and their combination on 3-NP-induced apoptosis

Treatment with 3-NP markedly decreased striatal BDNF levels and Bax expression, while increasing Bcl2 expression, resulting in a marked

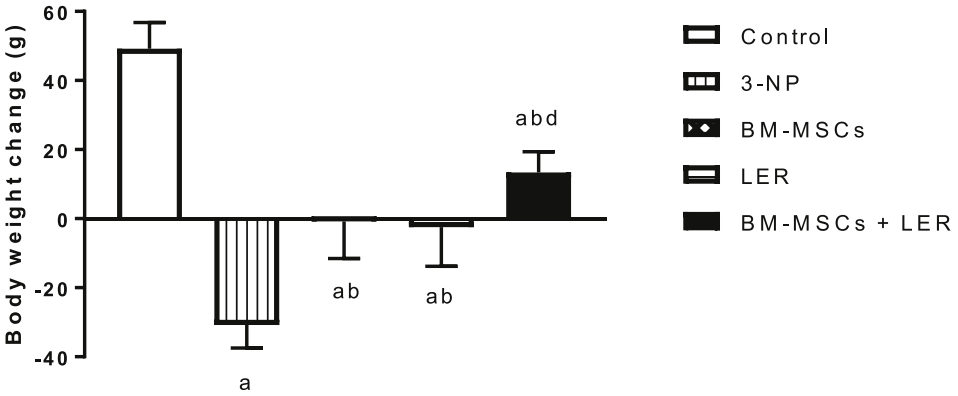

Fig. 2. Effect of LER, BM-MSCs and their combination on the 3-NP-induced change in rat body weight. Each bar with a vertical line represents the mean of experiments ± S. D (n = 6–8). a Compared with the control group, b compared with the 3-NP group, c compared with the BM-MSC group, and d compared with the LER group. All values are statistically significant at $p < 0.05$. Abbreviations: 3-NP: 3-nitropropionic acid, BM-MSCs: bone marrow-derived mesenchymal stem cells, LER: lercanidipine.

Table 1
Effect of LER, BM-MSCs and their combination on 3-NP-induced changes in mortality rate as well as performance on open field, rotarod, and grip strength tests.

	Mortality (%)	Open field test			Time on rotarod (s)	Grip strength (g)
		Ambulation frequency (No. of squares/ 3 min)	Rearing frequency (count/ 3 min)	Latency time (s)		
Control	0	49.17 ± 4	31.5 ± 1.3	1.667 ± 0.33	230 ± 17.89	548.6 ± 23.75
3-NP	33.33%	12.17 ± 3.66 [a]	9.667 ± 1.82 [a]	12.83 ± 1.14 [a]	14.67 ± 3.76 [a]	195.6 ± 11.88[a]
BM-MSCs	25%	23.83 ± 3.29 [ab]	21 ± 2 [b]	7.667 ± 1.17 [ab]	42.5 ± 6.92 [a]	347.4 ± 22.43 [ab]
LER	25%	30.67 ± 4 [ab]	23 ± 2.7 [b]	7.333 ± 1 [ab]	48.33 ± 7.38 [a]	342.7 ± 24.54 [ab]
BM-MSCs + LER	7.95%	39.83 ± 3.32 [b]	31.17 ± 3 [b]	4.167 ± 0.48 [b]	175 ± 12.32 [abcd]	446.7 ± 22.06 [abcd]

Each value represents the mean of experiments ± S.D. (n = 6–8). a Compared with the control group, b compared with the 3-NP group, c compared with the BM-MSC group, and d compared with the LER group. All values are statistically significant at $p < 0.05$. **Abbreviations:** 3-NP: 3-nitropropionic acid, BM-MSCs: bone marrow-derived mesenchymal stem cells, LER: lercanidipine.

decline in the Bax/Bcl2 ratio. Treatment with either BM-MSCs or LER significantly attenuated the elevation in the Bax/Bcl2 ratio; however, only BM-MSC treatment succeeded in ameliorating the 3-NP-induced alterations in BDNF levels. Notably, the combined therapy group exhibited significantly higher BDNF levels than the group treated with BM-MSCs alone and a normalized Bax/Bcl2 ratio (Fig. 5).

3.6. Effect of LER, BM-MSCs and their combination on 3-NP-induced histopathological alterations

Histopathological photomicrographs showed that the group administered 3-NP exhibited extensive neuronal necrosis, pyknosis, neurophagia, vascular congestion and cellular oedema compared with the control group (Fig. 6B). Furthermore, the striatum injury score was markedly increased in rats with 3-NP insults (Fig. 6R). However, extensive astroglia activation was also demonstrated by a marked increase in GFAP immunoreactivity as well as marked neuronal loss as evidenced by Nissl staining (Fig. 6G & L, respectively). On the other hand, the group given LER treatment showed moderate necrosis, neurophagia, vascular congestion and oedema as well as significantly ameliorated astroglia activation and neuronal loss (Fig. 6D, I & N, respectively). BM-MSCs, either alone or in combination with LER, retained striatal and neural structures with minimal morphological changes (Fig. 6C and E), as evidenced by the marked reduction in the striatum injury scores (Fig. 6R), astroglia activation (Fig. H, J & S) and neuronal loss (Fig. 6M, O & T).

3.7. Effect of LER on homing of BM-MSCs

As shown in Fig. 6P, Q & U, the use of LER with BM-MSCs enhanced the homing of implanted cells to the damaged striatum. Fluorescent microscopic examination revealed more PKH-26-labelled cells in the striatum of rats treated with LER and BM-MSCs than in the striatum of rats treated with BM-MSCs alone.

4. Discussion

The current study reveals a neuroprotective effect of LER against 3-NP-induced neurotoxicity in rats and compared it to that of BM-MSCs either alone or in combination with LER. Our study also confirms the involvement of the Ca^{+2}/CaN/NFATc4 and Wnt/β-catenin signalling pathways in 3-NP-mediated neurotoxicity and indicates that modulation of these signalling pathways is involved in the neuroprotective effect of LER and BM-MSCs.

Systemic administration of the mitochondrial toxin 3-NP to rats serves as a good experimental model of HD that closely mimics the biochemical manifestations and pathophysiological features of HD in humans (Alberch et al., 2010; Túnez et al., 2010). In the present study, 3-NP induced a significant increase in mortality rate together with a significant decrease in body weight. In addition, 3-NP administration resulted in reduced locomotor activity, loss of grip strength and coordination imbalance, all of which characterize the late stages of HD. Our results are in good agreement with previous studies demonstrating similar motor deficits following 3-NP administration (Ahmed et al., 2016; El-Abhar et al., 2018). Such movement disabilities are usually associated with anorexia and decreased food intake, which explains the significant body weight loss observed in the 3-NP group (Keene et al., 2001). Compared with monotherapy, co-treatment with LER and BM-MSCs mitigated 3-NP-induced mortality, body weight loss and motor impairment.

Changes in cytoplasmic Ca^{2+} concentration lead to disturbance of several signalling pathways (Mukherjee and Soto, 2011). CaN is highly expressed in the brain and is activated by increased cytosolic Ca^{2+} in neurological disorders (Klee and Krinks, 1978). CaN stimulation has

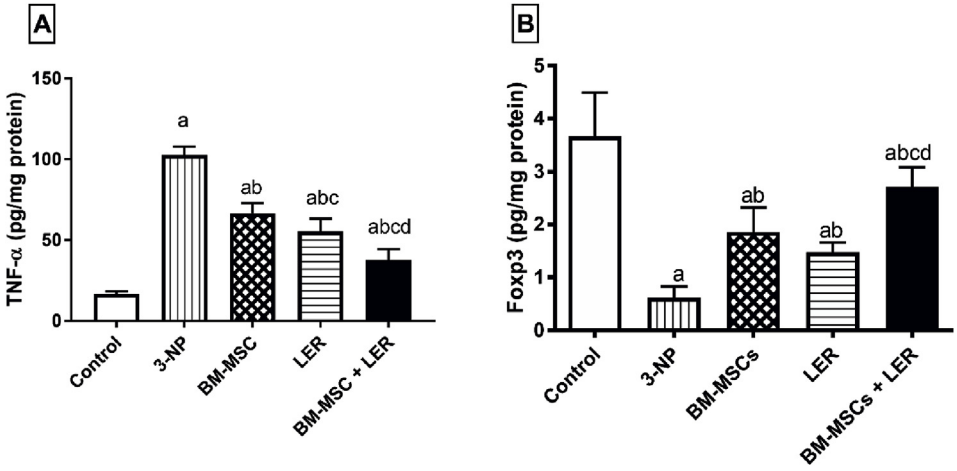

Fig. 3. Effect of LER, BM-MSCs and their combination on 3-NP-induced striatal inflammation indicated by TNF-α (A) and FOXP3 (B) expression. Each bar with a vertical line represents the mean of experiments ± S. D (n = 6–8). a Compared with the normal control group, b compared with the 3-NP group, c compared with the BM-MSC group, and d compared with the LER group. All values are statistically significant at $p < 0.05$. **Abbreviations:** 3-NP: 3-nitropropionic acid, BM-MSC: bone marrow-derived mesenchymal stem cell, LER: lercanidipine, TNF-α: tumour necrosis factor-alpha, FOXP3: forkhead box P3.

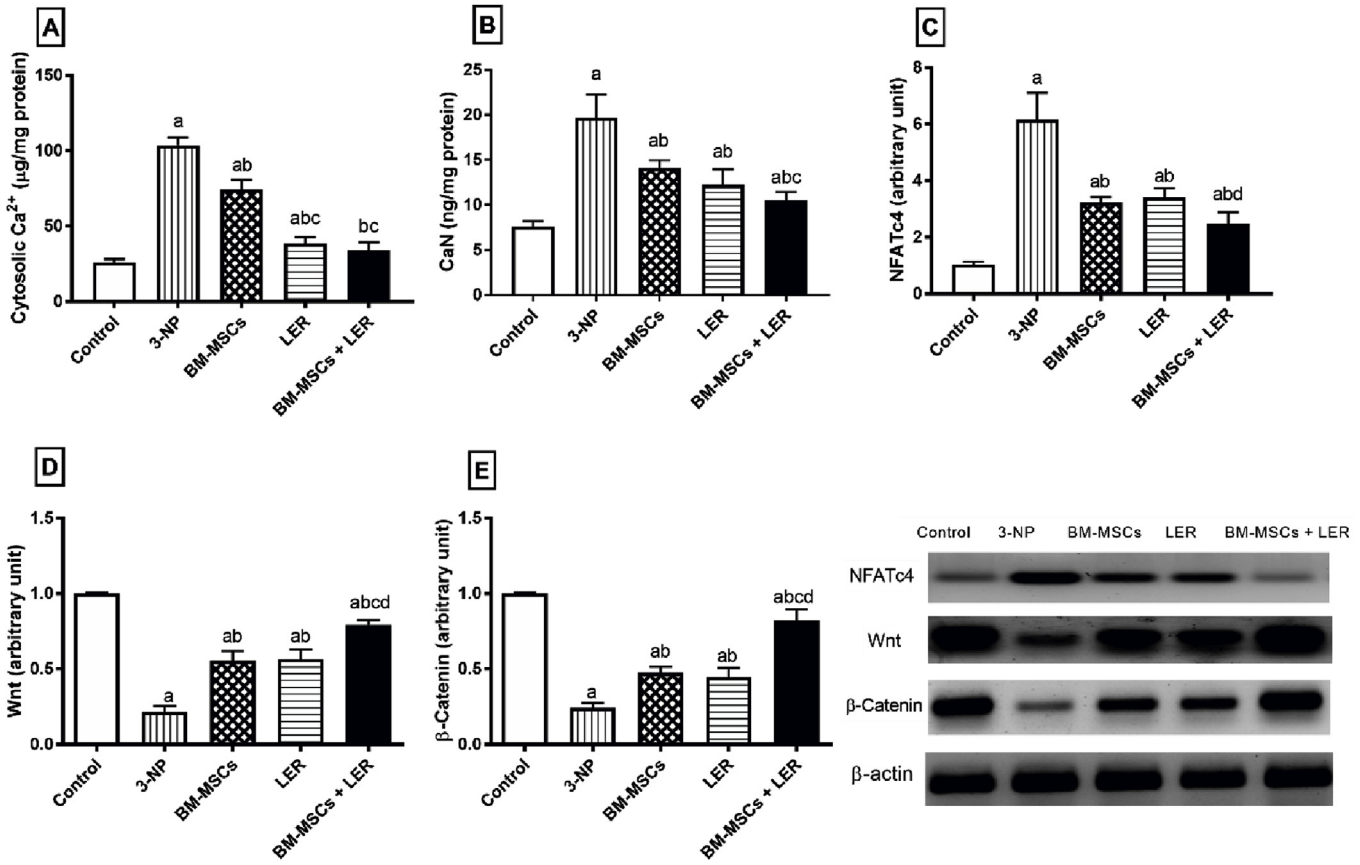

Fig. 4. Effect of LER, BM-MSCs and their combination on the 3-NP-induced alteration in cytosolic Ca^{2+} concentration (A), CaN level (B), NFATc4 (C), Wnt (D), and β-catenin (E) protein expression. Each bar with a vertical line represents the mean of experiments ± S. D (n = 6–8). a Compared with the control group, b compared with the 3-NP group, c compared with the BM-MSC group, and d compared with the LER group. All values are statistically significant at $p < 0.05$. **Abbreviations**: 3-NP: 3-nitropropionic acid, BM-MSCs: bone marrow-derived mesenchymal stem cells, LER: lercanidipine, CaN: calcineurin, NFATc4: nuclear factor of activated T cells c4.

been demonstrated in astrocytes surrounding amyloid deposits and aggravates neuroinflammation, resulting in pathological and morphological changes (Norris et al., 2005). Elevated CaN activity has been reported in neuronal cells immortalized from HD mice (Xifró et al., 2008). Furthermore, CaN inhibitors have been reported to improve 3-NP-induced cognitive dysfunction in rats (Kumar and Kumar, 2009). Hyperactivation of CaN results in dephosphorylation and nuclear translocation of cytoplasmic NFATc1-4, which translocates to the nucleus to induce sustained expression of genes encoding for many inflammatory modulators (Kipanyula et al., 2016). The NFATc4 isoform has been shown to be involved in neurodegenerative processes (Rojanathammanee et al., 2015). Here, 3-NP induced a significant increase in striatal cytosolic Ca^{2+} concentration, CaN level and NFATc4 expression, confirming the role of the Ca^{2+}/CaN/NFATc4 signalling pathway in 3-NP-induced neurotoxicity. However, treatment with either LER or BM-MSCs significantly suppressed 3-NP-induced activation of the Ca^{2+}/CaN/NFATc4 pathway, and their combination further suppressed its activation. In line with these results, BM-MSCs have been shown to have antihypertrophic effects and inhibit isoproterenol-induced deleterious changes in cardiomyocytes, most likely through the secretion of vascular endothelial growth factor, which inhibits the activation of Ca^{2+}/CaN/NFAT signalling (Cai et al., 2015). Various paracrine growth factors are observed secreted into the microenvironment by BMSCs and then lead to cellular regeneration via anti-inflammatory and anti-apoptotic effects (Mignone and Murry, 2011; Xu et al., 2007). Furthermore, LER has been reported to inhibit cardiomyocyte hypertrophy by suppressing the CaN/NFATc4 pathway (Chen et al., 2017). The further improvement in CaN and NFATc4 levels observed here with the combined therapy could be related to the CCB activity of LER and to its high lipophilicity and extensive storage in cell membranes (HERBETTE et al., 2016).

Increased CaN/NFATc4 activity in astrocytes leads to the induction and release of numerous immune/inflammatory factors (Kraner and Norris, 2018). TNF-α is a pro-inflammatory cytokine that was first described in the immune system but has since been found in significant amounts in the CNS (Golan et al., 2004). Canellada et al. (2006) reported the induction of TNF-α via CaN/NFAT signalling in PC12 neural cells, which may account for the 3-NP-induced increase in striatal TNF-α levels observed in the current study and other previous studies (El-Abhar et al., 2018; Khan et al., 2015). FOXP3 is the key transcription factor controlling T-regulatory cells (Lu et al., 2017). NFAT has been identified as an interaction partner of FOXP3, regulating its expression (Vaeth et al., 2012). Moreover, TNF-α has been shown to downregulate FOXP3 expression (Gao et al., 2015). In parallel, our results showed diminished striatal levels of FOXP3 in 3-NP-treated rats. Conversely, treatment with either LER or BM-MSCs significantly attenuated the elevation in TNF-α expression as well as the reduction in FOXP3 expression. However, combined therapy caused a more pronounced anti-inflammatory effect. Earlier studies have shown that BM-MSCs can repress inflammation via activating FOXP3 regulatory T cells, thus reducing the production of pro-inflammatory cytokines such as TNF-α (Le Blanc and Mougiakakos, 2012). Likewise, LER was reported to attenuate the production of TNF-α in vascular dysfunction in rats (Yeh et al., 2013).

Hyperactivated CaN/NFAT signalling has been associated with neuronal apoptotic pathways involving Bcl-2 family proteins

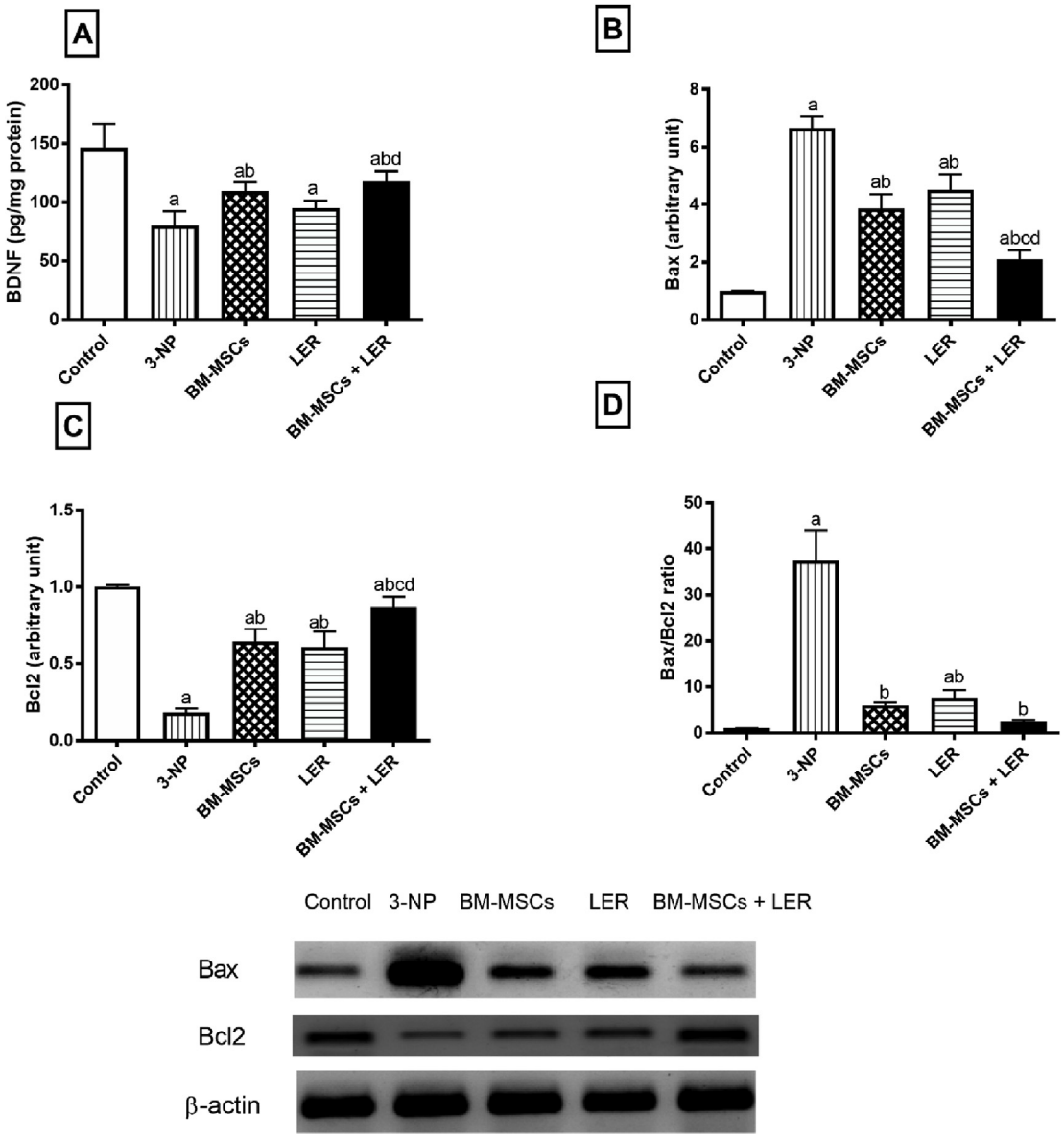

Fig. 5. Effect of LER, BM-MSCs and their combination on 3-NP-induced changes in BDNF (A), Bax (B), and Bcl2 (C) protein expression and the Bax/Bcl2 ratio (D). Each bar with a vertical line represents the mean of experiments ± S. D (n = 6–8). a Compared with the control group, b compared with the 3-NP group, c compared with the BM-MSC group, and d compared with the LER group. All values are statistically significant at $p < 0.05$. **Abbreviations:** 3-NP: 3-nitropropionic acid, BM-MSCs: bone marrow-derived mesenchymal stem cells, LER: lercanidipine.

(Mukherjee and Soto, 2011) and aberrant dephosphorylation of Htt protein, leading impaired vesicular transport of essential neurotrophins including BDNF (Pineda et al., 2009). In the same context, the present work showed a significant increase in the striatal Bax/Bcl2 ratio along with a prominent suppression of BDNF levels in the 3-NP-treated group. The anti-apoptotic and neurotrophic effects of combined therapy were manifested by increased BDNF levels and normalized Bax/Bcl2 ratios compared to monotherapy. Therefore, the improvement in the repair potential of BM-MSCs by the concomitant use of LER may partially rely on the anti-inflammatory, neurotrophic, and anti-apoptotic effects of both treatment, as well as the modulation of CaN/NFATc4 signalling, as demonstrated in the present study.

Finally, the canonical Wnt/β-catenin pathway contributes to the regulation of neuronal survival and homeostasis in the CNS (Caraci et al., 2008). Current evidence indicates that the downregulation of the Wnt/β-catenin pathway in HD is correlated with an increase in apoptosis (Libro et al., 2016). Herein, the elevation of apoptotic markers in 3-NP-treated rats was associated with a significant decrease in striatal Wnt and β-catenin protein expression. Interestingly, enhanced Wnt/β-Catenin signalling was previously demonstrated to promote the neuronal differentiation of BM-MSCs (Kondo et al., 2011). Moreover, Oh et al. (2015) reported that enhanced neurogenesis via the Wnt signalling pathway plays an important role in the effect of MSCs on mouse models of Alzheimer. However, the effect of combined therapy on the upregulation of Wnt and β-catenin protein expression was more pronounced than that of either treatment alone. These effects may be due to the inhibitory effect of LER on the CaN/NFATc4 pathway (Chen et al., 2017). CaN-activated NFAT has been reported to participate in switching off canonical Wnt/β-catenin signalling (Huang et al., 2011).

The findings of the present study were correlated with the histopathological changes evidenced by the increased striatal injury score and GFAP immunoreactivity as well as extensive neuronal loss shown by Nissl staining. Accumulating evidence supports a fundamental role for Ca^{2+}/CaN/NFAT signalling in astrocyte activation, which contributes to neuroinflammation, amyloid pathology and synapse dysfunction (Sompol and Norris, 2018). CaN inhibitors prevent astrocyte

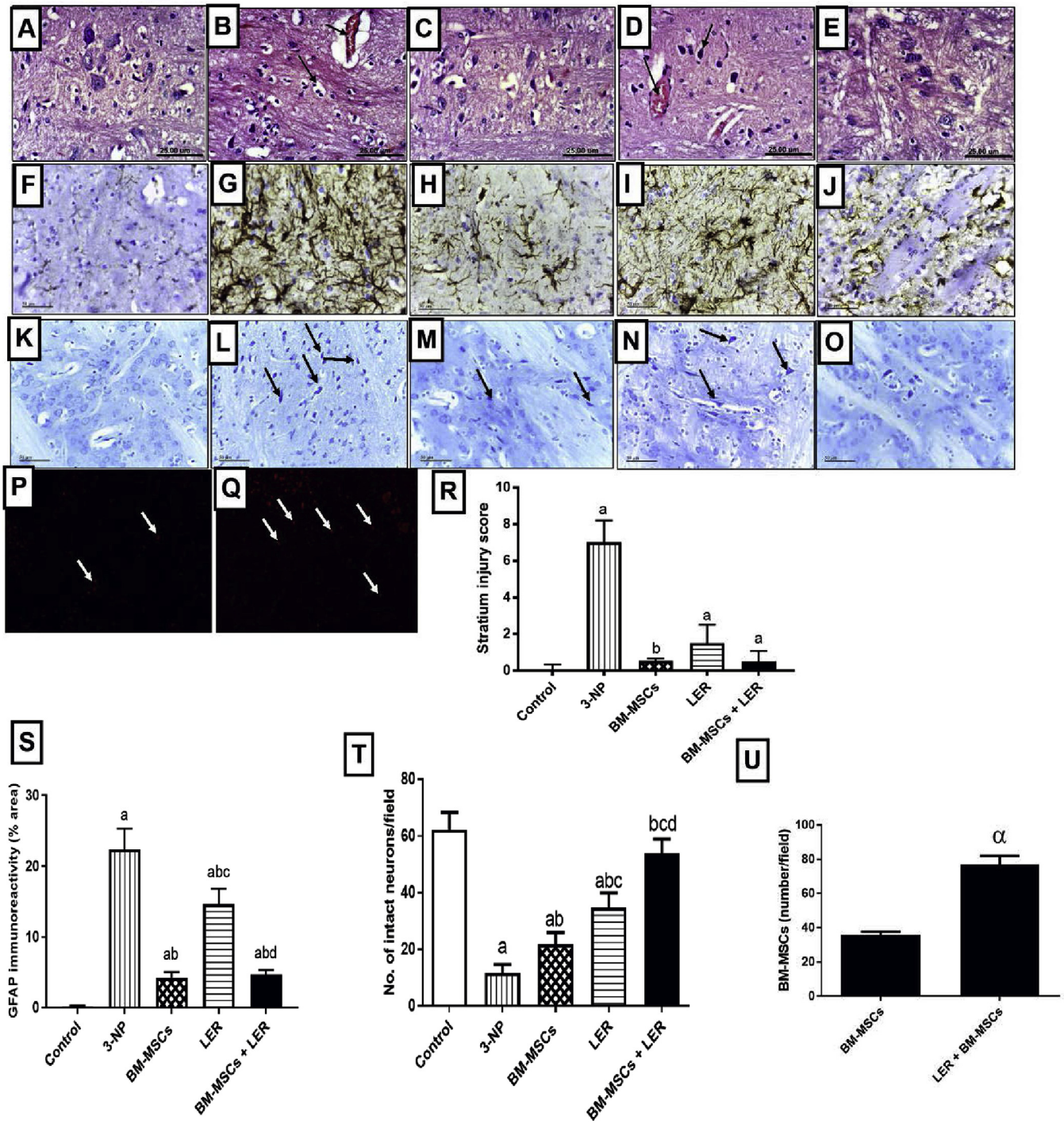

Fig. 6. Effect of LER, BM-MSCs, and their combination on 3-NP-induced histological changes. **A-E** Specimen stained with H&E (400 x magnification). **A** The control group showed normal striatal structure; **B** the 3-NP group showed extensive necrosis of neurons and congestion of blood vessels; **C** the BM-MSC group showed mild morphological changes; **D** the LER group showed moderate vascular congestion and mild necrosis of neurons, and **E** the LER + BM-MSC group showed no histological changes. **F–J** Specimens stained with GFAP (400 x magnification). **F** The control group showed normal GFAP immunostaining of inactivated astrocytes. **G** The 3-NP group showed a marked increase in GFAP-immunoreactive astrocytes. **H & I** The BM-MSC group and LER group showed a moderate increase in reactivity of the activated proliferated astrocytes. **J** The BM-MSC + LER combination group showed a mild increase in reactivity of the activated proliferated astrocytes. **K–O** Specimens stained with Nissl (400 x magnification). **P & Q** Sections for the determination of BM-MSC homing by fluorescence microscopy. **P** BM-MSC group. **Q** BM-MSC + LER group. **R** Striatum injury score. Statistical analysis was carried out using Kruskal–Wallis ANOVA followed by Dunn's multiple comparison test and expressed as the median and range (n = 3). **S** GFAP-immunoreactive astrocytes (% area). **T** Number of Nissl-stained cells (intact neurons). Statistical analysis was carried out using one-way ANOVA followed by the Tukey-Kramer multiple comparison test. a Compared with the control group, b compared with the 3-NP group, c compared with the BM-MSC group, and d compared with the LER group. **U** Number of BM-MSCs in the striatum. Statistical analysis was carried out using unpaired Student's *t*-test. α Compared to BM-MSCs. Values are expressed as the mean ± S. D (n = 3). All values are statistically significant at $p < 0.05$. **Abbreviations**: BM-MSC: bone marrow-derived mesenchymal stem cell; GFAP: glial fibrillary acidic protein; LER: lercanidipine.

activation and markedly decrease GFAP level (Liu et al., 2018). Furthermore, MSCs modulate reactivity of astrocytes thereby promoting neuroregeneration in the area of spinal cord injury (Mukhamedshina et al., 2019).

In conclusion, the current study provides evidence for a neuroprotective effect of LER and/or BM-MSCs in 3-NP-induced neurotoxicity in rats. Interestingly, LER augmented BM-MSC's effect in inhibiting 3-NP-induced neurological insults, via exerting synergistic modulatory effects on inflammation, apoptosis, and Ca^{2+}/CaN/NFATc4 and Wnt/β-catenin signalling pathways. LER/BM-MSC combined therapy may represent a feasible approach for improving the beneficial effects of stem cell therapy in HD.

Authors' contributions

Hebatullah S. Helmy, Ayman E. El-Sahar, Eman M. Elbaz: conceived and designed the experiments.

Muhammed A. Saad, Ayman E. El-Sahar, Rabab H. Sayed, Eman M. Elbaz, Hebatullah S. Helmy: performed the experiments.

Hebatullah S. Helmy, Eman M. Elbaz, Rabab H. Sayed: analysed the data and wrote the manuscript.

Rabab H. Sayed, Hebatullah S. Helmy, Muhammed A. Saad, Ayman E. El-Sahar: contributed reagents and analysis tools.

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Conflicts of interest

None to be declared.

References

Abdul, H.M., Furman, J.L., Sama, M. a, Mathis, D.M., Norris, C.M., 2010. NFATs and alzheimer's disease. Mol. Cell. Pharmacol. https://doi.org/10.4255/mcpharmacol.10.02.

Ahmed, L.A., Darwish, H.A., Abdelsalam, R.M., Amin, H.A.A., 2016. Role of rho kinase inhibition in the protective effect of fasudil and simvastatin against 3-nitropropionic acid-induced striatal neurodegeneration and mitochondrial dysfunction in rats. Mol. Neurobiol. https://doi.org/10.1007/s12035-015-9303-2.

Alberch, J., Pérez-Navarro, E., Canals, J.M., 2010. Animal models of huntington's disease. In: Encyclopedia of Neuroscience, https://doi.org/10.1016/B978-008045046-9.00502-7.

Alhadlaq, A., Mao, J.J., 2004. Mesenchymal stem cells: isolation and therapeutics. Stem Cells Dev. 13, 436–448. https://doi.org/10.1089/scd.2004.13.436.

Ali, E.H.A., Ahmed-Farid, O.A., Osman, A.A.E., 2017. Bone marrow-derived mesenchymal stem cells ameliorate sodium nitrite-induced hypoxic brain injury in a rat model. Neural Regen. Res. 12, 1990. https://doi.org/10.4103/1673-5374.221155.

Angers, S., Moon, R.T., 2009. Proximal events in Wnt signal transduction. Nat. Rev. Mol. Cell Biol. 10, 468–477. https://doi.org/10.1038/nrm2717.

Bancroft, J.D., Stevens, A., Pathologist), 1990. Theory and Practice of Histological Techniques. Churchill Livingstone.

Bradford, R., 1976. Determinación De Proteínas. Método De Bradford 254, 1976.

Brouillet, E., Jacquard, C., Bizat, N., Blum, D., 2005. 3-Nitropropionic acid: a mitochondrial toxin to uncover physiopathological mechanisms underlying striatal degeneration in Huntington's disease. J. Neurochem. 95, 1521–1540. https://doi.org/10.1111/j.1471-4159.2005.03515.x.

Cai, B., Tan, X., Zhang, Y., Li, X., Wang, X., Zhu, J., Wang, Y., Yang, F., Wang, B., Liu, Y., Xu, C., Pan, Z., Wang, N., Yang, B., Lu, Y., 2015. Mesenchymal stem cells and cardiomyocytes interplay to prevent myocardial hypertrophy. Stem Cells Transl. Med. https://doi.org/10.5966/sctm.2015-0032.

Canellada, A., Cano, E., Sánchez-Ruiloba, L., Zafra, F., Redondo, J.M., 2006. Calcium-dependent expression of TNF-α in neural cells is mediated by the calcineurin/NFAT pathway. Mol. Cell. Neurosci. https://doi.org/10.1016/j.mcn.2005.12.008.

Caraci, F., Busceti, C., Biagioni, F., Aronica, E., Mastroiacovo, F., Cappuccio, I., Battaglia, G., Bruno, V., Caricasole, A., Copani, A., Nicoletti, F., 2008. The Wnt antagonist, Dickkopf-1, as a target for the treatment of neurodegenerative disorders. Neurochem. Res. https://doi.org/10.1007/s11064-008-9710-0.

Chen, Y., Yuan, J., Jiang, G., Zhu, J., Zou, Y., Lv, Q., 2017. Lercanidipine attenuates angiotensin II-induced Cardiomyocyte hypertrophy by blocking calcineurin-NFAT3 and CaMKII-HDAC4 signaling. Mol. Med. Rep. https://doi.org/10.3892/mmr.2017.7211.

Ciani, L., Salinas, P.C., 2005. WNTs in the vertebrate nervous system: from patterning to neuronal connectivity. Nat. Rev. Neurosci. 6, 351–362. https://doi.org/10.1038/nrn1665.

Colpo, G.D., Furr Stimming, E., Teixeira, A.L., 2019. Stem cells in animal models of Huntington disease: a systematic review. Mol. Cell. Neurosci. https://doi.org/10.1016/j.mcn.2019.01.006.

El-Abhar, H., Abd El Fattah, M.A., Wadie, W., El-Tanbouly, D.M., 2018. Cilostazol disrupts TLR-4, Akt/GSK-3β/CREB, and IL-6/JAK-2/STAT-3/SOCS-3 crosstalk in a rat model of Huntington's disease. PLoS One. https://doi.org/10.1371/journal.pone.0203837.

Foraker, J.E., Oh, J.Y., Ylostalo, J.H., Lee, R.H., Watanabe, J., Prockop, D.J., 2011. Cross-talk between human mesenchymal stem/progenitor cells (MSCs) and rat hippocampal slices in LPS-stimulated cocultures: the MSCs are activated to secrete prostaglandin E2. J. Neurochem. https://doi.org/10.1111/j.1471-4159.2011.07511.x.

Gao, Y., Tang, J., Chen, W., Li, Q., Nie, J., Lin, F., Wu, Q., Chen, Z., Gao, Z., Fan, H., Tsun, A., Shen, J., Chen, G., Liu, Z., Lou, Z., Olsen, N.J., Zheng, S.G., Li, B., 2015. Inflammation negatively regulates FOXP3 and regulatory T-cell function via DBC1. Proc. Natl. Acad. Sci. https://doi.org/10.1073/pnas.1421463112.

Gögel, S., Gubernator, M., Minger, S.L., 2011. Progress and prospects: stem cells and neurological diseases. Gene Ther. 18, 1–6. https://doi.org/10.1038/gt.2010.130.

Golan, H., Levav, T., Mendelsohn, A., Huleihel, M., 2004. Involvement of tumor necrosis factor Alpha in hippocampal development and function. Cerebr. Cortex. https://doi.org/10.1093/cercor/bhg108.

Gupta, S., Sharma, U., Jagannathan, N.R., Gupta, Y.K., 2017. Neuroprotective effect of lercanidipine in middle cerebral artery occlusion model of stroke in rats. Exp. Neurol. 288, 25–37. https://doi.org/10.1016/j.expneurol.2016.10.014.

Herbette, L.G., Vecchiarelli, M., Leonardi, A., 2016. Lercanidipine: Short Plasma Half-Life, Long Duration of Action and High Cholesterol Tolerance. Updated Molecular Model to Rationalize its Pharmacokinetic Properties. Blood Presshttps://doi.org/10.1080/080370598438998.

Huang, T., Xie, Z., Wang, J., Li, M., Jing, N., Li, L., 2011. Nuclear factor of activated T cells (NFAT) proteins repress canonical Wnt signaling via its interaction with dishevelled (Dvl) protein and participate in regulating neural progenitor cell proliferation and differentiation. J. Biol. Chem. https://doi.org/10.1074/jbc.M111.251165.

Inestrosa, N.C., Montecinos-Oliva, C., Fuenzalida, M., 2012. Wnt signaling: role in alzheimer disease and schizophrenia. J. Neuroimmune Pharmacol. 7, 788–807. https://doi.org/10.1007/s11481-012-9417-5.

Inestrosa, N.C., Varela-nallar, L., 2014. Wnt signaling in the nervous system and in Alzheimer ' s disease. J. Mol. Cell Biol. 6, 64–74.

Jones, B.J., Roberts, D.J., 1968. The quantiative measurement of motor inco-ordination in naive mice using an acelerating rotarod. J. Pharm. Pharmacol. 20, 302–304.

Kean, et al., 2013. MSCs: Delivery routes and engraftment, cell-targeting strategies, and immune modulation. Stem Cells Int.

Keene, C.D., Rodrigues, C.M.P., Eich, T., Linehan-Stieers, C., Abt, A., Kren, B.T., Steer, C.J., Low, W.C., 2001. A bile acid protects against motor and cognitive deficits and reduces striatal degeneration in the 3-nitropropionic acid model of Huntington's disease. Exp. Neurol. https://doi.org/10.1006/exnr.2001.7755.

Khan, A., Jamwal, S., Bijjem, K.R.V., Prakash, A., Kumar, P., 2015. Neuroprotective effect of hemeoxygenase-1/glycogen synthase kinase-3β modulators in 3-nitropropionic acid-induced neurotoxicity in rats. Neuroscience. https://doi.org/10.1016/j.neuroscience.2014.12.018.

Kim, et al., 2015. In vivo near-infrared imaging for the tracking of systemically delivered mesenchymal stem cells: Tropism for brain tumors and biodistribution. Int. J. Nanomedicine.

Kipanyula, M.J., Kimaro, W.H., Etet, P.F.S., 2016. The emerging roles of the calcineurin-nuclear factor of activated T-lymphocytes pathway in nervous system functions and diseases. J. Aging Res. https://doi.org/10.1155/2016/5081021.

Klee, C.B., Krinks, M.H., 1978. Purification of cyclic 3′5′-nucleotide phosphodiesterase inhibitory protein by affinity chromatography on activator protein coupled to sepharose. Biochemistry. https://doi.org/10.1021/bi00594a017.

Kondo, T., Matsuoka, A.J., Shimomura, A., Koehler, K.R., Chan, R.J., Miller, J.M., Srour, E.F., Hashino, E., 2011. Wnt signaling promotes neuronal differentiation from mesenchymal stem cells through activation of Tlx3. Stem Cells. https://doi.org/10.1002/stem.624.

Kraner, S.D., Norris, C.M., 2018. Astrocyte activation and the calcineurin/NFAT pathway in cerebrovascular disease. Front. Aging Neurosci. https://doi.org/10.3389/fnagi.2018.00287.

Kumar, P., Kumar, A., 2009. Neuroprotective effect of cyclosporine and FK506 against 3-nitropropionic acid induced cognitive dysfunction and glutathione redox in rat: possible role of nitric oxide. Neurosci. Res. https://doi.org/10.1016/j.neures.2009.01.005.

Le Blanc, K., Mougiakakos, D., 2012. Multipotent mesenchymal stromal cells and the innate immune system. Nat. Rev. Immunol. https://doi.org/10.1038/nri3209.

Li, B., Zhang, H., Zeng, M., He, W., Li, M., Huang, X., Deng, D.Y.B., Wu, J., 2015. Bone marrow mesenchymal stem cells protect alveolar macrophages from lipopolysaccharide-induced apoptosis partially by inhibiting the Wnt/β-catenin pathway. Cell Biol. Int. https://doi.org/10.1002/cbin.10359.

Libro, R., Bramanti, P., Mazzon, E., 2016. The role of the Wnt canonical signaling in neurodegenerative diseases. Life Sci. https://doi.org/10.1016/j.lfs.2016.06.024.

Lindvall, O., Kokaia, Z., 2006. Stem cells for the treatment of neurological disorders. Nature 441, 1094–1096. https://doi.org/10.1038/nature04960.

Liu, J., Si, Z., Li, S., Huang, Z., He, Y., Zhang, T., Wang, A., 2018. The calcineurin inhibitor FK506 prevents cognitive impairment by inhibiting reactive astrogliosis in pilocarpine-induced status epilepticus rats. Front. Cell. Neurosci. https://doi.org/10.3389/fncel.2017.00428.

Liu, X.-J., Zhang, J.-F., Sun, B., Peng, H.-S., Kong, Q.-F., Bai, S.-S., Liu, Y.-M., Wang, G.-Y.,

Wang, J.-H., Li, H.-L., 2009. Reciprocal effect of mesenchymal stem cell on experimental autoimmune encephalomyelitis is mediated by transforming growth factor-β and interleukin-6. Clin. Exp. Immunol. 158, 37–44. https://doi.org/10.1111/j.1365-2249.2009.03995.x.

Lu, L., Barbi, J., Pan, F., 2017. The regulation of immune tolerance by FOXP3. Nat. Rev. Immunol. https://doi.org/10.1038/nri.2017.75.

Ludolph, A.C.C., he, F., Spencer, P.S.S., Hammerstad, J., Sabri, M., 1991. 3-Nitropropionic acid - exogenous animal neurotoxin and possible human striatal toxin. Can. J. Neurol. Sci./J. Can. des Sci. Neurol. 18, 492–498. https://doi.org/10.1017/S0317167100032212.

Massicotte, V.S., Frara, N., Harris, M.Y., Amin, M., Wade, C.K., Popoff, S.N., Barbe, M.F., 2015. Prolonged performance of a high repetition low force task induces bone adaptation in young adult rats, but loss in mature rats. Exp. Gerontol. https://doi.org/10.1016/j.exger.2015.10.014.

Mignone, J.L., Murry, C.E., 2011. A repair “kit” for the infarcted heart. Cell Stem Cell. https://doi.org/10.1016/j.stem.2011.03.005.

Mohamed, S.S., Ahmed, L.A., Attia, W.A., Khattab, M.M., 2015. Nicorandil enhances the efficacy of mesenchymal stem cell therapy in isoproterenol-induced heart failure in rats. Biochem. Pharmacol. 98, 403–411. https://doi.org/10.1016/j.bcp.2015.10.004.

Moreira, E.L.G., Rial, D., Duarte, F.S., De Carvalho, C.R., Horst, H., Pizzolatti, M.G., Prediger, R.D.S., Ribeiro-Do-Valle, R.M., 2010. Central nervous system activity of the proanthocyanidin-rich fraction obtained from Croton celtidifolius in rats. J. Pharm. Pharmacol. 62, 1061–1068. https://doi.org/10.1111/j.2042-7158.2010.01124.x.

Mukhamedshina, Y., Gracheva, O., Mukhutdinova, D., Chelyshev, Y., Rizvanov, A., 2019. Mesenchymal stem cells and the neuronal microenvironment in the area of spinal cord injury. Neural Regen. Res. https://doi.org/10.4103/1673-5374.244778.

Mukherjee, A., Soto, C., 2011. Role of calcineurin in neurodegeneration produced by misfolded proteins and endoplasmic reticulum stress. Curr. Opin. Cell Biol. https://doi.org/10.1016/j.ceb.2010.12.006.

Nagahara, A.H., Tuszynski, M.H., 2011. Potential Therapeutic Uses of BDNF in Neurological and Psychiatric Disorders. https://doi.org/10.1038/nrd3366.

Norris, C.M., Kadish, I., Blalock, E.M., Chen, K.-C., Thibault, V., Porter, N.M., Landfield, P.W., Kraner, S.D., 2005. Calcineurin triggers reactive/inflammatory processes in astrocytes and is upregulated in aging and alzheimer's models. J. Neurosci. 25, 4649–4658. https://doi.org/10.1523/JNEUROSCI.0365-05.2005.

Oh, S.H.e., Kim, H.N.a., Park, H.J., Shin, J.Y. oun, Lee, P.H.y., 2015. Mesenchymal stem cells increase hippocampal neurogenesis and neuronal differentiation by enhancing the Wnt signaling pathway in an alzheimer's disease model. Cell Transplant. https://doi.org/10.3727/096368914X679237.

Pineda, J.R., Pardo, R., Zala, D., Yu, H., Humbert, S., Saudou, F., 2009. Genetic and pharmacological inhibition of calcineurin corrects the BDNF transport defect in Huntington's disease. Mol. Brain. https://doi.org/10.1186/1756-6606-2-33.

Prockop, D.J., Oh, J.Y., 2012. Medical therapies with adult stem/progenitor cells (MSCs): a backward journey from dramatic results in vivo to the cellular and molecular explanations. J. Cell. Biochem. https://doi.org/10.1002/jcb.24046.

Purro, S.A., Galli, S., Salinas, P.C., 2014. Dysfunction of Wnt signaling and synaptic disassembly in neurodegenerative diseases. J. Mol. Cell Biol. https://doi.org/10.1093/jmcb/mjt049.

Quist, A., Doudevski, I., Lin, H., Azimova, R., Ng, D., Frangione, B., Kagan, B., Ghiso, J., Lal, R., 2005. Amyloid ion channels: a common structural link for protein-misfolding disease. Proc. Natl. Acad. Sci. 102, 10427–10432. https://doi.org/10.1073/pnas.0502066102.

Rojanathammanee, L., Floden, A.M., Manocha, G.D., Combs, C.K., 2015. Attenuation of microglial activation in a mouse model of Alzheimer's disease via NFAT inhibition. J. Neuroinflammation. https://doi.org/10.1186/s12974-015-0255-2.

Schwartz, N., Schohl, A., Ruthazer, E.S., 2009. Neural activity regulates synaptic properties and dendritic structure in vivo through calcineurin/NFAT signaling. Neuron 62, 655–669. https://doi.org/10.1016/j.neuron.2009.05.007.

Selvaraj, N., Adhimoolam, M., Perumal, D.K., Rajamohammed, M.A., 2015. Neuroprotective effect of lercanidipine- a novel calcium channel blocker in albino mice. J. Clin. Diagn. Res. 9, FF01–5. https://doi.org/10.7860/JCDR/2015/14085.6801.

Shalaby, H.N., El-Tanbouly, D.M., Zaki, H.F., 2018. Topiramate mitigates 3-nitropropionic acid-induced striatal neurotoxicity via modulation of AMPA receptors. Food Chem. Toxicol. https://doi.org/10.1016/j.fct.2018.05.022.

Silva-Alvarez, C., Arrázola, M.S., Godoy, J.A., Ordenes, D., Inestrosa, N.C., 2013. Canonical Wnt signaling protects hippocampal neurons from Aβ oligomers: role of non-canonical Wnt-5a/Ca(2+) in mitochondrial dynamics. Front. Cell. Neurosci. 7, 97. https://doi.org/10.3389/fncel.2013.00097.

Sompol, P., Norris, C.M., 2018. Ca2+, astrocyte activation and calcineurin/NFAT signaling in age-related neurodegenerative diseases. Front. Aging Neurosci. https://doi.org/10.3389/fnagi.2018.00199.

Subramanian, K.S., 1995. Storage and preservation of blood and urine for trace element analysis - a review. Biol. Trace Elem. Res. https://doi.org/10.1007/BF02788968.

Tao, H., Han, Z., Han, Z.C., Li, Z., 2015. Proangiogenic features of mesenchymal stem cells and their therapeutic applications. Stem Cell. Int. 1–11. https://doi.org/10.1155/2016/1314709. 2016.

Teixeira, F.G., Carvalho, M.M., Panchalingam, K.M., Rodrigues, A.J., Mendes-Pinheiro, B., Anjo, S., Manadas, B., Behie, L.A., Sousa, N., Salgado, A.J., 2017. Impact of the secretome of human mesenchymal stem cells on brain structure and animal behavior in a rat model of Parkinson's disease. Stem Cells Transl. Med. https://doi.org/10.5966/sctm.2016-0071.

Túnez, I., Tasset, I., La Cruz, V.P. De, Santamaría, A., 2010. 3-nitropropionic acid as a tool to study the mechanisms involved in huntington's disease: past, present and future. Molecules. https://doi.org/10.3390/molecules15020878.

Uccelli, A., Laroni, A., Freedman, M.S., 2011. Mesenchymal stem cells for the treatment of multiple sclerosis and other neurological diseases. Lancet Neurol. 10, 649–656. https://doi.org/10.1016/S1474-4422(11)70121-1.

Vaeth, M., Schliesser, U., Muller, G., Reissig, S., Satoh, K., Tuettenberg, A., Jonuleit, H., Waisman, A., Muller, M.R., Serfling, E., Sawitzki, B.S., Berberich-Siebelt, F., 2012. Dependence on nuclear factor of activated T-cells (NFAT) levels discriminates conventional T cells from Foxp3+ regulatory T cells. Proc. Natl. Acad. Sci. https://doi.org/10.1073/pnas.1203870109.

Wirtz, S., Herzig, S., 2004. Molecular mechanisms of vasoselectivity of the 1,4-dihydropyridine lercanidipine. Br. J. Pharmacol. 142, 275–284. https://doi.org/10.1038/sj.bjp.0705786.

Woodruff, T.M., Crane, J.W., Proctor, L.M., Buller, K.M., Shek, A.B., de Vos, K., Pollitt, S., Williams, H.M., Shiels, I.A., Monk, P.N., Taylor, S.M., 2006. Therapeutic activity of C5a receptor antagonists in a rat model of neurodegeneration. FASEB J. 20, 1407–1417. https://doi.org/10.1096/fj.05-5814com.

Wu, H., Hudry, E., Hashimoto, T., Kuchibhotla, K., Fan, Z., Spires-jones, T., Xie, H., Arbel-ornath, M., Cynthia, L., Bacskai, B.J., Hyman, B.T., 2010. NIH public access. Amyloid 30, 2636–2649. https://doi.org/10.1523/JNEUROSCI.4456-09.2010.

Xifró, X., García-Martínez, J.M., Del Toro, D., Alberch, J., Pérez-Navarro, E., 2008. Calcineurin is involved in the early activation of NMDA-mediated cell death in mutant huntingtin knock-in striatal cells. J. Neurochem. https://doi.org/10.1111/j.1471-4159.2008.05252.x.

Xu, M., UemAmyloidura, R., Dai, Y., Wang, Y., Pasha, Z., Ashraf, M., 2007. In vitro and in vivo effects of bone marrow stem cells on cardiac structure and function. J. Mol. Cell. Cardiol. https://doi.org/10.1016/j.yjmcc.2006.10.009.

Yeh, J.L., Hsu, J.H., Liang, J.C., Chen, I.J., Liou, S.F., 2013. Lercanidipine and labedipinedilol-A attenuate lipopolysaccharide/interferon-γ-induced inflammation in rat vascular smooth muscle cells through inhibition of HMGB1 release and MMP-2, 9 activities. Atherosclerosis. https://doi.org/10.1016/j.atherosclerosis.2012.12.005.