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Synergistic Effects of Mesenchymal Stem Cell Transplantation and Repetitive Transcranial Magnetic Stimulation on Promoting Autophagy and Synaptic Plasticity in Vascular Dementia

Fei Wang, PhD,[1] Chi Zhang, MS,[1] Siyuan Hou, BS,[2,3] and Xin Geng, PhD[2,3]

[1]Department of Neurology, General Hospital, [2]Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, and [3]Key Laboratory of Immune Microenvironment and Disease (Ministry of Education), Tianjin Medical University, China.

Address correspondence to: Xin Geng, PhD, Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Tianjin Medical University, Tianjin 300070, China. E-mail: gengx@tmu.edu.cn

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Abstract

Repetitive transcranial magnetic stimulation (rTMS) and mesenchymal stem cells (MSCs) transplantation both showed therapeutic effects on cognition impairment in vascular dementia (VD) model rats. However, whether these two therapies have synergistic effects and the molecular mechanisms remain unclear. In our present study, rats were randomly divided into six groups: control group, sham operation group, VD group, MSC group, rTMS group, and MSC+rTMS group. The VD model rats were prepared using a modified 2VO method. rTMS treatment was implemented at a frequency of 5 Hz, the stimulation intensity for 0.5 Tesla, 20 strings every day with 10 pulses per string and six treatment courses. The results of the Morris water maze test showed that the learning and memory abilities of the MSC group, rTMS group, and MSC+rTMS group were better than that of the VD group, and the MSC+rTMS group showed the most significant effect. The protein expression levels of brain-derived neurotrophic factor, NR1, LC3-II, and Beclin-1 were the highest and p62 protein was the lowest in the MSC+rTMS group. Our findings demonstrated that rTMS could further enhance the effect of MSC transplantation on VD rats and provided an important basis for the combined application of MSC transplantation and rTMS to treat VD or other neurological diseases.

Keywords: Vascular dementia, Repetitive transcranial magnetic stimulation, Brain-derived neurotrophic factor, Autophagy, Marrow mesenchymal stem cells

Vascular dementia (VD) is a chronic cerebrovascular syndrome caused by different etiologies, with vascular brain tissue damage being the main pathological manifestation (1). The symptoms of VD mainly include cognitive and behavioral disorders, which gradually worsen with the progression of the disease (2). Currently, VD is the second leading cause of dementia, second only to Alzheimer's disease. According to the different parts of the lesion, the pathological changes in VD are various. Brain damage caused by ischemia, such as multiple cortical and subcortical infarcts, is considered the main cause of VD (3,4). The pathogenic mechanisms of VD remain unclear and include damage of the cholinergic system, the toxicity effect of excitatory amino acids, neuroinflammation, the toxicity of free radicals, synaptic plasticity, and genetic alterations (5,6). The definite and effective treatments for VD remain in the stage of exploration (7).

Repetitive transcranial magnetic stimulation (rTMS) is a non-invasive technique that uses magnetic fields to act on the central nervous system based on the principle of rapid electromagnetic conversion (8). It causes the nerve cells of the cerebral cortex and subcortex to produce induced currents that affect the metabolic processes and electrical activity of nerve cells, thereby causing a series of physiological and biochemical effects (Figure 1). rTMS has been applied in many fields of neuroscience (9–11). rTMS cannot only affect the function of stimulating local and functional distant sites but also achieve regional reorganization of cortical function, producing subsequent biological effects. The stimulus is still present when the neurons are in the refractory period, generating the sum of excitatory postsynaptic potentials. Selecting different stimulation parameters will produce different biological effects (12,13). In

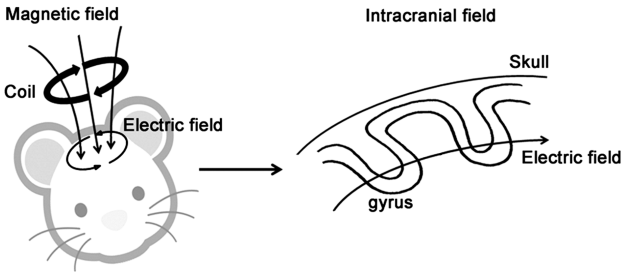

Figure 1. Basic principle of repetitive transcranial magnetic stimulation (rTMS). The rapidly changed current drives the coil to generate a time-varying magnetic field, which generates the induced electric field that has the opposite direction to the current inflowing the coil.

addition, rTMS can intervene in various electrostatic processes, such as phosphate transfer, ligand–receptor interactions, protein–protein interactions, and protein–nucleic acid interactions (14).

Bone marrow mesenchymal stem cells (MSCs) have multipotent properties and the ability to self-renew. The most common differentiation of MSCs is through the mesoderm as muscle, bone, cartilage, fat, and nonmesoderm tissue, including endothelial cells and nerve cells (15). MSCs release signals that regulate the immune response of the host and have the potential to enhance endogenous tissue repair by the release of nutritional factors (16). Compared with embryonic stem cells and neural stem cells, MSCs have the following advantages: autologous transplantation can be performed to avoid immune rejection; vein transplantation can be used to avoid more complications; the tissue sources are abundant and easy to culture; and ethical issues are not involved (17,18). In recent years, there have been some advances in the study of MSCs in nervous system diseases, but their mechanisms of action remain unclear. MSCs can differentiate into neuron-like cells and glial cells after they migrate to brain tissue, which is thought to be the main mechanism for MSCs (19,20). MSCs can secrete brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF), and cytokines and can reduce the number of apoptotic neural cells by secreting neurotrophic factors that downregulate the expression of Bax protein and upregulate the expression of Bcl-2 protein, thereby playing a neuroprotective role (21–25).

Autophagy is a unique phenomenon in eukaryotes. It degrades cytoplasmic components in lysosomes through the physiological and pathological processes (26). Macroautophagy, microautophagy, and chaperone-mediated autophagy are the three forms of autophagy (27–29). Autophagy plays an important role in starvation, microbial clearance, antigen presentation, cell death, development, aging, and tumor suppression (30,31). Two commonly used autophagy markers are LC3/Atg8 (microtubule-associated protein 1 light chain 3) and Beclin-1 (32). LC3-I and LC3-II are two forms of LC3 protein in cells. The intracellularly newly synthesized LC3 has its C-terminus cleaved by Atg4 protease and becomes a cytoplasmic soluble form of LC3-I. After autophagosomes are formed, LC3-I, modified by ubiquitination processing and coupled with phosphatidylethanolamine on the surface of the autophagosomal membrane, becomes a membrane-bound form of LC3-II and localizes to the inner and outer membranes of autophagosomes. Unlike other Atg proteins located on the membrane of autophagosomes, LC3-II remains stable on the autophagosome membrane until it fuses with lysosomes; therefore, it is used as a marker molecule for autophagosomes. The content of LC3-II or ratio of LC3-II/LC3-I is positively correlated with the number of autophagosomes, reflecting the autophagic activity of the cells to some extent (33–35). Beclin-1 is a homolog of the yeast autophagy-related gene ATG6 and is a specific gene involved in mammalian autophagy (36). Beclin-1 is involved in the formation of autophagosomes through the formation of complexes with type III phosphatidylinositol 3-phosphate kinase (PI3K) and regulates autophagic activity. Detecting the changes in the expression of LC3-II and Beclin-1 can reflect the changes in autophagy (37,38). The polyubiquitin-binding protein p62 (SQSTM1) is a receptor for cargo destined to be degraded by autophagy and includes ubiquitinated protein aggregates destined for clearance. p62 can bind to ubiquitin and LC3, thereby targeting the autophagosome and facilitating clearance of ubiquitinated proteins (39,40). Because p62 is degraded by autophagy, there is a general correlation between the inhibition of autophagy and increased levels of p62. Obviously, p62 can be used as another autophagic marker (41,42), functioning as a hub for intracellular signaling such as that in the mammalian target of rapamycin (mTOR) pathway (43).

Changes in the plasticity of the synaptic structure and function are the bases for the treatment of neurological diseases, and increasing the plasticity of the synaptic structure and function is one of the efforts of nervous system diseases (44,45). Our previous study found that rTMS and MSCs separately exert therapeutic effects on VD rats, but whether the two therapies have synergistic effects and the molecular mechanisms remain unclear (25,46,47). The aim of our present study is to explore the synergistic effects of rTMS and MSCs on VD rats and clarify the molecular mechanisms. Our hypothesis is that rTMS combined MSCs has the synergistic effects on improving the autophagy and synaptic plasticity on VD rats.

Materials and Methods

Animal Grouping and Generation of the VD Rat Models

This study was performed in strict accordance with the recommendations in the guidelines issued by the National Institutes of Health for care of laboratory animals. All experimental protocols were approved by the Committee on the Ethics of Animal Experiments of Tianjin Medical University. All surgery was performed under sodium pentobarbital anesthesia and all efforts were made to minimize suffering. Sixty healthy male Wistar rats weighing 260 ± 40 g were selected and provided by the Experimental Animal Center of the Academy of Military Medical Sciences. Rats were fed with sufficient water and feed daily. The light and darkness alternated for 12 hours, and the room temperature was maintained at 25 ± 1°C. The rats were randomly divided into the following six groups: normal control group, sham operation group, VD group, MSC group, rTMS group, and MSC+ rTMS group. In the VD group, MSC group, rTMS group, and MSC+rTMS group, the VD rats were prepared using a modified 2VO method. The rats were fasted for 8 hours before surgery and water deprived for 4 hours, and 10% chloral hydrate (3 mL/kg body weight) was intraperitoneally injected. The rats were positioned supine and fixed, and ventral midline neck incisions were performed. The VD model rats were prepared using a modified bilateral vessel occlusion method (2VO) referring to the published literatures (48,49). The common carotid artery was bilaterally detached, and the right common carotid artery was repeatedly clamped. The artery was clamped for 10 minutes and then reopened for 10 minutes. This was repeated three times, and the artery was ligated and cutoff. One week later, the other common carotid artery was treated in the same manner, and the skin was sutured. Intraoperative care was taken to prevent overstimulation or injury to the vagus nerve.

Morris Water Maze Test

The Morris water maze test was used to assess the cognitive function of rats. (i) Positioning navigation test: After 5 days of continuous training, the rats were allowed to swim freely for 2 minutes in a circular pool without a platform on the first day to familiarize themselves with the environment. The swimming speeds were recorded, eliminating the rats with poor swimming ability. From the second day, the rats were placed into the water maze from the four quadrants (I, II, III, and IV) of the pool. The rats' heads were all facing the same side, and the time in which the rats were in the water and climbed onto the platform quadrant (the third quadrant) was recorded, called the escape latency. Two training sessions (at 9 am and 5 pm) were tested daily, and the training interval was 8 hours. Each session was performed four times for 4 days. The swimming distance was from the beginning of the experiment to the end of the experiment. If the rats did not find the platform within 2 minutes, they were guided to the platform and stayed for 30 seconds to become familiar with the environment and platform position, and the escape latency was counted as 2 minutes. (ii) Space exploration test: After the positioning navigation test was completed, the space exploration test was conducted on the next day. When the platform was removed, the rats were put into the water facing the pool, and the same entry points were selected in the third quadrant. The number of times crossing the original quadrant platform was recorded in 2 minutes. The swimming time in the third quadrant and percentage of the swimming distances in the third quadrant of the whole distances was calculated, determining the abilities of spatial cognition and memory storage. The criteria for judging the VD model were as follows: the mean value of the escape latency of the rats in the sham-operated group was taken as a reference value on the fifth day, and then the average escape latency time of the rats in the VD group was obtained on the fifth day. After subtracting the reference value, the difference was divided by the average escape latency time. Next, a corresponding ratio was obtained. The ratio of the successful VD model should be greater than 20%.

Isolation, Identification, and Transplantation of Rat MSCs

The tibia and femur of the rat hind limb were aseptically separated to ensure the bone integrity. The separated tibia and femur were wiped off with sterile gauze to remove the adhered tissue and were soaked in Dulbecco's modified Eagle's medium (DMEM) with antibiotics. The bone marrow cavity was washed repeatedly with DMEM containing 10% fetal bovine serum, and the cell suspension was collected in a sterile centrifuge tube, centrifuged at 1,000 r/min for 10 minutes at 4°C and incubated in a 37°C/5% CO_2 incubator. MSCs were passaged to the fifth generation, 0.25% trypsinized to collect cells, and washed with phosphate-buffered saline (PBS), and then approximately 1×10^6 cells were added to 100 µL of PBS, and the appropriate fluorescein isothiocyanate -labeled CD29, CD31, CD34, CD44, CD45, and CD90 antibodies were added. Next, the cells were placed in the dark at room temperature for 30 minutes, washed with PBS, resuspended in 500 µL of PBS to make a single-cell suspension, and analyzed by flow cytometry. After 10 weeks of in vitro culture, MSCs were inoculated into six-well plates. When the adherent cells grew to 80% confluency, they were preincubated with 10% fetal bovine serum, 10 ng/mL basic fibroblast growth factor, and L-DMEM for 24 hours and washed with PBS three times. They were then induced with 200 mmol/L butylated hydroxyanisole, 2% dimethyl sulfoxide, and L-DMEM for 6 hours. According to the methods in our previous study (25), the telomerase reverse transcriptase (TERT) gene was transfected into MSCs using the pLXSN retroviral vector. Reverse transcriptase polymerase chain reaction (RT-PCR) was used to detect the expression of TERT mRNA in MSCs. Telomerase repeated amplification protocol was used to detect telomerase activity. MSCs with a concentration of 4×10^6/mL in a 1-mL volume were injected into the MSC group and MSC+rTMS rat groups via the tail vein.

Parameter Settings of rTMS

The Pro R30-type magnetic stimulator and a "figure-8-shaped" coil manufactured by Danish Medtronic Company were used in the rTMS treatment. The internal diameter of the coil was 1 cm and outer diameter was 8 cm. The "figure-8-shaped" coil was centered on the central point of the rat's sagittal suture. The coil surface was in close contact with the scalp and parallel to the rat parietal bone. The parameter settings were decided and adopted according to the previously published papers of our lab (46,47,50) and other groups (51,52). In the published papers of our lab and other groups, the figure-of-eight coils rather than dedicated TMS coils were used for rat models. Therefore, in the present study, we still used the figure-of-eight coil in our experiments. The stimulation frequency was 5 Hz and the stimulation intensity was 0.5 Tesla. There were 20 strings of stimulation every day with 10 pulses per string, and the stimulation interval per pulse was 2–3 seconds. Six courses were implemented in the rTMS treatment, each course lasting for 5 consecutive days, and the interval was 2 days.

Immunohistochemical Analysis of BDNF and NR1 Protein

The brain tissues were fixed in 4% paraformaldehyde. After conventional gradient alcohol dehydration and paraffin embedding, 4-µm-thick continuous coronal slices were fixed on poly-L-lysine-treated clean slides and were then oven baked at 60°C for 60 minutes to prevent it from coming off. The standard streptavidin-peroxidase method and diaminobenzidine color were used to detect the expression of BDNF and NR1 protein. The Leica Mias 2000 image analysis system was used to observe the results of immunohistochemical staining. BioMias image analysis software was used to measure the average grayscale value of immune products in the hippocampal CA1 region. Each rat was assayed for three sections, and each section was assayed for five visual fields in the hippocampus CA1 regions. Next, the data of the average grayscale value was analyzed.

RT-PCR Detection

Trizol was used to extract RNA from the hippocampal CA1 region of rats in each group. RT-PCR was used to detect the expression of BDNF and NR1 mRNA. Glyceraldehyde 3-phosphate dehydrogenase was used as the internal control. The total volume of the system was 20 µL containing 2 µL of cDNA, 0.5 µmol/L primer and SYBR Green I. The reaction conditions were as follows: 94°C for 30 seconds, 59°C for 30 seconds, 72°C for 30 seconds, for 30 cycles. The PCR products were subjected to 1% agarose gel electrophoresis. The BDNF primer sequences were as follows: 5′-GTG ACA GTA GCG AGT GGG-3′ and 5′-TAT CCT TAT GAA CCG CCA GCA-3′, and the primer sequences of NR1 were as follows: 5′-AAGGTGCACGCCTTTATCTG-3′ and 5′-TTCTCATGGGACTTGAGTATGGA-3′. The internal reference GAPDH primer sequences were as follows: 5′-CCTGGAGAAACCTGCCAAG-3′ and 5′-CACAGGAGACAA CCTGGTCC-3′.

Western Blotting Analysis

The brain tissues of the hippocampal CA1 region were taken in each group, and 1 mL of total protein extraction reagent with protease inhibitor was added and homogenized after extraction of total protein. The sample concentration was determined by BCA protein quantitation. The proteins were separated by denaturing polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes overnight at 4°C at 30 mA. The membranes were then incubated for 1 hour at room temperature in 5% nonfat dry milk solution to block nonspecific binding on the membrane. Antibodies against BDNF, NR1, P62, LC3, and Beclin-1 were incubated for 1.5 hours. Horseradish peroxidase-labeled secondary antibody was added and incubated for 1 hour at room temperature. Chemiluminescence was used to detect the proteins. The gray values of the target proteins were divided by the gray value of the internal reference actin for error correction. The obtained results were the relative expression of the target proteins.

Transmission Electron Microscope Analysis

According to the Pellegrino rat brain stereotaxic map, the brain tissues of the hippocampal CA1 region of each group were rapidly excised. The tissues were washed with the PBS solution three times, were immediately placed into 2% glutaraldehyde fixation solution, and were fixed with 1% citric acid for 1 hour. They were dehydrated with acetone and fixed and were embedded with epoxy resin Epon 812. An ultrathin microtome was used for sectioning. The samples were stained with 20 g/L of uranyl acetate and lead citrate, and the synapse structures were observed using the transmission electron microscope (JEM-1010).

Bioinformatics Analysis of the Interacting Proteins of the BDNF-p62-mTOR Network

The STRING database (http://string-db.org/) was used to query the interacting proteins of BDNF, p62 (SQSTM1), and mTOR protein, and Cytoscape software was used to analyze the protein interaction network to explore the relationship between autophagy-related proteins and neurotrophic factors. The STRING database can be used to annotate the structure, function, and evolutionary properties of proteins. It can also explore and predict interaction networks, provide new research directions for future experiments, and provide efficient mapping of cross-species predictions. Cytoscape is the software that graphically displays, analyzes, and edits the networks of genes or proteins. The GeneMANIA, DAVID, KEGG, and GO databases were then used to analyze the signaling pathways and functions of the interacting proteins of BDNF, p62, and mTOR.

Statistical Analysis

All data were represented as the means ± *SD* ($\bar{x}$ ± s), analyzed by SPSS 19.0 software. Multiple groups were compared using one-way analysis of variance analysis, Student's *t* test was used for multiple comparisons, and $p < .05$ was considered statistically significant.

Results

MSC Transplantation and rTMS Have a Synergistic Effect on the Improvement of Learning and Memory Abilities

In the positioning navigation test, the average escape latency and swimming distance in the MSC group, rTMS group, and MSC+rTMS group were shorter than those in the VD group (Figure 2A and B). In the space exploration test, the swimming time in the quadrant of the original platform, the number of times crossing the platforms within 120 seconds, and percentage of the total distance through the original platform quadrant in the MSC group, rTMS group, and the MSC+rTMS group were increased compared with those in the VD group (Figure 2C,E, and F), while the time of crossing the platform for the first time was shortened compared with that in the VD group (Figure 2D). These findings demonstrated that separate and combined application of MSC transplantation and rTMS both improved the learning and memory abilities of VD rats. The improvement was more significant in the MSC+rTMS group than in the MSC group and rTMS group alone, and the combined application of MSCs transplantation and rTMS had a synergistic effect.

The mRNA and Protein Expression Levels of BDNF and NR1 Are Increased Significantly by the Treatment of MSC Transplantation Combined With rTMS

The results of RT-PCR and Western blotting showed that the mRNA and protein expression levels of BDNF and NR1 in the hippocampus CA1 region in the MSC group, rTMS group, and MSC+rTMS group were higher than those in the VD group, and they were highest in the MSC+rTMS group. The difference was statistically significant (Figure 3A–F).

The results of immunohistochemistry analysis showed that BDNF-positive cells were stained in the hippocampus and cortex in

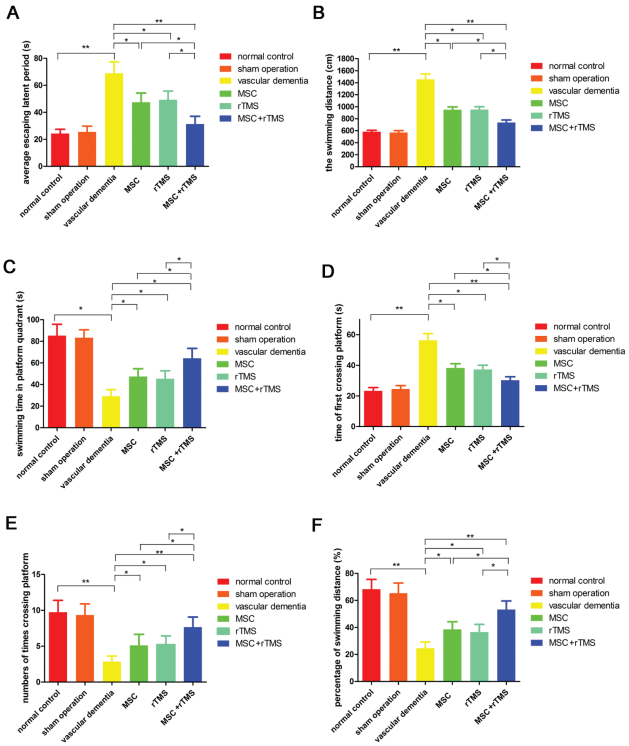

Figure 2. Results of the Morris water maze test in six groups. In the positioning navigation test, the average escape latency and swimming distance are shown in **A** and **B**. In the space exploration test, the swimming time in the quadrant of the original platform, number of times crossing the platforms within 120 seconds and percentage of the total distance through the original platform quadrant are shown in **C**, **E**, and **F**. The time of crossing the platform for the first time is shown in **D**. *$p < .05$, **$p < .01$.

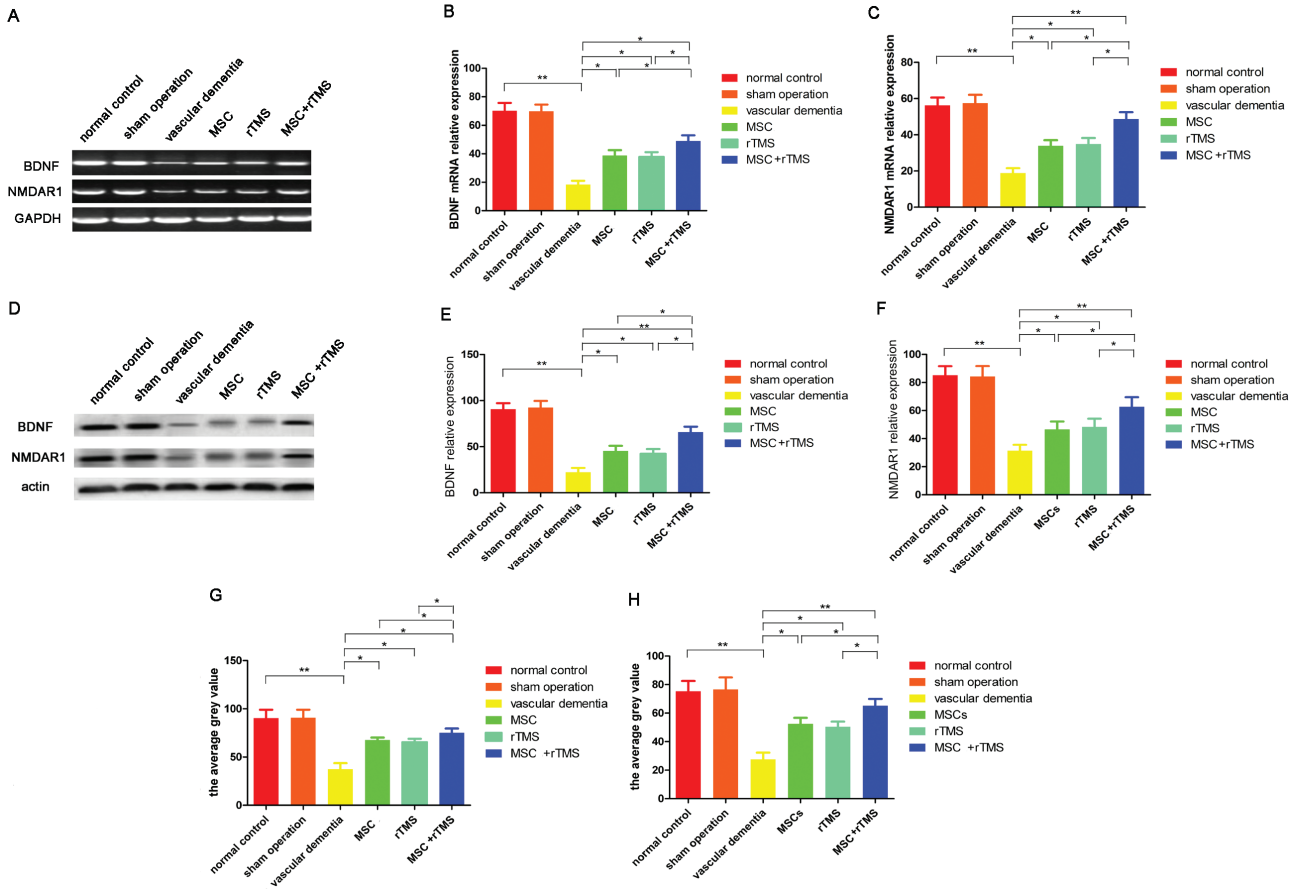

Figure 3. Results of the mRNA and protein expression levels of BDNF and NR1 in the hippocampus CA1 region in the six groups. The results of RT-PCR analysis are shown in **A–C**. The results of Western blotting analysis are shown in **D–F**. The results of the immunohistochemistry analysis are shown in **G** and **H**. *$p < .05$, **$p < .01$. BDNF = Brain-derived neurotrophic factor; RT-PCR = Reverse transcriptase polymerase chain reaction.

the cytoplasm in all groups. The NR1 protein showed strong staining in the pyramidal cell membrane and pale staining in the cytoplasm in the hippocampal CA1 region. The staining in the radiography and lacunar molecular layer was as strong as that in the pyramidal cell membrane. The average gray values of BDNF- and NR1-positive cells in the MSC group, rTMS group, and MSC+rTMS group were higher than those in the VD group, and they increased more significantly in the MSC+rTMS group than in the MSC group and rTMS group. The difference was statistically significant (Figure 3G and H).

Combined Treatment of MSC Transplantation and rTMS Has a Synergistic Effect on the Improvement of Autophagic Levels

Western blotting results showed that the levels of LC3-II and Beclin-1 in the MSC group, rTMS group, and MSC+rTMS group were higher than those in the VD group, and the levels were significantly highest in the MSC+rTMS group. The expression level of p62 protein in the MSC group, rTMS group, and MSC+rTMS group was lowest in the MSC+rTMS group. This indicated that the autophagic levels of the MSC group, rTMS group, and MSC+rTMS group were increased, and the autophagic level of the MSC+rTMS group was increased more significantly (Figure 4A–D).

Changes in the Ultrastructure of the Hippocampal CA1 Regions

Under transmission electron microscope, the synapses of the hippocampal CA1 region in the VD group showed long or irregular shapes. The presynaptic areas were significantly deformed, and mitochondria were condensed. The synaptic vesicles and volumes of the postsynaptic areas were reduced. The presynaptic and postsynaptic membranes were thickened, and the synaptic gaps were disappeared. In the MSC group, rTMS group, and MSC+rTMS group, the synaptic morphology was more regular than that in the VD group. The synaptic volumes were increased, indicating that the synaptic damage was restored. The distribution of vesicles in the presynaptic area was uniform, the synaptic cleft was visible, and the degree of mitochondrial agglomeration was significantly reduced in the MSC+rTMS group, indicating that the MSC+rTMS group was better than the MSC group and rTMS group (data was not shown). The results of the changes in the width of the synaptic cleft, thickness of postsynaptic density, length of the synaptic active zone, and synaptic curvature are shown in Figure 5A–D. These findings demonstrated that the combined treatment of MSC transplantation and rTMS has a more pronounced recovery effect on the treatment of damaged synapses than MSC transplantation and rTMS treatment alone.

BDNF and Autophagic Proteins Share Some Common Pathways and Similar Functions

The BDNF-P62-mTOR interaction network was analyzed by STRING and Cytoscape software (Figure 6). Twenty-seven common proteins were found in the BDNF-P62-mTOR network: AKT1, ATG5, ATG7, ATG12, BECN1, CDC42, CHUK, GABARAP, GABARAPL1, GABARAPL2, HTT, IKBKB, LCK, MAP1LC3A, MAP1LC3B, MAP3K3, NFKB1, NFKBIA, NGF, NGFR, PARK2, PIK3R1, RELA,

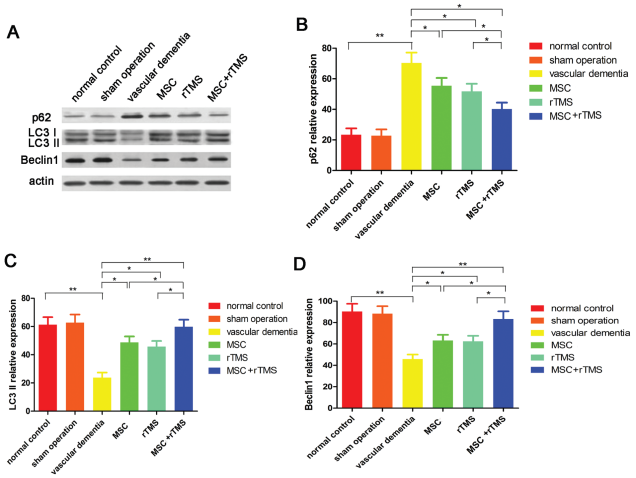

Figure 4. Protein expression levels of autophagy-related proteins p62, LC3-II, and Beclin-1. (**A**) Western blotting results of p62, LC3-II, and Beclin-1; (**B–D**) relative expression levels of proteins p62, LC3-II, and Beclin-1. $*p < .05$, $**p < .01$.

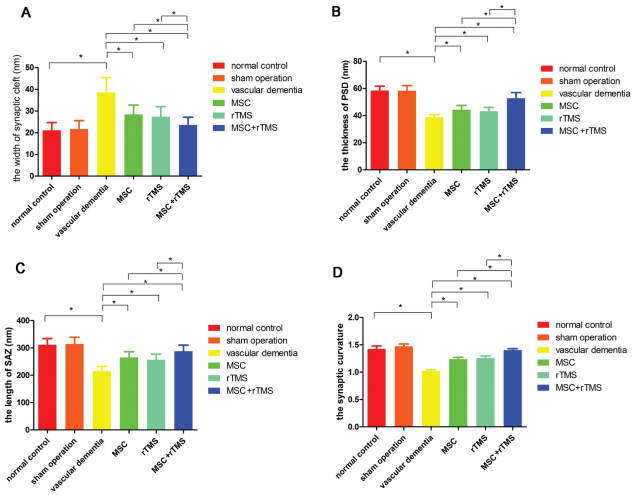

Figure 5. The results of changes in the ultrastructure in the hippocampal CA1 region. (**A**) Width of the synaptic cleft; (**B**) Thickness of PSD; (**C**) Length of SAZ; (**D**) Synaptic curvature. PSD = Postsynaptic density; SAZ = Synaptic active zone.

RPTOR, TRAF2, TRAF6, and ULK1 (listed in Table 1). They might be the bridges between neurotrophic factors and autophagy-related proteins. The pathways and functions of these common proteins were further analyzed by searching the GeneMANIA, DAVID, KEGG, and GO databases. The related diseases of these common proteins included type-2 diabetes, HIV, and Alzheimer's disease. The common pathways of these proteins included the neurotrophin pathway, osteoclast differentiation, and MAPK signaling pathway. The biological processes included macroautophagic, negative regulation of apoptosis, and positive regulation of transcription, and the molecular functions included protein binding, ubiquitin protein ligase binding, and identical protein binding. The cellular components of these common proteins included the cytosol, cytoplasm, autophagosome, and mitochondria (Figure 7A–F).

Discussion

Our previous study showed that rTMS plays an important and beneficial role in the restoration treatment of VD that may be related

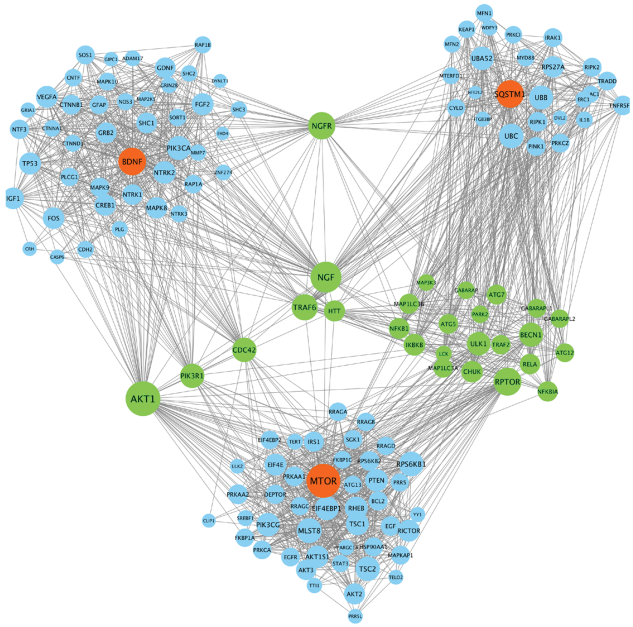

Figure 6. Interaction proteins in the BDNF-P62-mTOR network are analyzed by STRING and Cytoscape software. The 27 common proteins are shown in green.

to increasing the mRNA and protein expressions levels of BDNF, NR1, and synapsin (SYN) and affecting the synaptic plasticity in the hippocampal CA1 region (46,47). In our present study, we found that the mRNA and protein expression levels of BDNF and NR1 were increased significantly by MSC transplantation combined with rTMS. The Morris water maze test showed that rTMS combined with MSC transplantation could significantly increase the learning and memory abilities of VD rats, and the effect was superior to MSCs and rTMS alone. Therefore, rTMS combined with MSC transplantation showed the synergetic effect on the recovery of cognitive function of VD rats.

Our findings also showed that the expression level of p62 protein in the MSC group, rTMS group, and MSC+rTMS group was lower, and the expression levels of LC3-II and Beclin-1 were higher than those in the VD group. The decreased expression level of p62 protein and increased expression levels of LC3-II and Beclin-1 were more pronounced in the MSC+rTMS group than in the MSC group and rTMS group. It was indicated that the autophagic levels in the MSC group, rTMS group, and MSC+rTMS group were increased and that of the MSC+rTMS group was increased more significantly. We speculated that rTMS could enhance the therapeutic effect of MSC transplantation on cognitive dysfunction in VD, and the mechanism might be related to the following reasons. rTMS increased the localization and accumulation of MSCs in the damaged regions of the brain, thereby enhancing the repair effect and increasing the expression of BDNF and other neurotrophic factors by promoting the autophagic levels. The phosphorylation of NR1 on postsynaptic densities was specifically enhanced, and the permeability of calcium was increased. Next, the intracellular calcium ion-dependent enzymes were activated, causing a series of biochemical reactions, promoting the production of NR1-dependent LTP and enhancing synaptic transmission efficiency. Ultimately, the learning and memory abilities of the MSC- and rTMS-treated VD rats were improved. Based on these observations, we were further interested in the relationship between neurotrophin BDNF and autophagy-related proteins. Thus, we analyzed the pathways and functions of those common proteins

Table 1. List of Common Proteins in BDNF-p62-mTOR Interaction Network

Abbreviation of Proteins	Uniprot Accession	Full Names of Proteins
AKT1	P31749	AKT serine/threonine kinase 1
ATG5	Q9H1Y0	Autophagy-related 5
ATG7	O95352	Autophagy-related 7
ATG12	O94817	Autophagy-related 12
BECN1	Q14457	Beclin 1
CDC42	P60953	Cell division cycle 42
CHUK	O15111	Conserved helix-loop-helix ubiquitous kinase
GABARAP	O95166	GABA type A receptor-associated protein
GABARAPL1	Q9H0R8	GABA type A receptor-associated protein like 1
GABARAPL2	P60520	GABA type A receptor-associated protein like 2
HTT	P42858	Huntingtin
IKBKB	O14920	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta
LCK	P06239	LCK proto-oncogene, Src family tyrosine kinase
MAP1LC3A	Q9H492	Microtubule-associated protein 1 light chain 3 alpha(MAP1LC3A)
MAP1LC3B	Q9GZQ8	Microtubule-associated protein 1 light chain 3 beta
MAP3K3	Q99759	Mitogen-activated protein kinase kinase kinase 3
NFKB1	P19838	Nuclear factor kappa B subunit 1
NFKBIA	P25963	NFKB inhibitor alpha
NGF	P01138	Nerve growth factor
NGFR	P08138	Nerve growth factor receptor
PARK2	O60260	Parkin RBR E3 ubiquitin protein ligase
PIK3R1	P27986	Phosphoinositide-3-kinase regulatory subunit 1
RELA	Q04206	RELA proto-oncogene, NF-kB subunit
RPTOR	Q8N122	Regulatory-associated protein of MTOR complex 1
TRAF2	Q12933	TNF receptor-associated factor 2
TRAF6	Q9Y4K3	TNF receptor-associated factor 6 (TRAF6)

in the BDNF-p62-mTOR interaction network, which shared common pathways and similar functions.

The basic working principle of rTMS is to store a high-current charge on a group of capacitors and release a large amount of charge in an extremely short time through the action of an induction coil so that the pulsed current flows rapidly to generate a magnetic field. When the intensity of the induced current exceeds the excitability threshold of the nervous tissue, it will cause depolarization of the local cerebral nerve cells. rTMS does not directly stimulate the nerves but uses the induced current effects to affect the central nervous system and treat neurological diseases (53,54). The effect of rTMS depends on parameters such as the stimulation frequency, intensity, shape of the coil, and direction. rTMS can be treated with the subject fully awake and does not need to be performed with the assistance of anesthesia. rTMS is easy to use in clinical practice and has been widely used to treat stroke, depression, insomnia, and other neuropsychiatric diseases, as well as neurological rehabilitation (55). In our previous study, we found that rTMS improved the ability of learning and memory in VD rats and increased synaptic plasticity (46,47). Synaptic plasticity includes plasticity of the synapse morphological structure, plasticity of synaptic development, and plasticity of synaptic transmission (56). Transmission electron microscopy can be used to observe the changes in the synapse morphological structure, and the measured parameters can represent the morphological plasticity (57). Actually, there were some published articles showed that a global pattern of hyperexcitability was a feature stably reported in both vascular and degenerative dementia (58–60) or in patients with vascular depression at risk for future dementia (61–63). However, emerging studies (46,50,51,64) have shown that high-frequency rTMS can improve the recovery of cognitive function in rats, and its mechanism may be that it increases the expression of BDNF, NMDAR1, SYN, or VEGF proteins, improves synaptic plasticity, enhances neurogenesis, and reduces apoptosis. Therefore, in our present study, we used high-frequency rTMS to explore whether it could improve the recovery of cognitive function in VD rats via enhancing autophagy and the synergistic effect of rTMS and MSCs transplantation on VD rats.

The pathogenic factors of VD include hypertension, diabetes, heart disease, hyperlipidemia, and cerebral amyloidosis. Cerebral blood flow is closely related to the severity of dementia (65,66). The ideal method of generating the VD animal models should have the characteristics of simple operation, high survival rate, high success rate of model replication, clear behavioral abnormalities, and histopathological changes. Currently, a few methods exist to generate VD rat models and include classic 2VO, 4VO (four-vessel occlusion), vascular embolization, and middle cerebral artery occlusion (MCAO). Classical 2VO and 4VO methods lead to obvious abnormalities in learning, memory, spatial cognition, and motor function, but these two methods can lead to severe hippocampal neuronal necrosis and excessive damage to the animal models. The postoperative mortality is high, and it is quite different from the pathogenesis of VD in the clinic (67,68). After the experimental trials and comparisons, we believe that the improved 2VO method was a relatively ideal method to construct the VD rat models (48,49). In the improved 2VO method, the right common carotid artery was clamped for 10 minutes and was reconnected for 10 minutes, repeating three times, and was then cutoff after the ligation. The other common carotid artery was treated in the same manner 1 week later. After the VD models were made, the Morris water maze was used to test their learning and memory abilities to judge whether the models were successful.

MSCs can penetrate the blood–brain barrier and migrate to brain tissue to play a role in neuroprotection. MSCs migrate to localized areas of brain lesions and secrete peripheral paracrine neurotrophic

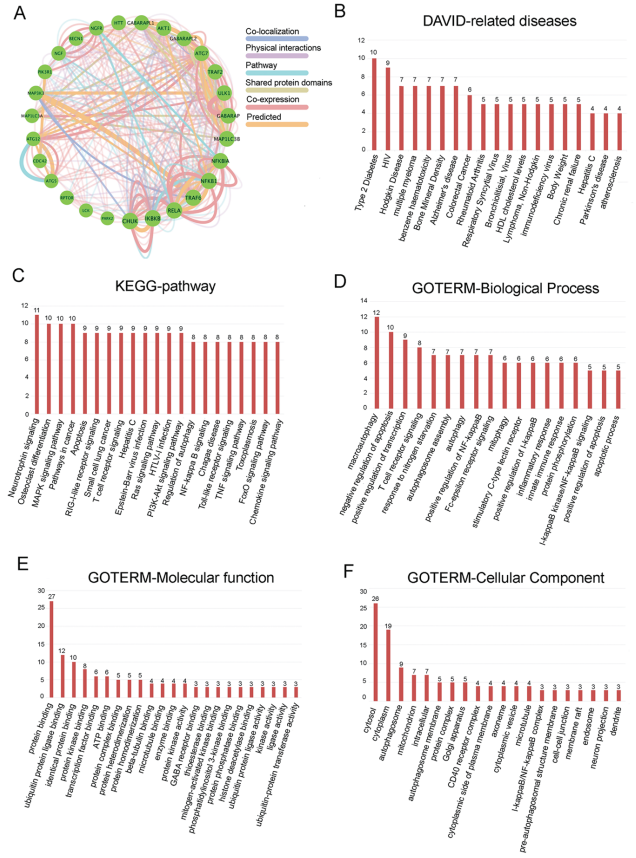

Figure 7. Pathway and function analyses of 27 common proteins of the BDNF-p62-mTOR network. (**A**) Comprehensive analysis by the GeneMANIA database. (**B**) Related disease analysis by the DAVID database. (**C**) Pathway analysis by the KEGG database. (**D–F**) Analyses of biological process, molecular function, and cellular component by the GO database.

factors such as BDNF, VEGF, and cytokines, which bind to receptors on damaged cells and activate intracellular signaling systems, promoting the proliferation and survival of nerve cells in the brain (69,70). Although the percentage of MSCs induced to differentiate into neuron-like cells is high in vitro, there are many deaths of MSCs during the induction process, and there are few normal functions in the induced differentiation of nerve cells in vivo. Thus, the therapeutic effect is very low (71). Telomerase is a ribozyme that plays an important role in chromosome replication in most eukaryotes. TERT is a catalytic subunit that has reverse transcriptase activity and is an important component of telomerase (72,73). Our previous study showed that the rate of proliferation in TERT-transfected MSCs was significantly increased compared with that in untransfected MSCs and empty vector-transfected MSCs after long-term cell culture (25). Tang et al. reported that the transfection of lentivirus-mediated TERT and VEGF into bone marrow mesenchymal stem cells revealed that the proliferative activity and angiogenic capacity of bone marrow mesenchymal stem cells were significantly increased and did not promote tumorigenesis (74). In our present study, according to flow cytometry analysis, surface markers CD31, CD34, and CD45 were negative (4.36%, 2.54%, and 3.87%, respectively), and CD29, CD44, and CD90 were positive (95.65%, 96.45%, and 94.23%, respectively). After MSCs were transfected with pLXSN-TERT, the expression of TERT mRNA was significantly increased and telomerase activity was increased. The proliferation rate of TERT-MSCs was significantly higher than that of untransfected MSCs. After long-term culture, TERT-MSCs still had high proliferation rate (25).

BDNF is an important neurotrophic factor with a neuroprotective effect and plays an important role in synaptic plasticity (75). Studies have reported that BDNF can specifically enhance the phosphorylation of NR1 (N-methyl-D-aspartate glutamatergic receptor 1 [NMDAR1]) and affect synaptic plasticity (76). NMDA receptors have a bidirectional role in the development of VD. In the acute phase of cerebral ischemia and hypoxia, it can mediate the cytotoxicity of excitatory amino acids, leading to neuronal damage and cognitive impairment (77). However, in the chronic phase, the cytotoxicity of NMDA receptor-mediated excitatory amino acids is no longer present, and further cognitive impairment may be related to the reduction of NMDA receptor-induced synaptic transmission dysfunction. NR1 and NR2 are two subunits of NMDA receptors. NR1 is a functional subunit with full functional properties of the NMDA receptor. NR2 is a regulatory subunit that itself does not have the functional activity of NMDA receptors and only affects the functional properties of NR1 after polymerization with NR1. The activation of NR1 plays an important role in long-term potentiation (LTP) induction and spatial learning and memory (78,79).

Actually, our present study has some limitations. For example, we only studied the effect of high-frequency rTMS combined with MSCs transplantation on the recovery of cognitive function in VD rats. According to our current experiments, we cannot determine whether the low-frequency rTMS has a stronger effect on enhancing autophagy and synaptic plasticity, which is more meaningful for guiding clinical application of rTMS. In addition, the detailed relationship between neurotrophin such as BDNF, NGF, neurotrophin-3 (NT-3), and autophagy-related proteins will be further explored in our future work.

Taken together, for the first time, our findings revealed that rTMS could synergistically enhance the transplantation effect of MSCs on VD rats. The mechanism may be related to increasing autophagy and synaptic plasticity. Our findings provide an important basis of the combined application of MSC transplantation and rTMS treatment to treat VD. The specific mechanisms of the synergetic effects of MSCs and rTMS on the treatment of other neurological diseases such as stroke, epilepsy, and depression remain to be further studied in the future.

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Conflict of Interest

None reported.

References

1. Lang B, Kindy MS, Kozel FA, Schultz SK, Taheri S. Multi-parametric classification of vascular cognitive impairment and dementia: the impact of diverse cerebrovascular injury biomarkers. *J Alzheimers Dis*. 2018;62:39–60. doi:10.3233/JAD-170733
2. Santos MAO, Bezerra LS, Correia CDC, Bruscky IS. Neuropsychiatric symptoms in vascular dementia: epidemiologic and clinical aspects. *Dement Neuropsychol*.2018;12:40–44.doi:10.1590/1980-57642018dn12-010006

3. Llorens F, Schmitz M, Ferrer I, Zerr I. CSF biomarkers in neurodegenerative and vascular dementias. *Prog Neurobiol*. 2016;138–140:36–53. doi:10.1016/j.pneurobio.2016.03.003
4. Snowden JS, Thompson JC, Stopford CL, et al. The clinical diagnosis of early-onset dementias: diagnostic accuracy and clinicopathological relationships. *Brain*. 2011;134(Pt 9):2478–2492. doi:10.1093/brain/awr189
5. Li S, Shao Y, Li K, et al. Vascular cognitive impairment and the gut microbiota. *J Alzheimers Dis*. 2018;63:1209–1222. doi:10.3233/JAD-171103
6. Ikram MA, Bersano A, Manso-Calderón R, et al. Genetics of vascular dementia - review from the ICVD working group. *BMC Med*. 2017;15:48. doi:10.1186/s12916-017-0813-9
7. Bordet R, Ihl R, Korczyn AD, et al. Towards the concept of disease-modifier in post-stroke or vascular cognitive impairment: a consensus report. *BMC Med*. 2017;15:107. doi:10.1186/s12916-017-0869-6
8. Wessel MJ, Hummel FC. Non-invasive cerebellar stimulation: a promising approach for stroke recovery? *Cerebellum*. 2018;17:359–371. doi:10.1007/s12311-017-0906-1
9. Concerto C, Lanza G, Cantone M, et al. Repetitive transcranial magnetic stimulation in patients with drug-resistant major depression: a six-month clinical follow-up study. *Int J Psychiatry Clin Pract*. 2015;19:252–258. doi :10.3109/13651501.2015.1084329
10. Cantone M, Bramanti A, Lanza G, et al. Cortical plasticity in depression. *ASN Neuro*. 2017;9:1759091417711512. doi:10.1177/1759091417711512
11. Lanza G, Cantone M, Aricò D, et al. Clinical and electrophysiological impact of repetitive low-frequency transcranial magnetic stimulation on the sensory-motor network in patients with restless legs syndrome. *Ther Adv Neurol Disord*. 2018;11:1756286418759973. doi:10.1177/1756286418759973
12. De Pisapia N, Barchiesi G, Jovicich J, Cattaneo L. The role of medial prefrontal cortex in processing emotional self-referential information: a combined TMS/fMRI study. *Brain Imaging Behav*. 2018. doi:10.1007/s11682-018-9867-3 (Epub ahead of print)
13. Yang CC, Völlm B, Khalifa N. The effects of rTMS on impulsivity in normal adults: a systematic review and meta-analysis. *Neuropsychol Rev*. 2018;28:377–392. doi:10.1007/s11065-018-9376-6
14. To WT, De Ridder D, Hart J Jr, Vanneste S. Changing brain networks through non-invasive neuromodulation. *Front Hum Neurosci*. 2018;12:128. doi:10.3389/fnhum.2018.00128
15. Bruna F, Plaza A, Arango M, Espinoza I, Conget P. Systemically administered allogeneic mesenchymal stem cells do not aggravate the progression of precancerous lesions: a new biosafety insight. *Stem Cell Res Ther*. 2018;9:137. doi:10.1186/s13287-018-0878-1
16. Henao Agudelo JS, Braga TT, Amano MT, et al. Mesenchymal stromal cell-derived microvesicles regulate an internal pro-inflammatory program in activated macrophages. *Front Immunol*. 2017;8:881. doi:10.3389/fimmu.2017.00881
17. Zhang Y, Qiu B, Wang J, Yao Y, Wang C, Liu J. Effects of BDNF-transfected BMSCs on neural functional recovery and synaptophysin expression in rats with cerebral infarction. *Mol Neurobiol*. 2017;54:3813–3824. doi:10.1007/s12035-016-9946-7
18. Zhao H, Cheng L, Du X, et al. Transplantation of cerebral dopamine neurotrophic factor transducted BMSCs in contusion spinal cord injury of rats: promotion of nerve regeneration by alleviating neuroinflammation. *Mol Neurobiol*. 2016;53:187–199. doi:10.1007/s12035-014-9000-6
19. Yang EZ, Zhang GW, Xu JG, et al. Multichannel polymer scaffold seeded with activated Schwann cells and bone mesenchymal stem cells improves axonal regeneration and functional recovery after rat spinal cord injury. *Acta Pharmacol Sin*. 2017;38:623–637. doi:10.1038/aps.2017.11
20. Saxena M, Prashar P, Yadav PS, Sen J. Mouse bone marrow stromal cells differentiate to neuron-like cells upon inhibition of BMP signaling. *Differentiation*. 2016;92:1–9. doi:10.1016/j.diff.2016.03.003
21. Sassoli C, Pierucci F, Tani A, et al. Sphingosine 1-phosphate receptor 1 is required for MMP-2 function in bone marrow mesenchymal stromal cells: implications for cytoskeleton assembly and proliferation. *Stem Cells Int*. 2018;2018:5034679. doi:10.1155/2018/5034679
22. Ward MR, Abadeh A, Connelly KA. Concise review: rational use of mesenchymal stem cells in the treatment of ischemic heart disease. *Stem Cells Transl Med*. 2018. doi:10.1002/sctm.17–0210 (Epub ahead of print)
23. Xie Z, Tang S, Ye G, et al. Interleukin-6/interleukin-6 receptor complex promotes osteogenic differentiation of bone marrow-derived mesenchymal stem cells. *Stem Cell Res Ther*. 2018;9:13. doi:10.1186/s13287-017-0766-0
24. Zhou S, Glowacki J. Chronic kidney disease and vitamin D metabolism in human bone marrow-derived MSCs. *Ann N Y Acad Sci*. 2017;1402:43–55. doi:10.1111/nyas.13464
25. Wang F, Chang G, Geng X. NGF and TERT co-transfected BMSCs improve the restoration of cognitive impairment in vascular dementia rats. *PLoS One*. 2014;9:e98774. doi:10.1371/journal.pone.0098774
26. Pawlowska E, Szczepanska J, Szatkowska M, Blasiak J. An interplay between senescence, apoptosis and autophagy in glioblastoma multiforme-role in pathogenesis and therapeutic perspective. *Int J Mol Sci*. 2018;19:pii: E889. doi:10.3390/ijms19030889
27. Tekirdag K, Cuervo AM. Chaperone-mediated autophagy and endosomal microautophagy: joint by a chaperone. *J Biol Chem*. 2018;293:5414–5424. doi:10.1074/jbc.R117.818237
28. Vizza D, Perri A, Toteda G, et al. Rapamycin-induced autophagy protects proximal tubular renal cells against proteinuric damage through the transcriptional activation of the nerve growth factor receptor NGFR. *Autophagy*. 2018;14:1028–1042. doi:10.1080/15548627.2018.1448740
29. Burgoyne JR. Oxidative stress impairs autophagy through oxidation of ATG3 and ATG7. *Autophagy*. 2018;14:1092–1093. doi:10.1080/15548627.2018.1444311
30. Pua HH, Dzhagalov I, Chuck M, Mizushima N, He YW. A critical role for the autophagy gene Atg5 in T cell survival and proliferation. *J Exp Med*. 2007;204:25–31. doi:10.1084/jem.20061303
31. Dupont N, Chauhan S, Arko-Mensah J, et al. Neutral lipid stores and lipase PNPLA5 contribute to autophagosome biogenesis. *Curr Biol*. 2014;24:609–620. doi:10.1016/j.cub.2014.02.008
32. Seillier M, Peuget S, Gayet O, et al. TP53INP1, a tumor suppressor, interacts with LC3 and ATG8-family proteins through the LC3-interacting region (LIR) and promotes autophagy-dependent cell death. *Cell Death Differ*. 2012;19:1525–1535. doi:10.1038/cdd.2012.30
33. Dou Z, Xu C, Donahue G, et al. Autophagy mediates degradation of nuclear lamina. *Nature*. 2015;527:105–109. doi:10.1038/nature15548
34. Dagda RK, Cherra SJ 3rd, Kulich SM, Tandon A, Park D, Chu CT. Loss of PINK1 function promotes mitophagy through effects on oxidative stress and mitochondrial fission. *J Biol Chem*. 2009;284:13843–13855. doi:10.1074/jbc.M808515200
35. Li N, Wang H, Jiang C, Zhang M. Renal ischemia/reperfusion-induced mitophagy protects against renal dysfunction via Drp1-dependent-pathway. *Exp Cell Res*. 2018;369:27–33. doi:10.1016/j.yexcr.2018.04.025
36. Shen H, Yin L, Deng G, et al. Knockdown of Beclin-1 impairs epithelial-mesenchymal transition of colon cancer cells. *J Cell Biochem*. 2018;119:7022–7031. doi:10.1002/jcb.26912
37. Liu B, Gao JM, Li F, Gong QH, Shi JS. Gastrodin attenuates bilateral common carotid artery occlusion-induced cognitive deficits via regulating Aβ-related proteins and reducing autophagy and apoptosis in rats. *Front Pharmacol*. 2018;9:405. doi:10.3389/fphar.2018.00405
38. Levine B, Liu R, Dong X, Zhong Q. Beclin orthologs: integrative hubs of cell signaling, membrane trafficking, and physiology. *Trends Cell Biol*. 2015;25:533–544. doi:10.1016/j.tcb.2015.05.004
39. Rodriguez-Muela N, Parkhitko A, Grass T, et al. Blocking p62-dependent SMN degradation ameliorates spinal muscular atrophy disease phenotypes. *J Clin Invest*. 2018;128:3008–3023. doi:10.1172/JCI95231
40. Siedlecka-Kroplewska K, Wrońska A, Stasiłojć G, Kmieć Z. The designer drug 3-fluoromethcathinone induces oxidative stress and activates autophagy in HT22 neuronal cells. *Neurotox Res*. 2018;34:388–400. doi:10.1007/s12640-018-9898-y
41. Alizadeh S, Mazloom H, Sadeghi A, et al. Evidence for the link between defective autophagy and inflammation in peripheral blood mononuclear cells of type 2 diabetic patients. *J Physiol Biochem*. 2018;74:369–379. doi:10.1007/s13105-018-0624-2

42. Herhaus L, Dikic I. Ubiquitin-induced phase separation of p62/SQSTM1. *Cell Res*. 2018;28:389–390. doi:10.1038/s41422-018-0030-x
43. Katsuragi Y, Ichimura Y, Komatsu M. p62/SQSTM1 functions as a signaling hub and an autophagy adaptor. *FEBS J*. 2015;282:4672–4678. doi:10.1111/febs.13540
44. Forrest MP, Parnell E, Penzes P. Dendritic structural plasticity and neuropsychiatric disease. *Nat Rev Neurosci*. 2018;19:215–234. doi:10.1038/nrn.2018.16
45. Norimoto H, Makino K, Gao M, et al. Hippocampal ripples downregulate synapses. *Science*. 2018;359:1524–1527. doi:10.1126/science.aao0702
46. Wang F, Geng X, Tao HY, Cheng Y. The restoration after repetitive transcranial magnetic stimulation treatment on cognitive ability of vascular dementia rats and its impacts on synaptic plasticity in hippocampal CA1 area. *J Mol Neurosci*. 2010;41:145–155. doi:10.1007/s12031-009-9311-7
47. Wang F, Chang GM, Yu Q, Geng X. The neuroprotection of repetitive transcranial magnetic stimulation pre-treatment in vascular dementia rats. *J Mol Neurosci*. 2015;56:198–204. doi:10.1007/s12031-014-0480-7
48. Sarti C, Pantoni L, Bartolini L, Inzitari D. Persistent impairment of gait performances and working memory after bilateral common carotid artery occlusion in the adult Wistar rat. *Behav Brain Res*. 2002;136:13–20.
49. Jiwa NS, Garrard P, Hainsworth AH. Experimental models of vascular dementia and vascular cognitive impairment: a systematic review. *J Neurochem*. 2010;115:814–828. doi:10.1111/j.1471-4159.2010.06958.x
50. Zhang N, Xing M, Wang Y, Tao H, Cheng Y. Repetitive transcranial magnetic stimulation enhances spatial learning and synaptic plasticity via the VEGF and BDNF-NMDAR pathways in a rat model of vascular dementia. *Neuroscience*. 2015;311:284–291. doi:10.1016/j.neuroscience.2015.10.038
51. Luo J, Zheng H, Zhang L, Zhang Q, Li L, Pei Z, Hu X. High-frequency repetitive transcranial magnetic stimulation (rTMS) improves functional recovery by enhancing neurogenesis and activating BDNF/TrkB signaling in ischemic rats. *Int J Mol Sci*. 2017;18:pii: E455. doi:10.3390/ijms18020455
52. Lu X, Bao X, Li J, et al. High-frequency repetitive transcranial magnetic stimulation for treating moderate traumatic brain injury in rats: a pilot study. *Exp Ther Med*. 2017;13:2247–2254. doi:10.3892/etm.2017.4283
53. Baek A, Kim JH, Pyo S, et al. The differential effects of repetitive magnetic stimulation in an in vitro neuronal model of ischemia/reperfusion injury. *Front Neurol*. 2018;9:50. doi:10.3389/fneur.2018.00050
54. Dagan M, Herman T, Mirelman A, Giladi N, Hausdorff JM. The role of the prefrontal cortex in freezing of gait in Parkinson's disease: insights from a deep repetitive transcranial magnetic stimulation exploratory study. *Exp Brain Res*. 2017;235:2463–2472. doi:10.1007/s00221-017-4981-9
55. Weigand A, Horn A, Caballero R, et al. Prospective validation that subgenual connectivity predicts antidepressant efficacy of transcranial magnetic stimulation sites. *Biol Psychiatry*. 2018;84:28–37. doi:10.1016/j.biopsych.2017.10.028
56. Bittner KC, Milstein AD, Grienberger C, Romani S, Magee JC. Behavioral time scale synaptic plasticity underlies CA1 place fields. *Science*. 2017;357:1033–1036. doi:10.1126/science.aan3846
57. Pannasch U, Freche D, Dallérac G, et al. Connexin 30 sets synaptic strength by controlling astroglial synapse invasion. *Nat Neurosci*. 2014;17:549–558. doi:10.1038/nn.3662
58. Pennisi G, Bella R, Lanza G. Motor cortex plasticity in subcortical ischemic vascular dementia: what can TMS say? *Clin Neurophysiol*. 2015;126:851–852. doi:10.1016/j.clinph.2014.09.001
59. Bella R, Cantone M, Lanza G, et al. Cholinergic circuitry functioning in patients with vascular cognitive impairment–no dementia. *Brain Stimul*. 2016;9:225–233. doi:10.1016/j.brs.2015.09.013
60. Lanza G, Bramanti P, Cantone M, Pennisi M, Pennisi G, Bella R. Vascular cognitive impairment through the looking glass of transcranial magnetic stimulation. *Behav Neurol*. 2017;2017:1421326. doi:10.1155/2017/1421326
61. Bella R, Ferri R, Cantone M, et al. Motor cortex excitability in vascular depression. *Int J Psychophysiol*. 2011;82:248–253. doi:10.1016/j.ijpsycho.2011.09.006
62. Concerto C, Lanza G, Cantone M, et al. Different patterns of cortical excitability in major depression and vascular depression: a transcranial magnetic stimulation study. *BMC Psychiatry*. 2013;13:300. doi:10.1186/1471-244X-13-300
63. Pennisi M, Lanza G, Cantone M, et al. Correlation between motor cortex excitability changes and cognitive impairment in vascular depression: pathophysiological insights from a longitudinal TMS study. *Neural Plast*. 2016;2016:8154969. doi:10.1155/2016/8154969
64. Guo F, Lou J, Han X, Deng Y, Huang X. Repetitive transcranial magnetic stimulation ameliorates cognitive impairment by enhancing neurogenesis and suppressing apoptosis in the hippocampus in rats with ischemic stroke. *Front Physiol*. 2017;8:559. doi:10.3389/fphys.2017.00559
65. Dominguez J, Fe de Guzman M, Reandelar M, Thi Phung TK. Prevalence of dementia and associated risk factors: a population-based study in the Philippines. *J Alzheimers Dis*. 2018;63:1065–1073. doi:10.3233/JAD-180095
66. Fotuhi M, Hachinski V, Whitehouse PJ. Changing perspectives regarding late-life dementia. *Nat Rev Neurol*. 2009;5:649–658. doi:10.1038/nrneurol.2009.175
67. Kalaria RN. Neuropathological diagnosis of vascular cognitive impairment and vascular dementia with implications for Alzheimer's disease. *Acta Neuropathol*. 2016;131:659–685. doi:10.1007/s00401-016-1571-z
68. Venkat P, Chopp M, Chen J. Models and mechanisms of vascular dementia. *Exp Neurol*. 2015;272:97–108. doi:10.1016/j.expneurol.2015.05.006
69. Wei L, Fraser JL, Lu ZY, Hu X, Yu SP. Transplantation of hypoxia preconditioned bone marrow mesenchymal stem cells enhances angiogenesis and neurogenesis after cerebral ischemia in rats. *Neurobiol Dis*. 2012;46:635–645. doi:10.1016/j.nbd.2012.03.002
70. Chen J, Yang Y, Shen L, et al. Hypoxic preconditioning augments the therapeutic efficacy of bone marrow stromal cells in a rat ischemic stroke model. *Cell Mol Neurobiol*. 2017;37:1115–1129. doi:10.1007/s10571-016-0445-1
71. Lu H, Liu X, Zhang N, et al. Neuroprotective effects of brain-derived neurotrophic factor and noggin-modified bone mesenchymal stem cells in focal cerebral ischemia in rats. *J Stroke Cerebrovasc Dis*. 2016;25:410–418. doi:10.1016/j.jstrokecerebrovasdis.2015.10.013
72. Lin S, Nascimento EM, Gajera CR, et al. Distributed hepatocytes expressing telomerase repopulate the liver in homeostasis and injury. *Nature*. 2018;556:244–248. doi:10.1038/s41586-018-0004-7
73. Lu AT, Xue L, Salfati EL, et al. GWAS of epigenetic aging rates in blood reveals a critical role for TERT. *Nat Commun*. 2018;9:387. doi:10.1038/s41467-017-02697-5
74. Tang H, Xiang Y, Jiang X, et al. Dual expression of hTERT and VEGF prolongs life span and enhances angiogenic ability of aged BMSCs. *Biochem Biophys Res Commun*. 2013;440:502–508. doi:10.1016/j.bbrc.2013.09.053
75. Ding X, Liang YJ, Su L, et al. BDNF contributes to the neonatal incision-induced facilitation of spinal long-term potentiation and the exacerbation of incisional pain in adult rats. *Neuropharmacology*. 2018;137:114–132. doi:10.1016/j.neuropharm.2018.04.032
76. Liu M, Kay JC, Shen S, Qiao LY. Endogenous BDNF augments NMDA receptor phosphorylation in the spinal cord via PLCγ, PKC, and PI3K/Akt pathways during colitis. *J Neuroinflammation*. 2015;12:151. doi:10.1186/s12974-015-0371-z
77. Xing M, Sun Q, Wang Y, Cheng Y, Zhang N. Hydroxysafflor yellow A increases BDNF and NMDARs in the hippocampus in a vascular dementia rat model. *Brain Res*. 2016;1642:419–425. doi:10.1016/j.brainres.2016.04.030
78. Sasaki-Hamada S, Suzuki A, Sanai E, Matsumoto K, Oka JI. Neuroprotection by chotosan, a Kampo formula, against glutamate excitotoxicity involves the inhibition of GluN2B-, but not GluN2A-containing NMDA receptor-mediated responses in primary cultured cortical neurons. *J Pharmacol Sci*. 2017;135:134–137. doi:10.1016/j.jphs.2017.10.009
79. Xu Q, Ji XF, Chi TY, et al. Sigma-1 receptor in brain ischemia/reperfusion: Possible role in the NR2A-induced pathway to regulate brain-derived neurotrophic factor. *J Neurol Sci*. 2017;376:166–175. doi:10.1016/j.jns.2017.03.027