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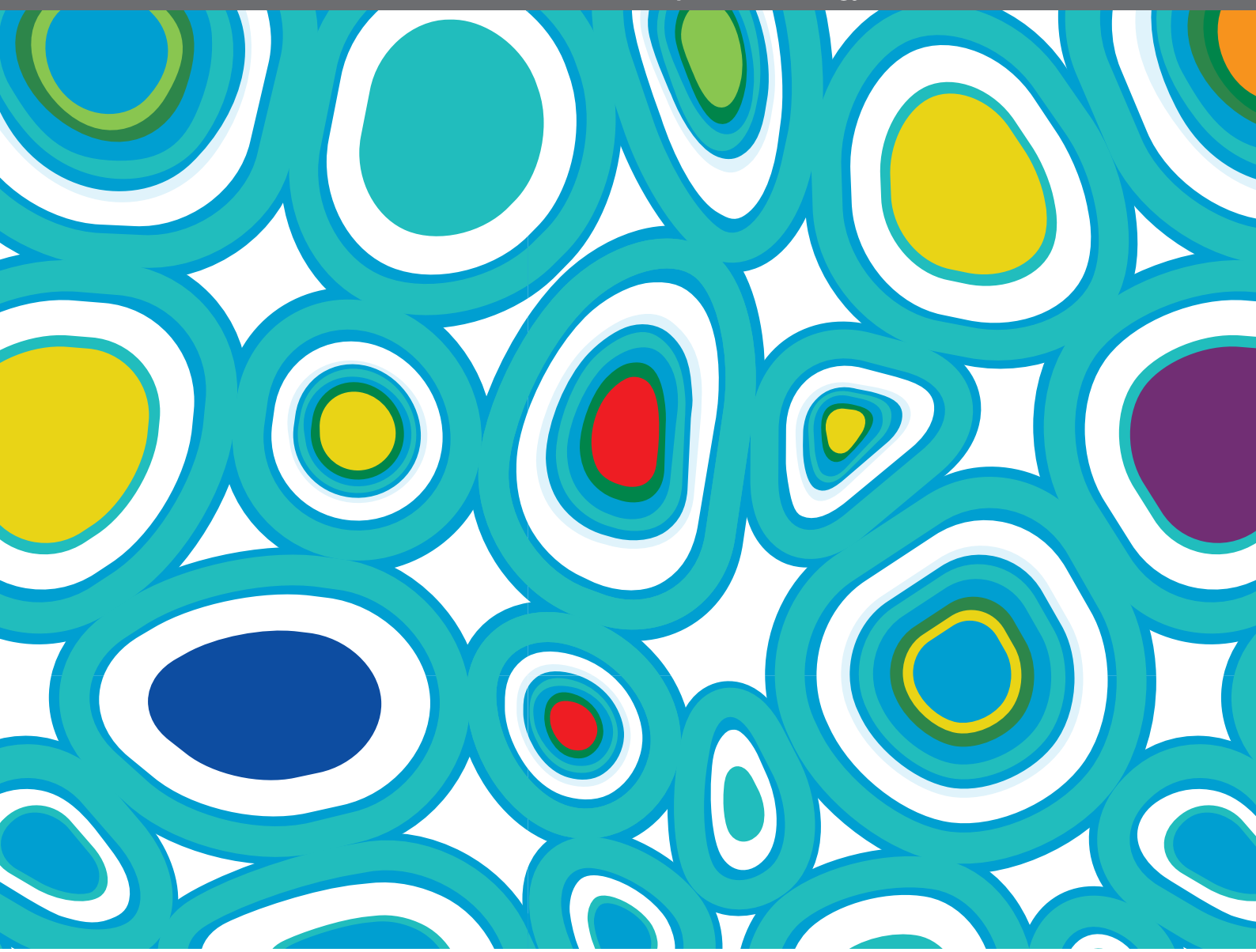

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CELL THERAPY FOR NEUROLOGICAL DISORDERS

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Olfactory Mucosa Mesenchymal Stem Cells Alleviate Cerebral Ischemia/Reperfusion Injury *Via* Golgi Apparatus Secretory Pathway Ca^{2+}-ATPase Isoform1

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Olfactory mucosa mesenchymal stem cells (OM-MSCs) have exhibited their effectiveness in central nervous system diseases and provided an appealing candidate for the treatment of ischemic stroke. Previous evidence have shown that Golgi apparatus (GA) secretory pathway Ca^{2+}-ATPase isoform1 (SPCA1) was a potential therapeutic target for ischemic stroke. In this study, we explored the neuroprotective mechanism of OM-MSCs and its effect on the expression and function of SPCA1 during cerebral ischemia/reperfusion. Based on *in vitro* and *in vivo* experiments, we discovered that OM-MSCs attenuated apoptosis and oxidative stress in ischemic stroke models, reduced the cerebral infarction volume, and improved the neurologic deficits of rats. OM-MSCs also upregulated SPCA1 expression and alleviated Ca^{2+} overload and decreased the edema and dissolution of the GA in neurons. Moreover, we discovered that SPCA1 depletion in oxygen and glucose deprivation/reoxygenation (OGD/R)-treated N2a cells mitigated the protective effects of OM-MSCs. Altogether, OM-MSCs exerted neuroprotective effects in ischemic stroke probably *via* modulating SPCA1 and reducing the edema and dissolution of the GA in neurons.

Keywords: ischemic stroke, SPCA1, olfactory mucosa mesenchymal stem cells, Golgi apparatus stress, Ca^{2+} overload

Abbreviations: *CCK-8*, Cell Counting Kit-8; *GA*, Golgi apparatus; *HSC*, hematopoietic stem cell; *iPSCs*, induced pluripotent stem cells; *I/R*, ischemia/reperfusion; *IRI*, ischemia/reperfusion injury; *LDH*, lactate dehydrogenase; *LPO*, lipid peroxidation; *MCAO*, middle cerebral artery occlusion; *mNSS*, modified neurologic severity score; *MSCs*, mesenchymal stem cells; *NSC*, neural stem cells; *OGD/R*, oxygen and glucose deprivation/reoxygenation; *OM-MSCs*, olfactory mucosa mesenchymal stem cells; *ROS*, reactive oxygen species; *SPCA*, secretory pathway Ca^{2+}-ATPase; *SPCA1*, secretory pathway Ca^{2+}-ATPase isoform1; *T-SOD*, total superoxide dismutase.

INTRODUCTION

Stroke is a leading cause of death and disability worldwide, in which ischemic stroke accounts for approximately 71% of all stroke types. In 2017, the global incidence of ischemic stroke events was about 7.7 million, with 2.7 million deaths (GBD 2017 Disease and Injury Incidence and Prevalence Collaborators, 2018; Campbell et al., 2019). However, the available therapy options regarding ischemic stroke have limited effects (Huang et al., 2018). Current treatments for acute ischemic stroke are based on reperfusion through thrombolysis or endovascular therapy, but both therapies are limited by the therapeutic time window: thrombolysis by the recombinant tissue plasminogen activator (rtPA) is required within 4.5 h of onset; endovascular therapy, 6 h. Although endovascular therapy can be extended to 24 h if the patient meets the inclusion criteria (Nogueira et al., 2018), only a few patients can actually benefit from it on account of the strict inclusion criteria and the narrow therapeutic time windows. Therefore, many researchers are actively looking for other effective treatments for ischemic stroke, such as cell therapy.

Previous preclinical researches have shown that stem cell transplantation could lead to functional improvement of ischemic stroke animal models (Wei et al., 2017; Boncoraglio et al., 2019). The stem cells involved in these studies included neural stem cells (NSCs; Boese et al., 2018), mesenchymal stem cells (MSCs; Kranz et al., 2010; Oshita et al., 2020), induced pluripotent stem cells (iPSCs; Gervois et al., 2016), and so on. Due to the diversity of access sources, multiple differentiation potential, and the plasticity of function, MSCs have become an appealing stem cell candidate for the treatment of ischemic stroke (Stonesifer et al., 2017; Wang et al., 2018).

Olfactory mucosa mesenchymal stem cells (OM-MSCs), first identified by Tome et al. (2009), are a type of Nestin-positive MSCs that reside in the lamina propria of the olfactory mucosa, having the potential to differentiate into osteocytes, adipocytes, smooth muscle cells, and neurons (Delorme et al., 2010). OM-MSCs are easily accessible, exhibit an extensive proliferation rate, and eliminate ethical concerns compared with the other stem cell types (Lindsay et al., 2020). Moreover, OM-MSCs promoted central nervous system myelination *in vitro* by secretion of the chemokine CXCL12, which was not related to bone marrow mesenchymal stem cells (BM-MSCs; Lindsay and Barnett, 2017). Another research demonstrated that OM-MSCs had a stronger secretion of immunosuppressive cytokines than adipose-derived mesenchymal stem cells (AD-MSCs; Jafari et al., 2020). The aforementioned advantages supported that OM-MSCs may be an appealing candidate of cell therapies for the treatment of human diseases.

Accumulating evidence showed that OM-MSCs exhibited effectiveness and potential in central nervous system diseases, including spinal cord injury, early-onset sensorineural hearing loss, and hippocampal lesions (Lindsay and Barnett, 2017; Lindsay et al., 2017; Zhuo et al., 2017). Huang et al. have concluded that OM-MSCs could inhibit pyroptotic and apoptotic death of microglial cells during ischemia/reperfusion (Huang et al., 2020). However, the impact of OM-MSCs on neuronal injury in ischemic stroke remains unclear.

At present, the inhibition of reperfusion injury is the key to the treatment of ischemic stroke. Intracellular oxidative stress and Ca^{2+} overload are the pivotal pathological processes of cerebral ischemia/reperfusion injury (IRI), leading to irreversible neuronal damage. Apart from mitochondria and lysosomes, Golgi apparatus (GA) also participates in the process of oxidative stress. Jiang et al. (2011) have presented the concept of "GA stress," which consisted of the activity of Ca^{2+}-ATPase in GA; the morphology and membrane surface components of the GA would change correspondingly under oxidative stress.

There are Ca^{2+} release channels and Ca^{2+} uptake mechanisms in the GA (Li et al., 2013). The Golgi-resident secretory pathway Ca^{2+}-ATPase (SPCA), which is highly expressed in the brain, is mainly responsible for transporting Ca^{2+} from the cytoplasm to the Golgi lumen and is involved in cytosolic and intra-Golgi Ca^{2+} homeostasis (He and Hu, 2012; Li et al., 2013). SPCA comprises secretory pathway Ca^{2+}-ATPase isoform1 (SPCA1) and SPCA2, encoded by ATP2C1 and ATP2C2, respectively, (Hu et al., 2000; Xiang et al., 2005). SPCA1 is well understood, while the function of SPCA2 is rarely studied.

Previous studies have found that oxidative stress may exert the ability to downregulate the expression of SPCA1 in ischemia/reperfusion rats (Pavlíková et al., 2009; Li et al., 2015; Fan et al., 2016). Besides, SPCA1 was found to be able to protect cells from oxidative stress by interacting with the *HSP60* gene (Uccelletti et al., 2005), while the inhibition of SPCA1 function could lead to apoptosis in N2a cells (Sepulveda et al., 2009) and mice (Okunade et al., 2007). Furthermore, the inactivation of SPCA1 could induce the alteration of the mitochondrial structure and metabolism, which would make the mitochondria more sensitive to oxidative stress (He and Hu, 2012). Based on existing evidences, improving the expression and function of SPCA1 was expected to be a therapeutic target for cerebral IRI.

In the present study, we explored the neuroprotective mechanism of OM-MSCs during cerebral ischemia/reperfusion and its effect on the expression as well as function of SPCA1 and further investigated the role of SPCA1 knockdown in the neuroprotective effect of OM-MSCs on cerebral IRI.

MATERIALS AND METHODS

Ethic Statement

Olfactory mucosa mesenchymal stem cells were obtained from two healthy male volunteers for scientific purposes (21 and 28 years old, respectively) at the Second Affiliated Hospital of Hunan Normal University. Human nasal mucosa biopsies were performed by otolaryngology endoscopy operation at the Department of Otolaryngologic Surgery, the second affiliated hospital of Hunan Normal University (Changsha, China). Written informed consent was given by each individual participating in the study before the operation, in accordance with the Helsinki Convention (1964). The investigators and all procedures were approved by the ethics committee of Hunan Normal University (ethical approval document no. 2018–30).

Isolation and Characterization of OM-MSCs

The isolation and culture of OM-MSCs were carried out using a protocol from a previous study (Ge et al., 2016). Briefly, olfactory tissue samples were obtained from the root of the medial aspect of the middle turbinate undergoing endoscopic nasal surgery, washed three times at room temperature with penicillin streptomycin solution (Invitrogen, Carlsbad, CA, United States), and then cultured in Dulbecco's modified Eagle's medium/nutrient mixture F12 (DMEM/F12; Invitrogen, United States) containing 10% fetal bovine serum (FBS; Gibco, Australia) and incubated at 37°C in 5% CO_2. OM-MSCs at passages 3 and 4 were used for further experiments. Cell surface markers were used to characterize OM-MSCs by flow cytometric analysis.

Oxygen and Glucose Deprivation/Reoxygenation

Mouse N2a cells were purchased from the Cell Storage Center of the Chinese Academy of Sciences (Shanghai, China). N2a cells were cultured in DMEM (Sigma, United States) supplemented with 10% FBS (Gibco, Australia) at 37°C in 5% CO_2.

To achieve ischemic-like conditions *in vitro*, the oxygen and glucose deprivation/reoxygenation (OGD/R) model was performed as previously described (Huang and Hu, 2018). Simply, the N2a cells were placed in a modular incubator chamber (Billups Rothenberg, Inc., Del Mar, CA, United States), which kept the pO_2 value consistently below 0.5%. The culture medium was replaced with deoxygenated glucose-free Hanks' Balanced Salt Solution (Sigma). The cells were maintained in the hypoxic and glucose-free chamber for 4 h. After OGD, the N2a cells were quickly maintained in DMEM without FBS and incubated under normoxic conditions for 0, 4, 12, and 24 h. N2a cells cultured with DMEM containing 10% FBS in normoxia (5% CO_2, 37°C) were used as normal controls.

Co-culture of N2a Cells and OM-MSCs

The co-culture system was set up as previously described (Wei et al., 2019). In brief, the N2a cells (1×10^5) grown in six-well plates were subjected to stress by the OGD method, as described above. At the same time as reoxygenation begins, the N2a cells were rescued by plating 1×10^5 OM-MSCs (OM-MSCs : N2a = 1:1) on the Transwell membrane inserts (pore size, 0.4 μm; polycarbonate membrane, Corning, United States) and incubating for 24 h. During reperfusion, DMEM without FBS conditioning media were used.

Animals

All animal procedures were approved by the Laboratory Animal Ethics Committee of the Second Affiliated Hospital of Hunan Normal University (ethical approval document no. 2020–164). All experimental procedures were performed in accordance with the Guide for the Care and Use of Experimental Animals. Male Sprague–Dawley rats weighing 250–300 *g* were kept under controlled housing conditions with a 12-h light/dark cycle with food and water *ad libitum*.

Rats Reversible Middle Cerebral Artery Occlusion Model and OM-MSC Transplantation

The right reversible middle cerebral artery occlusion (MCAO) model was performed as previously described (Longa et al., 1989). Briefly, rats were fasted for 12 h before surgery with water accessible. Rats were initially anesthetized with 3.5% isoflurane and maintained with 1.0–2.0% isoflurane in 2:1 N_2O/O_2 using a face mask. The right common carotid artery (CCA), internal carotid artery (ICA), and external carotid artery (ECA) were separated, and an incision was made on the carotid artery using ophthalmic scissors. A surgical filament (0.26-mm diameter; Beijing Cinontech Co. Ltd.) was inserted into the ICA from the incision of CCA, with the length of the line being 18–20 mm. Resistance implied that the line had reached the beginning of the right middle cerebral artery (MCA), thus blocking the blood flow of the vessel. The filament was withdrawn after 120 min, after which the skin wound was sutured. The body temperature of the rats was maintained at 37 ± 0.5°C during the whole procedure. For analgesia, post-surgery rats were given a subcutaneous injection of morphine (2.5 mg/kg) every 4 h for 1 day following MCAO.

In total, 72 adult male Sprague–Dawley rats were randomly divided into three groups: sham operation group (sham), MCAO group (ischemia/reperfusion, I/R), and MCAO + OM-MSC group (transplantation; n = 24 animals per group). In the transplantation group, the rats received tail vein injection of 5.0×10^6 OM-MSCs dissolved in 1 ml saline at 24 h after MCAO model induction, while the rats received tail vein injection of 1 ml saline in the I/R group. Rats in each group were sacrificed after anesthesia for experiment on day 7 after reperfusion. All experimental procedures were performed by investigators blinded to group allocation.

Inclusion and Exclusion Criteria

The inclusion and exclusion criteria of the I/R and transplantation groups were based on the Zea-Longa score when the rats were awake after operation (Longa et al., 1989). The following are the scoring criteria: 0 point = there is no any neurological symptom; 1 point = the left forelimb of the rats cannot entirely stretch; 2 points = Sprague–Dawley (SD) rats rotate to the ischemic side while walking, moderate neurological deficit; 3 points = SD rats dump to the ischemic side when standing; and 4 points = SD rats cannot walk on their own and lose consciousness. Specifically, SD rats with a score of 1–3 were used in the subsequent experiment, while SD rats that died, or with a score of 0 or 4, were dropped. To compensate for dropouts, three additional animals were enrolled to the study population, resulting in an overall study population of 75 rats.

CCK-8 Assay

Cell viability in each group was measured by using the Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Dojindo, Japan) according to the manufacturer's protocol.

LDH Measurement

Immediately following OGD/R, the culture supernatants were collected and, subsequently, the level of lactate dehydrogenase (LDH) was detected using the LDH Cytotoxicity Assay Kit (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) according to the manufacturer's protocol.

Apoptosis Measurement

Apoptosis of N2a cells and the ipsilateral cortex of SD rats were detected *via* annexin V–fluorescein isothiocyanate (FITC) and propidium iodide (PI) double staining using a FITC Annexin V Apoptosis Detection Kit I (KeyGen Biotech, Jiangsu, China) according to the manufacturer's instructions. The fluorescence was measured by flow cytometry (Beckman, United States).

TUNEL and NeuN Double Immunostaining

Apoptosis of neurons in the ipsilateral cortex of SD rats was evaluated by terminal-deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) and NeuN double immunostaining according to the manufacturer's protocol. The brain sections from each group were incubated with TUNEL reaction mixture (Beyotime, Shanghai, China) for 1 h at room temperature and then stained with anti-NeuN (ab177487, Abcam, Cambridge, United Kingdom) and DAPI (Wellbio, China). The slides were observed using a fluorescence microscope (Motic, China).

ROS Measurement

Intracellular reactive oxygen species (ROS) was detected using an oxidation-sensitive fluorescent probe (2′,7′-dichlorodihydrofluorescein diacetate, DCFH-DA). Following OGD/R, reactive oxygen species detection was performed using a fluorescent probe DCFH-DA kit (Beyotime). The cells were washed twice with phosphate-buffered saline (PBS) and subsequently incubated with 10 μmol/L DCFH-DA at 37°C for 20 min. After washing three times, the fluorescence was measured by flow cytometry (Beckman).

LPO and T-SOD Measurements

The ipsilateral cortex of the SD rats from each group was used for total superoxide dismutase (T-SOD) and lipid peroxidation (LPO) measurements. The levels of LPO and T-SOD were detected using lipid peroxidation and T-SOD assay kits (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instructions.

Quantitative Real-Time PCR

Total RNA was isolated from N2a cells and ipsilateral brain samples of SD rats using the TRIzol™ Reagent (Thermo Fisher Scientific, United States). Reverse transcription was performed using a reverse transcription kit (Beijing ComWin Biotech Co., Ltd., China). Quantitative PCR (qPCR) was performed using UltraSYBR Mixture (Beijing ComWin Biotech Co., Ltd.). The following qPCR primer sequences were used for messenger RNA (mRNA) quantification: 5′-AGAACTTATTGCCTCCGTCC-3′ and 5′-ATCTTTTGAAATCGTGCAACCTG-3′ for mouse SPCA1, 5′-ACATCCGTAAAGACCTCTATGCC-3′ and 5′-TACTCCTGCTTGCTGATCCAC-3′ for mouse β-actin.

Western Blot Assay

N2a cells and the ipsilateral cortex of SD rats were processed for Western blot as described (Fan et al., 2016). Immunoblot analyses were performed using the following primary antibodies: anti-SPCA1 (ab126171, Abcam) and anti-β-actin (60008-1-Ig, Proteintech, United States). The anti-rabbit IgG and anti-mouse IgG secondary antibodies were obtained from Proteintech. The proteins were visualized using an enhanced chemiluminescent (ECL) detection kit (Advansta Inc., United States).

Intracellular Ca^{2+} Measurement

For N2a cells, Golgi vesicles were isolated by a GA protein extraction kit (BestBio, Hunan, China) according to the manufacturer's instructions. The concentrations of Ca^{2+} in the cytoplasm and Golgi vesicles were detected using the Ca^{2+} Assay Kit (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's protocol.

For the ipsilateral brain samples of SD rats, intracellular Ca^{2+} was measured in Fluo-3/acetoxymethyl (AM)-loaded cells by flow cytometry. Briefly, the brain tissues were digested and then incubated with 5 μM Fluo-3/AM (Beyotime) at 37°C for 0.5 h according to the instructions of the manufacturer. After washing and resuspension in PBS, intracellular Ca^{2+} levels were measured at an excitation wavelength of 488 nm and an emission wavelength of 530 nm using a flow cytometer (Beckman).

Electron Microscope Test

An electron microscope specimen was prepared as previously described (Fan et al., 2016) and then observed with a Hitachi HT7700 transmission electron microscope (Tokyo, Japan). Section analyses were all under the same intensity condition and the same magnification of the electron microscope.

Infarct Volume Analysis

The mice were sacrificed on day 7 after reperfusion and the brains were removed quickly. Infarct volumes were measured by 2,3,5-triphenyltetrazolium chloride (TTC) staining. All brain slices of mice from each group (n = 3 animals per group) were used to perform TTC staining. Slices were incubated in 2% TTC solution for 30 min at 37.0°C, then fixed in 10% formalin in the border zone of infarction, and were outlined with Image-Pro Plus Analysis Software (Media Cybernetics, Bethesda, MD, United States). The analysis was done by investigators who were blinded to the experimental groups.

Behavioral Analysis

The modified neurologic severity score (mNSS) and rotarod treadmill were used to evaluate the neurological deficits of rats in each group before they were killed. All rats in each group received behavioral analysis on days 0 (pre-MCAO), 1, 3, and 7 after reperfusion. The mNSS consists of motor, sensory, reflex, and balance tests and was used to grade the neurological function on a scale of 0–18 (Zhao et al., 2020). For the rotarod treadmill, the

rats were placed on rotating rods which accelerated at 3–20 rpm for 5 min. The time that the animal remained on the rod was the measured parameter. Two observers blinded to the treatment and grouping were assigned to conduct behavioral analysis.

shRNA Knockdown of SPCA1

For short hairpin RNA (shRNA) knockdown, we chose the shRNA target sequence 5′-ccggccTGCGGACTTACGCTTATTT ctcgagAAATAAGCGTAAGTCCGCAggtttttg-3′. N2a cells were silenced with SPCA1 shRNA by using a shRNA transfection kit according to the manufacturer's instruction (GIEE, China). The efficiency of the knockdown of SPCA1 in N2a cells was verified by qPCR and Western blot.

Statistical Analysis

All statistical analyses were performed using SPSS statistical software (SPSS, Inc., Chicago, IL, United States). After testing for normal distribution, the data of two independent variables were analyzed using Mann–Whitney test. For three or more variables, Kruskal–Wallis test was performed followed by *post hoc* analysis using Tukey's test. All data are expressed as mean ± SEM. Differences between the mean values were considered significant at $P < 0.05$.

RESULTS

Characterization of OM-MSCs

The morphology of OM-MSCs was typically fibroblastic or in spindle form, as shown in **Figure 1A**. The immunophenotype of OM-MSCs identified by flow cytometry exhibited positive expression of MSC markers (CD44, CD73, CD90, CD105, CD133, and CD146) and negative expression of hematopoietic stem cell (HSC) markers (CD34 and CD45; **Figure 1B**).

OM-MSCs Ameliorated OGD/R-Induced Apoptosis and Oxidative Stress in N2a Cells

Oxygen and glucose deprivation/reoxygenation-induced N2a cell injury was performed as a classical model to mimic cerebral IRI *in vitro*. After 4 h OGD exposure, we treated N2a cells with different time courses of reoxygenation. The cell viability was significantly decreased with the time development compared with the normal group (**Figure 2A**), while the LDH, apoptosis rate, and ROS level were apparently increased and reached the highest changes at the 24-h reoxygenation time point (**Figures 2B–F**). Thereby, a 4-h OGD treatment followed by a 24-h reoxygenation therapy were applied for further experiments.

We then used a Transwell device to co-culture N2a cells with OM-MSCs to investigate whether OM-MSCs could rescue N2a cells from OGD/R injury. The results are shown in **Figure 3**. OM-MSC co-culture notably reversed the decline in cell viability after OGD/R insult; meanwhile, the upregulated LDH production, apoptosis rate, as well as ROS level were also markedly reduced *via* OM-MSC co-culture upon OGD/R-induced injury (**Figures 3A–F**).

OM-MSCs Alleviated Neuronal Apoptosis and Oxidative Stress in I/R Rats

We then established the MCAO rat model to achieve cerebral IRI *in vivo*. The apoptosis rate in the I/R group was significantly higher than that in the sham group. NeuN and TUNEL double immunostaining further verified the increased neuronal apoptosis in I/R rats. Meanwhile, neuronal apoptosis induced by cerebral ischemia/reperfusion was notably alleviated by OM-MSC injection in the transplantation group rats (**Figures 4A–C**).

We also examined the oxidative stress level in each group of rats. Compared with the sham group, the LPO level was elevated while the T-SOD level was reduced in the I/R group. The opposite alterations of these two indicators supported that cerebral ischemia/reperfusion could indeed lead to increased levels of oxidative stress. Likewise, OM-MSC injection could downregulate the LPO level and upregulate the T-SOD level in I/R rats (**Figures 4D,E**).

OM-MSCs Reduced Cerebral Infarction Volume and Improved Neurologic Deficits in I/R Rats

The cerebral infarction volume in each group was examined by TTC staining. No infarction was observed in the sham group, while a white infarct lesion occurred in the I/R and transplantation groups, suggesting successful establishment of the MCAO model in rats. The infarction size in the transplantation group was significantly diminished, indicating that OM-MSC injection was able to reduce the cerebral infarction volume (**Figures 5A,B**).

Behavioral function was evaluated by the mNSS and rotarod treadmill. The mNSS was remarkably increased in the I/R 1-day, I/R 3-day, and I/R 7-day groups compared with that of the sham group and notably decreased in the transplantation 3-day and transplantation 7-day groups compared with that in the I/R group (**Figure 5C**). The rotarod treadmill results were also obviously improved by OM-MSC transplantation at 3 and 7 days (**Figure 5D**). In brief, our results confirmed that OM-MSC injection could improve neurologic deficits in I/R rats.

OM-MSCs Upregulated SPCA1 Expression in OGD/R-Treated N2a Cells and I/R Rats

The expression level of SPCA1 in N2a cells was identified to be decreased after OGD/R insult compared to the normal group, the alteration reaching a maximum in the 24-h reoxygenation at both the mRNA and protein levels (**Figures 6A–C**). OM-MSCs were able to upregulate the expression of SPCA1 in N2a cells after OGD/R injury (**Figures 6D–F**).

Similarly, cerebral ischemia/reperfusion contributed to a remarkable decline in the mRNA and protein levels of SPCA1 in the I/R group compared with the sham group, and OM-MSC transplantation was capable of upregulating the SPCA1 protein expression in I/R rats (**Figures 6G–I**).

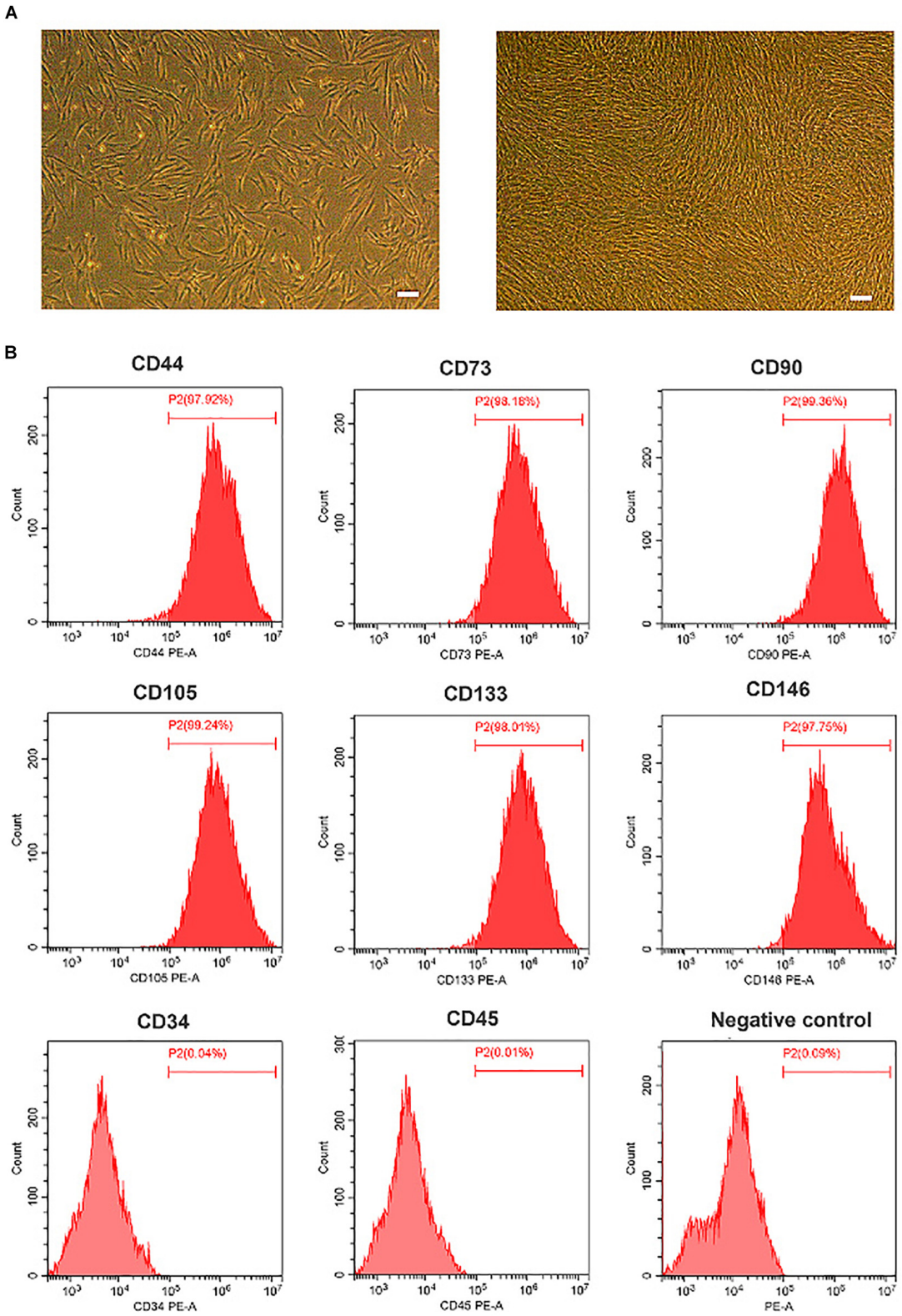

FIGURE 1 | Characterization of olfactory mucosa mesenchymal stem cells (OM-MSCs). **(A)** Morphology of OM-MSCs in cell culture. Cultured cells [day 1 after passage (*left*), day 3 after passage (*right*)] showed typical fibroblastic or spindle morphology (*scale bar*, 200 μm). **(B)** Immunophenotype of OM-MSCs at passage 3 identified by flow cytometry analysis.

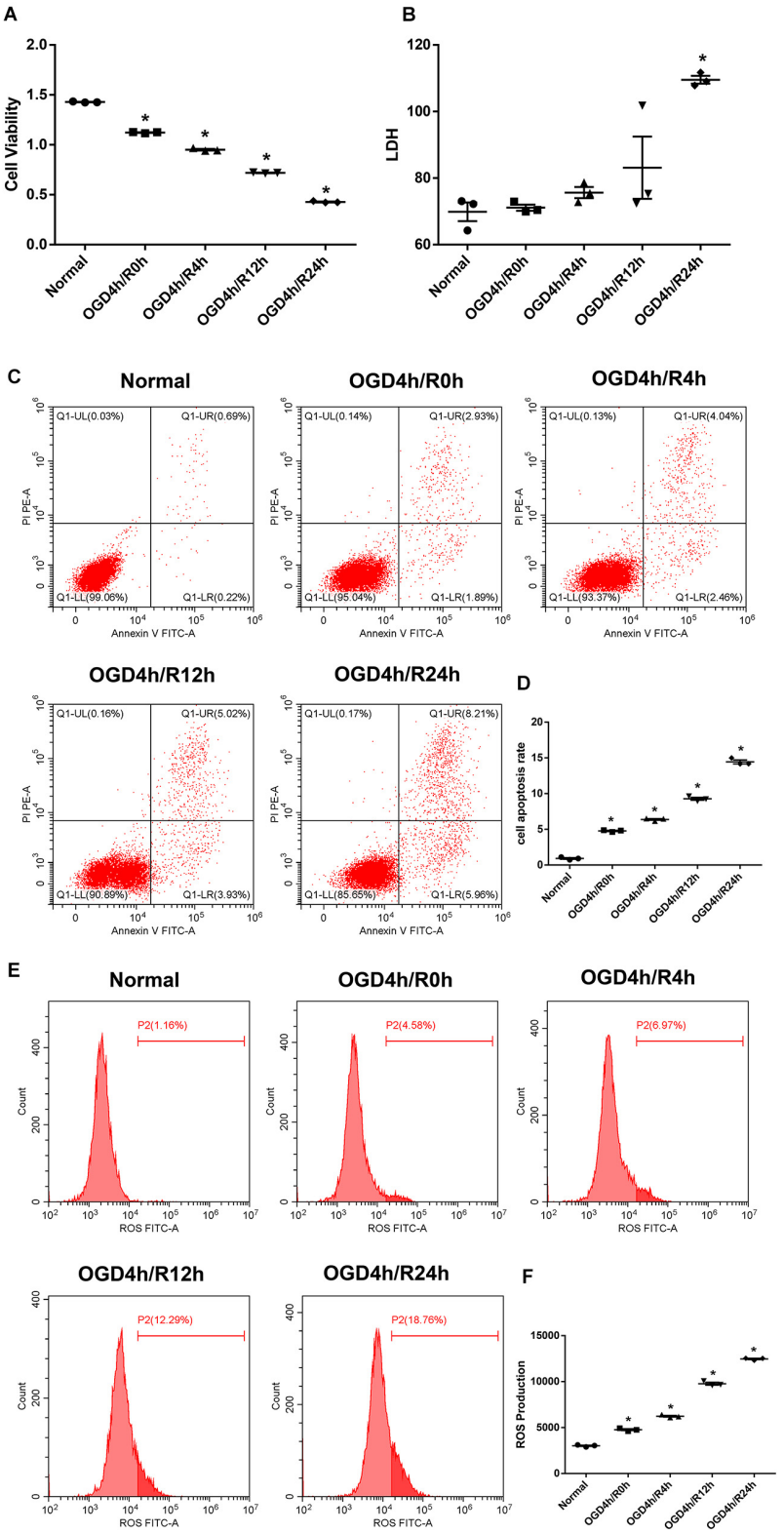

FIGURE 2 | Oxygen and glucose deprivation/reoxygenation (OGD/R) induce cell injury in N2a cells. **(A)** Cell viability was determined by the Cell Counting Kit-8 (CCK-8) assay. **(B)** Cell death was determined by the lactate dehydrogenase (LDH) assay. **(C,D)** Apoptosis was evaluated by flow cytometry analysis. **(E,F)** Intracellular reactive oxygen species (ROS) was detected using an oxidation-sensitive fluorescent probe (DCFH-DA). Data are shown as the mean ± SEM based on three independent experiments. $^{*}p \leq 0.05$, compared with the normal group.

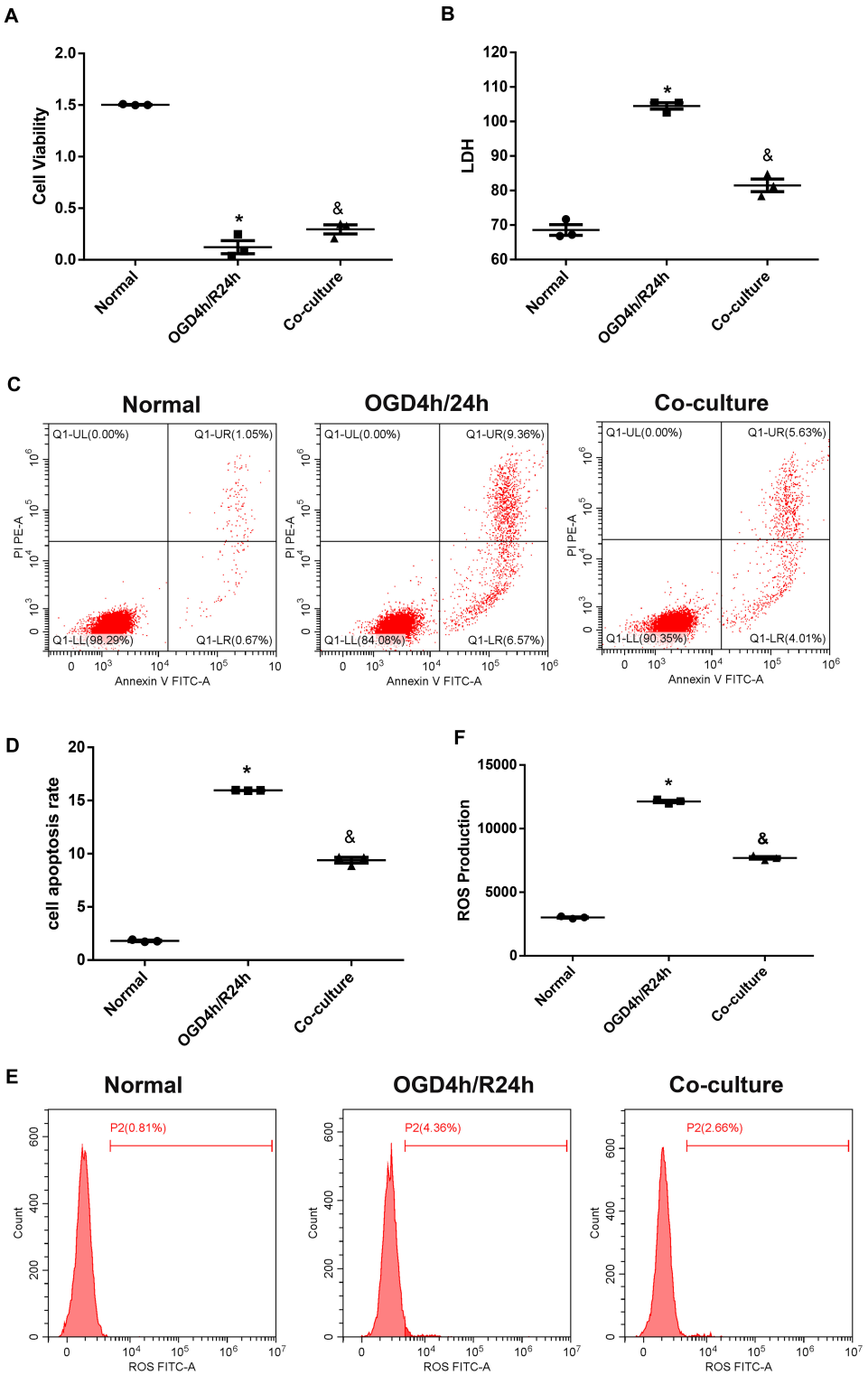

FIGURE 3 | Olfactory mucosa mesenchymal stem cells (OM-MSCs) attenuated oxygen and glucose deprivation/reoxygenation (OGD/R)-induced apoptosis and oxidative stress in N2a cells. **(A)** Cell viability was determined by the Cell Counting Kit-8 (CCK-8) assay. **(B)** Cell death was determined by the lactate dehydrogenase (LDH) assay. **(C,D)** Apoptosis was evaluated by flow cytometry analysis. **(E,F)** Intracellular reactive oxygen species (ROS) was detected using an oxidation-sensitive fluorescent probe (DCFH-DA). Data are shown as the mean ± SEM based on three independent experiments. $^{*}p \leq 0.05$, compared with the normal group and $^{\&}p \leq 0.05$, compared with the OGD4h/R24h group.

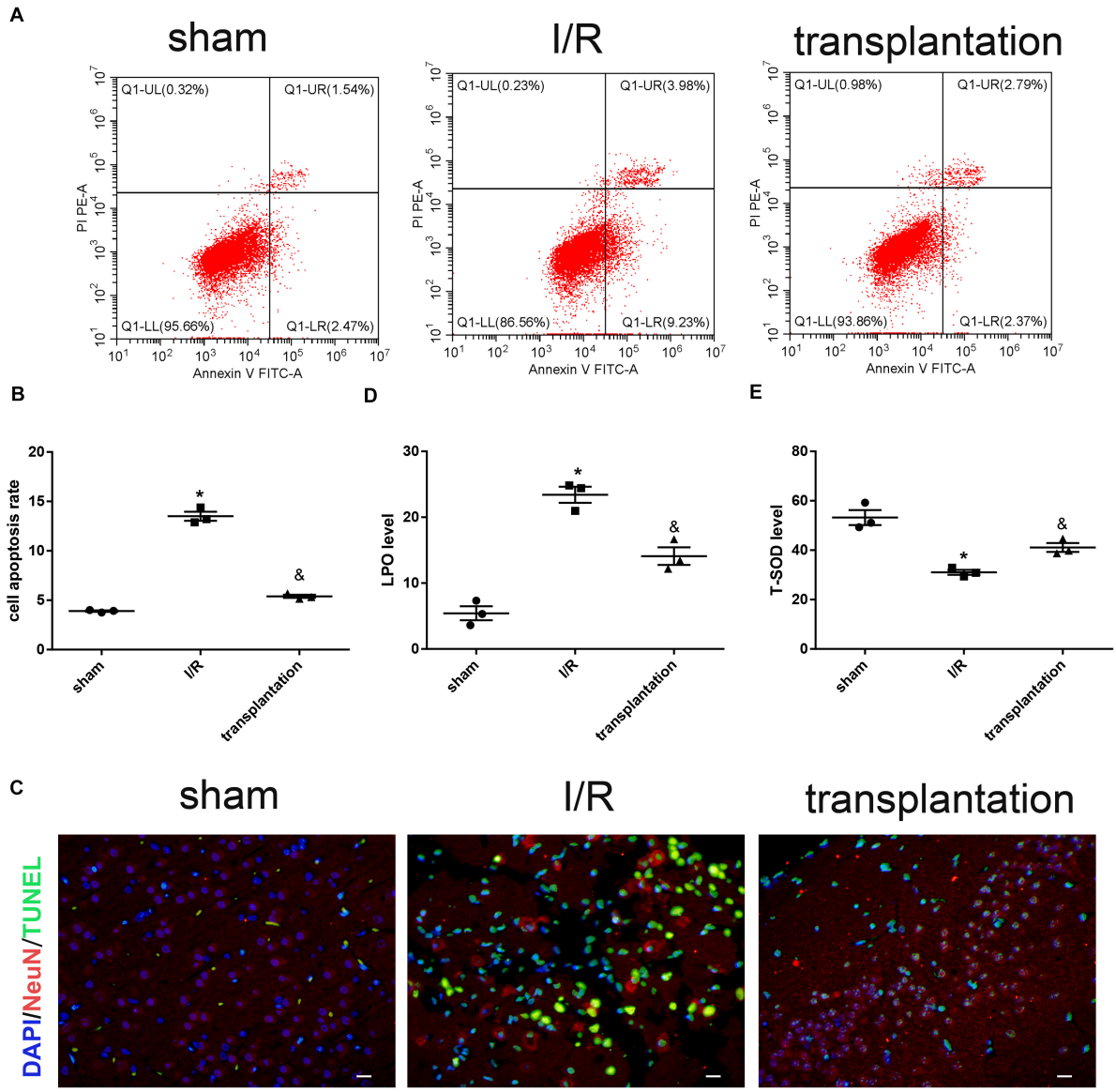

FIGURE 4 | Olfactory mucosa mesenchymal stem cells (OM-MSCs) alleviated neuronal apoptosis and oxidative stress in ischemia/reperfusion (I/R) rats. **(A,B)** Apoptosis of ipsilateral brain samples was evaluated by flow cytometry analysis. **(C)** Representative images of NeuN and TUNEL double immunostaining (*scale bar*, 40 μm). **(D,E)** Lipid peroxidation (LPO) and total superoxide dismutase (T-SOD) levels of ipsilateral brain samples were measured by the LPO assay and T-SOD assay kits, respectively. Data are shown as the mean ± SEM (n = 3 animals per group). $^{*}p \leq 0.05$, compared with the sham group and $^{\&}p \leq 0.05$, compared with the I/R group.

OM-MSCs Attenuated Ca^{2+} Overload and Improved GA Morphology in OGD/R-Treated N2a Cells and I/R Rats

Due to the function of SPCA1 being associated with intracellular Ca^{2+} homeostasis, we subsequently measured the intracellular Ca^{2+} concentrations and discovered a notably increased Ca^{2+} concentration in the cytoplasm after OGD/R exposure while a decreased Ca^{2+} concentration in GA, both of which reached the highest changes in the 24-h time point (**Figures 7A,B**). Interestingly, after OM-MSC co-culture following OGD/R insult, the increased Ca^{2+} concentration in the cytoplasm was obviously alleviated (**Figure 7C**), while the Ca^{2+} concentration in GA was upregulated (**Figure 7D**).

We also assayed the intracellular Ca^{2+} concentration in the rats from each group and observed an elevated Ca^{2+} concentration in I/R rats; likewise, OM-MSC transplantation could notably repress the increase of Ca^{2+} concentration in I/R rats (**Figures 7E,F**).

Moreover, we examined the GA ultramicrostructure changes of neurons using an electron microscope. As visualized in **Figure 7G**, neurons in the sham group had GA with normal

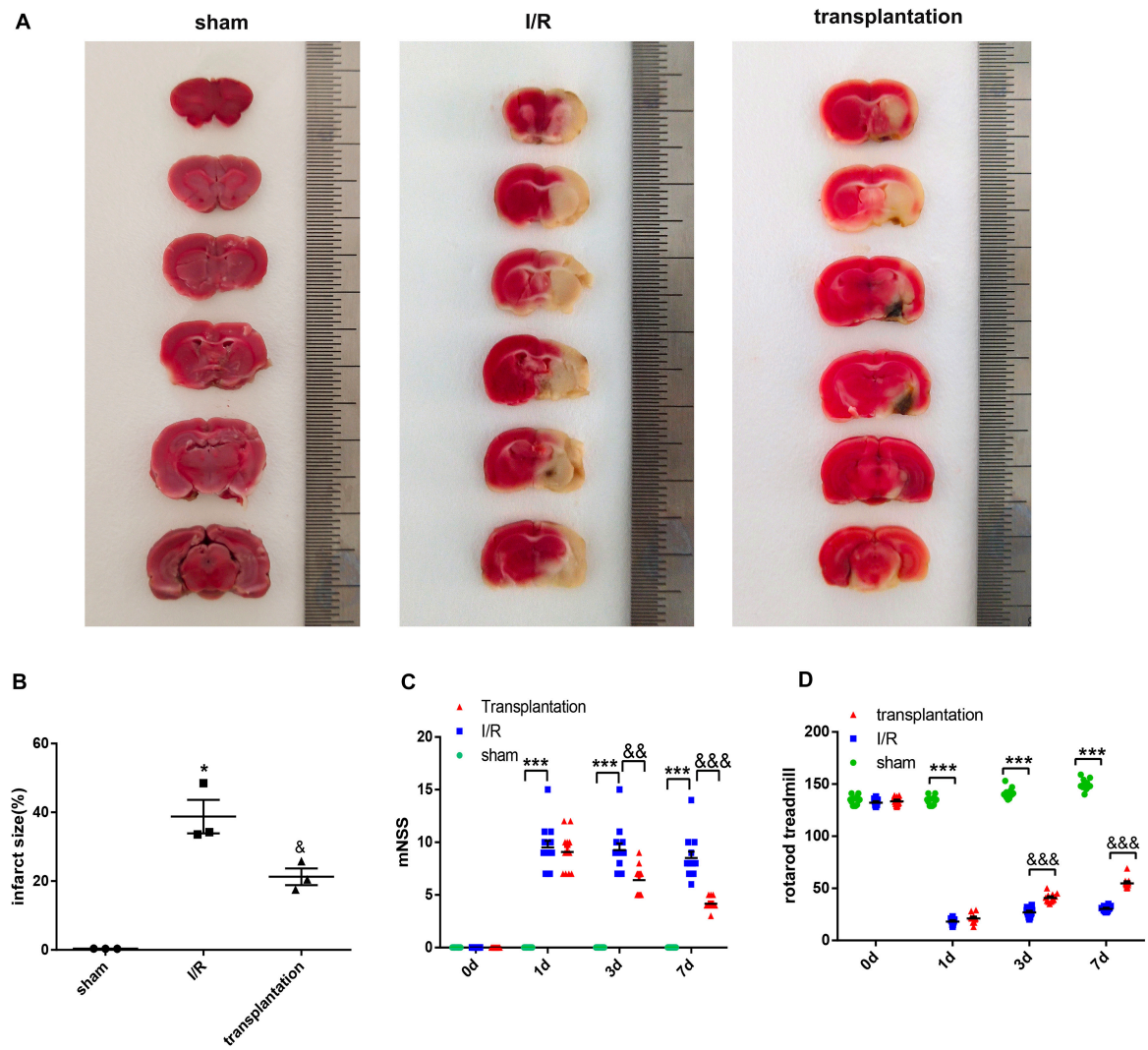

FIGURE 5 | Olfactory mucosa mesenchymal stem cell (OM-MSC) injection reduced the cerebral infarction volume and alleviated neurologic deficits in ischemia/reperfusion (I/R) rats. **(A,B)** The infarct volume was determined by 2,3,5-triphenyltetrazolium chloride (TTC) staining (n = 3 animals per group). **(C,D)** The *line charts* show the results of the modified neurologic severity score (mNSS; n = 12 animals per group) and rotarod treadmill (n = 12 animals per group). Data are shown as the mean ± SEM. $^{*}p \leq 0.05$, $^{***}p < 0.001$, compared with the sham group and $^{\&}p \leq 0.05$, $^{\&\&}p < 0.01$, and $^{\&\&\&}p < 0.001$, compared with the I/R group.

morphology and structure, accompanied by the endoplasmic reticulum, lysosomes, mitochondria, nerve microfilaments, neural tubes, and a complete double nuclear membrane. In the I/R group, the GA was swollen and dissolved, other organelles were also fractured, and the nuclear membrane became blurred. However, the GA was less edematous in the transplantation group. Collectively, the ultramicropathological changes of the GA in the transplantation groups were less significant compared with those in the I/R group.

OM-MSCs Protected N2a Cells From OGD/R-Induced Injury Through Modulating SPCA1

To explore the role of SPCA1 in the neuroprotective effect of OM-MSCs against cerebral IRI, plasmid containing SPCA1 shRNA sequence was constructed and transfected into N2a cells before the experiment. The transduction results were verified by PCR as well as Western blot analysis, which are shown in **Figures 8A–C**. Transfection with SPCA1 shRNA contributed to a notable decrease in the mRNA and protein levels compared with control shRNA.

Compared with the control shRNA group, the apoptosis rate, ROS levels, LDH production, as well as the Ca^{2+} concentration in the cytoplasm of N2a cells induced by OGD/R insult were apparently increased in the SPCA1 shRNA group, and SPCA1 depletion in N2a cells mitigated the protective effects of OM-MSCs following OGD/R injury (**Figures 8D–I**). Meanwhile, after OGD/R injury, the Ca^{2+} concentration in the GA of N2a cells was significantly lower in the SPCA1 shRNA group than in the control shRNA group, and SPCA1 knockdown in N2a cells restricted the

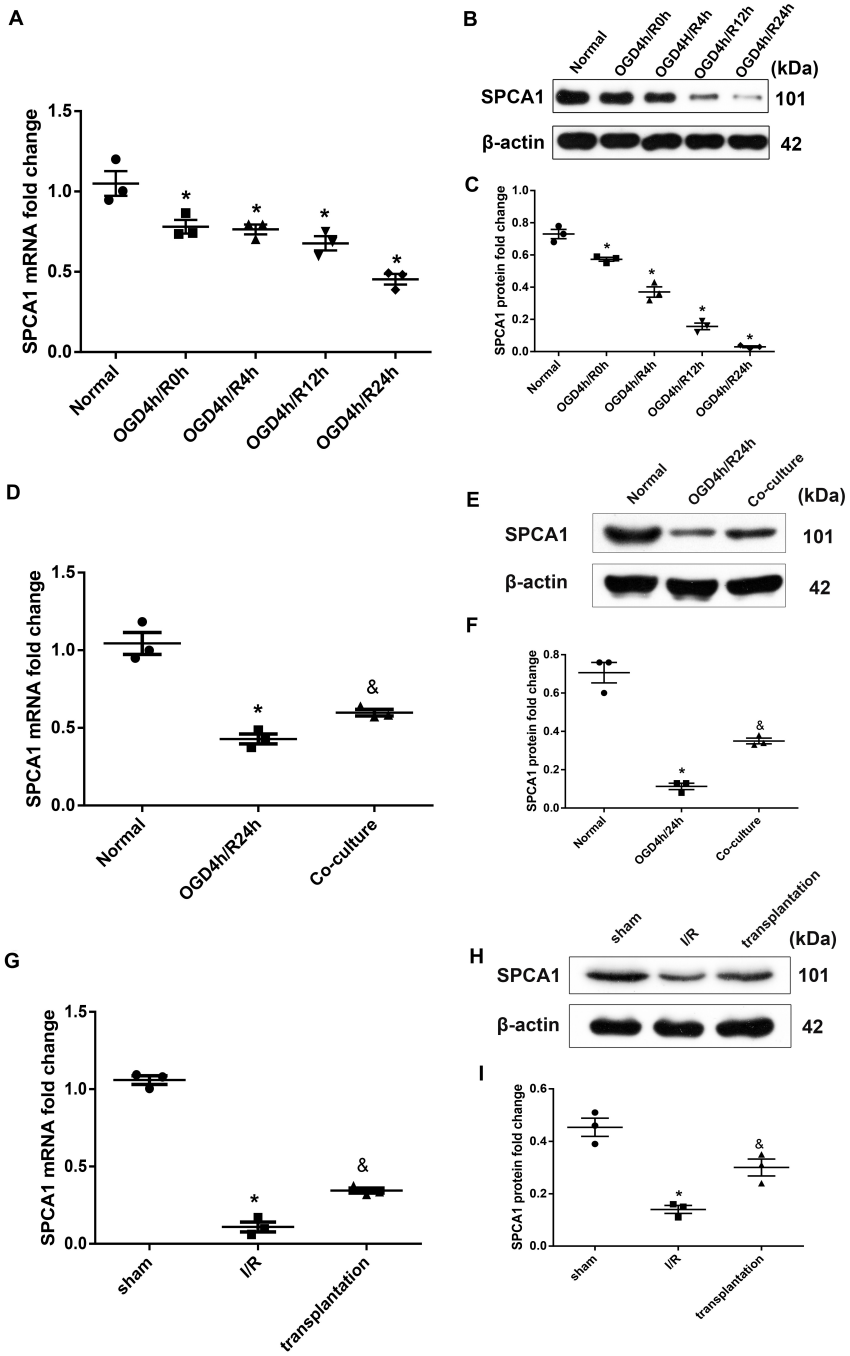

FIGURE 6 | Olfactory mucosa mesenchymal stem cells (OM-MSCs) upregulated SPCA1 expression in oxygen and glucose deprivation/reoxygenation (OGD/R)-treated N2a cells and ischemia/reperfusion (I/R) rats. **(A–C)** SPCA1 messenger RNA (mRNA) and protein expressions of OGD/R-treated N2a cells at different time points were visualized by quantitative PCR (qPCR) and Western blot. **(D–F)** SPCA1 mRNA and protein expressions of N2a cells in the normal, OGD4h/R24h, and OM-MSC co-culture groups were measured by qPCR and Western blot. **(G–I)** SPCA1 mRNA and protein expressions of rats' ipsilateral brain samples in the sham, I/R, and OM-MSC transplantation groups were detected by qPCR and Western blot. Data are shown as the mean ± SEM based on three independent experiments. $^{*}p \leq 0.05$, compared with the normal or sham group and $^{\&}p \leq 0.05$, compared with the OGD4h/R24h or I/R group.

capacity of OM-MSCs to upregulate the Ca^{2+} concentration in the GA of N2a cells after OGD/R insult (**Figure 8J**). Overall, the above findings showed that OM-MSCs protected N2a cells from OGD/R-induced injury probably through modulating SPCA1.

DISCUSSION

We successfully established *in vivo* and *in vitro* models of cerebral IRI, as previously described (Longa et al., 1989; Huang and Hu,

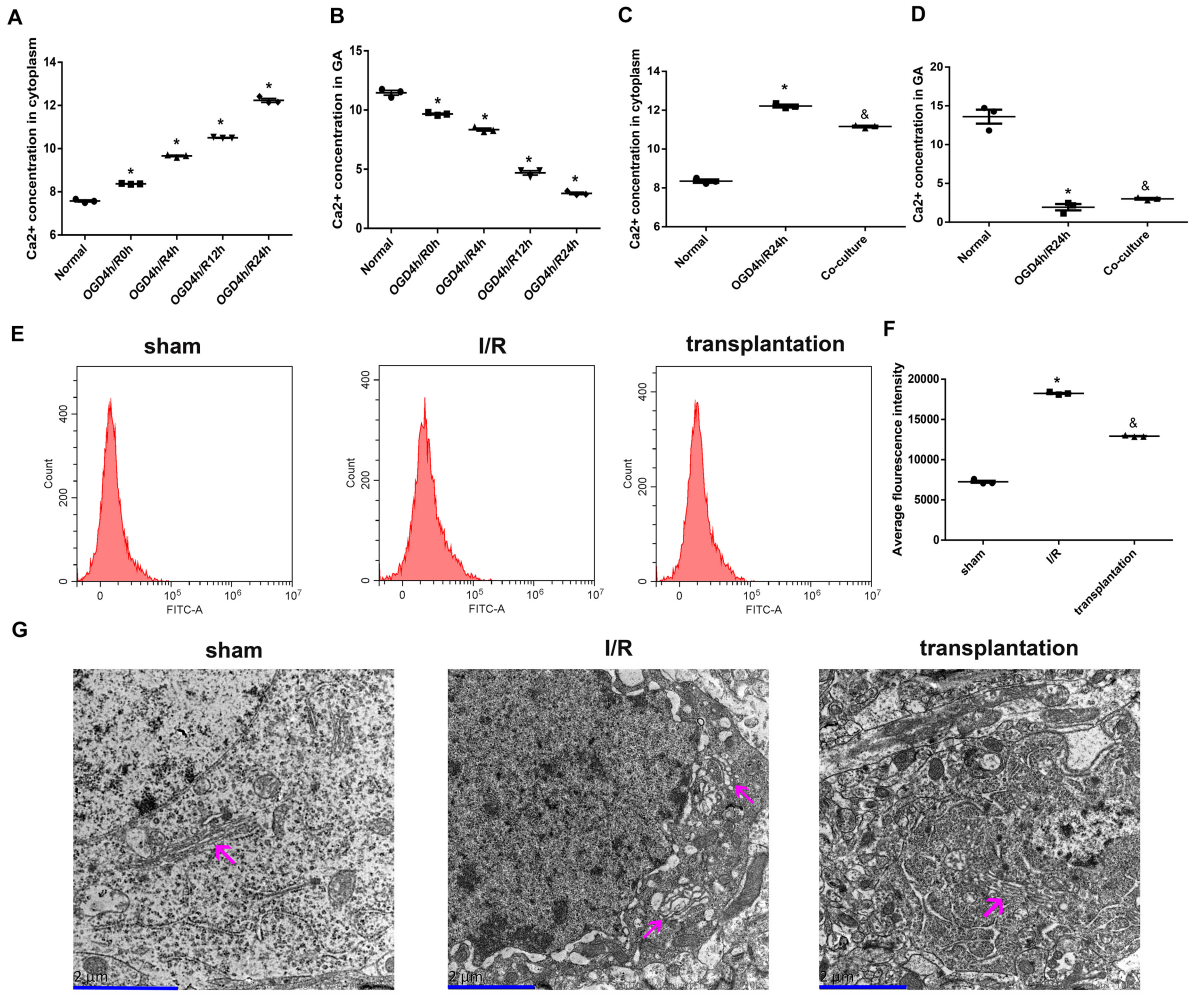

FIGURE 7 | Olfactory mucosa mesenchymal stem cells (OM-MSCs) attenuated Ca^{2+} overload and improved Golgi apparatus (GA) morphology in oxygen and glucose deprivation/reoxygenation (OGD/R)-treated N2a cells and ischemia/reperfusion (I/R) rats. **(A,B)** Ca^{2+} concentrations in the cytoplasm and GA of OGD/R-treated N2a cells at different time points were determined by the Ca^{2+} Assay Kit. **(C,D)** Ca^{2+} concentrations in the cytoplasm and GA of N2a cells in the normal, OGD4h/R24h, and OM-MSC co-culture groups were measured by the Ca^{2+} Assay Kit. **(E,F)** Intracellular Ca^{2+} of rats' ipsilateral brain samples in the sham, I/R, and OM-MSC transplantation groups were detected by flow cytometry analysis using a Fluo-3/AM kit. **(G)** Representative image of GA ultramicrostructure changes by using an electron microscope (*scale bar*, 2 μm). The GA was indicated by the *magenta arrow*. Data are shown as the mean ± SEM based on three independent experiments. $^{*}p \leq 0.05$, compared with the normal or sham group and $^{\&}p \leq 0.05$, compared with the OGD4h/R24h or I/R group.

2018). It is well known that OGD/R-induced cell injury is mainly characterized by a decreased cell viability and increased apoptosis rate and LDH release level (Huang and Hu, 2018; Ma et al., 2020), and our results were consistent with previous studies. Meanwhile, significant cerebral infarction lesions were observed by TTC staining of the rats' brain tissues, suggesting the establishment of a successful *in vivo* model.

Oxidative stress, induced by ROS during cerebral ischemia and especially reperfusion, is important in the pathological process of ischemic stroke and is critical for the cascade development of cerebral IRI (Wu et al., 2020). It results in LPO, apoptosis, and, ultimately, neuronal death together with other pathophysiological mechanisms. We also found that the ROS and LPO levels were increased while the SOD levels were decreased in our models of cerebral IRI.

Mesenchymal stem cells have exhibited therapeutic properties on IRI because of their paracrine activity, cell–cell interaction, anti-inflammatory activity, and immunomodulatory effects (Souidi et al., 2013; Barzegar et al., 2019; Oliva, 2019; Tobin et al., 2020). Leu et al. (2010) have demonstrated that intravenous injection of AD-MSCs significantly attenuated oxidative stress in an experimental ischemic stroke model. Calio et al. (2014) have concluded that the transplantation of BM-MSCs decreases oxidative stress and apoptosis in the brain of stroke rats. Alhazzani et al. (2018) have found that MSC co-culture could protect Ca^{2+} and oxidant-mediated damage in SH-SY5Y-differentiated neuronal cells. Similarly, our results also showed that OM-MSCs were able to downregulate ROS as well as LPO levels and upregulate antioxidase SOD levels in the cerebral IRI models, eventually reducing neuronal apoptosis and infarction

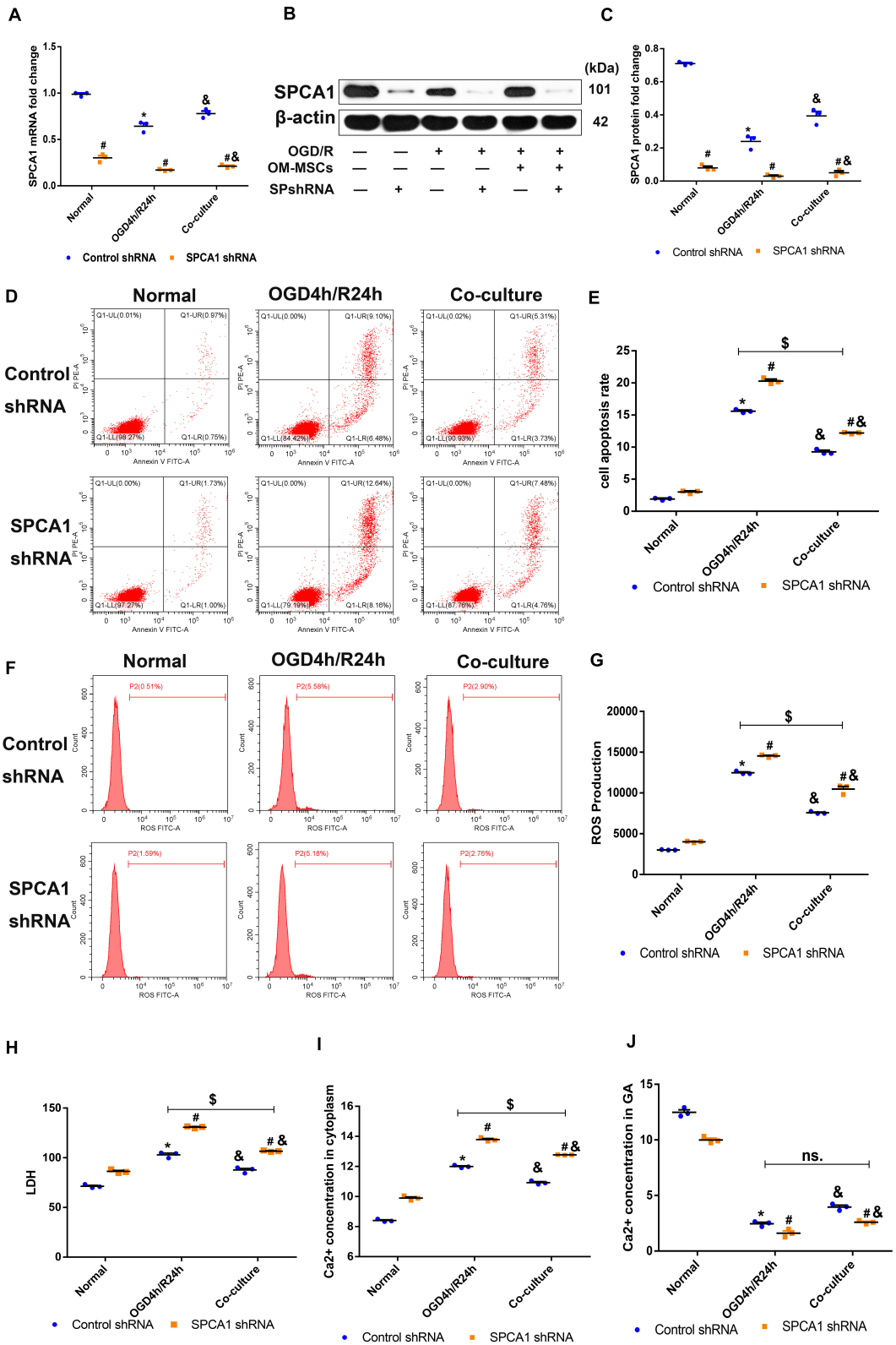

FIGURE 8 | Olfactory mucosa mesenchymal stem cells (OM-MSCs) protected N2a cells from oxygen and glucose deprivation/reoxygenation (OGD/R)-induced injury through modulating SPCA1. **(A–C)** SPCA1 messenger RNA (mRNA) and protein expressions in both the control short hairpin RNA (shRNA) group and the SPCA1 shRNA group were visualized by quantitative PCR (qPCR) and Western blot. **(D,E)** Apoptosis in both the control shRNA group and the SPCA1 shRNA group was

(Continued)

FIGURE 8 | Continued
evaluated by flow cytometry analysis. **(F,G)** Intracellular reactive oxygen species (ROS) in both the control shRNA group and the SPCA1 shRNA group was detected using an oxidation-sensitive fluorescent probe (DCFH-DA). **(H)** Cell death in both the control shRNA group and the SPCA1 shRNA group was determined by the lactate dehydrogenase (LDH) assay. **(I,J)** Ca^{2+} concentrations in the cytoplasm **(I)** and the Golgi apparatus (GA; **J)** in both the control shRNA group and the SPCA1 shRNA group were determined by the Ca^{2+} Assay Kit. Data are shown as the mean ± SD based on three independent experiments. $^{*}p \leq 0.05$, compared with the normal group; $^{\&}p \leq 0.05$, compared with the OGD4h/R24h group; and $^{\#}p \leq 0.05$, compared with the control shRNA group. $^{\$}p \leq 0.05$, ns. $p > 0.05$, compared with OGD4h/R24h of the control shRNA group.

volume. Consequently, we fully believe that OM-MSCs could also confer cerebral protection against IRI by suppressing oxidative stress.

In recent years, with the concept of "GA stress" proposed, the complex role of GA in oxidative stress has been gradually recognized (Jiang et al., 2011; Li et al., 2016). Based on the findings of Jiang et al. (2011) and He and Hu (2012), as one of the Ca^{2+} transporters of GA, SPCA1 played an important role in the process of GA maintaining intracellular Ca^{2+} homeostasis under physiological conditions. However, the activity and expression of SPCA1 were decreased during cerebral ischemia/reperfusion, and its ability to uptake intracellular Ca^{2+} was also impaired, leading to intracellular Ca^{2+} overload (Pavlíková et al., 2009; Li et al., 2015; Fan et al., 2016). It is well known that Ca^{2+} overload is another fatal molecular event in cerebral IRI (Kalogeris et al., 2016; Radak et al., 2017). Sustained excessive intracellular calcium levels often cause neuronal cell hypercontracture, proteolysis, and, eventually, death (Pittas et al., 2019). During reperfusion, the metabolites produced by oxidative stress destroy the integrity of the cell membrane and organelle membrane, and Ca^{2+} release channels on the cell membranes and organelle membranes are opened, resulting in Ca^{2+} influx into the cytoplasm from the extracellular environment and the endoplasmic reticulum or sarcoplasmic reticulum (Li et al., 2015). And more importantly, Ca^{2+} overload could also enhance oxidative stress, their interaction promoting the pathological process of an IRI cascade (Jiang et al., 2011). In this paper, we discovered a decrease in SPCA1 expression, an increase in cytoplasmic Ca^{2+} levels, and a decrease in GA Ca^{2+} levels in the ischemic stroke model, which were in line with previous studies.

Besides, the GA fragment, another typical manifestation of GA stress in ischemic stroke, was often induced by oxidative stress and apoptosis (Zhong et al., 2015; Zhang et al., 2019). The damage of microtubule proteins mainly contributed to the fragmentation and even dissolution of the GA during oxidative stress and apoptosis. Our results presented that the GA was swollen and dissolved in the neuron of I/R rats, which was in accordance with existing evidences.

Previous researches on the neuroprotective role of MSCs at the subcellular organelle level in ischemic stroke always focused on the mitochondria and endoplasmic reticulum (Xing et al., 2016; Mahrouf-Yorgov et al., 2017). The impact of stem cell therapy on the function and morphology of GA after cerebral IRI was uncovered. Based on the results above, our results firstly demonstrated that OM-MSCs were able to upregulate SPCA1 expression, rescue its function of maintaining intra-Golgi Ca^{2+} homeostasis, and reduce the edema and dissolution of GA in neurons of ischemic stroke models.

Since SPCA1 has previously been shown to exhibit anti-oxidative stress and anti-apoptotic effects in ischemic stroke (Uccelletti et al., 2005; Sepulveda et al., 2009), the upregulation of SPCA1 expression and other neuroprotective effects of OM-MSCs in an ischemia/reperfusion model have also been confirmed according to our results. As a result, we speculated whether the neuroprotective effect of OM-MSCs on cerebral IRI was associated with its ability to upregulate SPCA1 expression and rescue its function in neurons. Subsequently, we used SPCA1 shRNA to construct a SPCA1 knockout model in N2a cells and found that SPCA1 shRNA partly restricted the capacity of OM-MSCs to alleviate OGD/R-induced apoptosis and elevated the ROS levels and LDH production as well as the intracellular Ca^{2+} overload. The above findings suggested that the expression and function of SPCA1 in neurons were relevant to the neuroprotective effect of OM-MSCs on cerebral IRI.

Nevertheless, we also observed that OM-MSCs still had a partial protective effect, including reduced apoptosis and ROS production and regulated Ca^{2+} concentration in the cytoplasm, on OGD/R-induced cell injury in the case of SPCA1 being knocked down. We considered that this outcome was related to the function diversity and plasticity of OM-MSCs. Previous studies have concluded that MSCs exhibited anti-oxidative, anti-apoptotic, endogenous neurogenesis, synaptogenesis, angiogenesis, anti-inflammatory, and immunomodulatory effects in ischemic stroke (Souidi et al., 2013; Hao et al., 2014), and MSCs exerted their neuroprotective effects partly by secreting neurotrophic factors, such as VEGF, NGF, BDNF, and bFGF, etc. (Stonesifer et al., 2017; Barzegar et al., 2019). The secretome of OM-MSCs has been identified by Ge et al. (2016), and their results showed that these secreted proteins were associated with neurotrophy, angiogenesis, cell growth, differentiation, and apoptosis. Consequently, OM-MSCs may be capable of attenuating cerebral IRI partly by secreting a series of neurotrophic factors. Those molecules may reduce apoptosis and oxidative stress levels by acting on the mitochondria and endoplasmic reticulum when SPCA1 was blocked. That the function of the injured mitochondria and endoplasmic reticulum could be rescued by other types of MSCs has been confirmed by previous researchers (Chi et al., 2018; Tseng et al., 2020). In terms of the regulation of intracellular Ca^{2+} by OM-MSCs during cerebral ischemia/reperfusion when SPCA1 was knocked down, the possible mechanisms were as follows: firstly, OM-MSCs reduced the oxidative stress level through other feasible pathways, which inhibited the Ca^{2+} influx from extracellular stores and the endoplasmic reticulum, eventually leading to a decline in the Ca^{2+} concentration of the cytoplasm. Secondly, in addition to GA, there were also Ca^{2+} uptake channels in the

endoplasmic reticulum membrane, which were also impaired by IRI (Jiang et al., 2011). OM-MSCs may be able to rescue these channels and subsequently reduce the Ca^{2+} overload in the cytoplasm to a certain extent. Accordingly, OM-MSCs could still exhibit part of the ability to reduce apoptosis as well as ROS production and regulate the Ca^{2+} concentration after SPCA1 knockdown.

Nowadays, cell-based therapies are considered to be one of the most promising options to radically advance ischemic stroke treatment (Boltze et al., 2019), and animal models of ischemic stroke are indispensable for their translation into clinical trials. Hence, the establishment of a highly efficient and predictable animal model is conductive to improve the quality of preclinical researches regarding cell therapy (Kringe et al., 2020). In the present study, the choice of male rats effectively eliminated the influence of female sex hormones on the effect of cell therapy, and the standardization of the animal housing conditions greatly reduced its impact on the neurological endpoint. Randomized grouping and allocation concealment also avoided the limitations of other confounding factors to some degree (Boltze et al., 2017; Bosetti et al., 2017). Additionally, the reperfusion model we used here was in line with the recommendations of the guidelines for the study of neuroprotective therapies in recanalization scenarios (Savitz et al., 2019). Consequently, we believed that these advantages would make our results more credible.

However, there are still some limitations regarding this study, which are expected to be improved on in subsequent studies. The first is the small sample size. It would be significant to perform the examination of this treatment in a large cohort for subsequent confirmation. Secondly, the mNSS and rotarod treadmill are widely used neurofunctional assessments in experimental stroke, but other evidences indicated that the mNSS and rotarod are not perfectly fit for neurofunctional assessments after stroke in the context of MSC-based therapies since these two behavioral tests could not distinguish recovery from compensatory behavior well (Boltze et al., 2014; Balkaya et al., 2018). Therefore, it is recommended to choose behavioral tests that are minimally affected by behavior compensation in future experimental stroke, such as Montoya's staircase and the cylinder test. Thirdly, studies on other types of cells have found that cryopreservation limited the effectiveness of those cell types (Weise et al., 2014). A similar exploration should also be carried out in cryopreserved OM-MSCs. Fourthly, no immunosuppressive agent was used, although in xenotransplantation, the possible graft rejection could directly influence the therapeutic outcome of OM-MSCs. However, other investigators recommended MSCs as a novel immunomodulatory strategy in preclinical transplantation studies due to their immunosuppressive properties (Diehl et al., 2017), suggesting that MSCs could be applied relatively safely in non-autologous approaches. Lastly, the establishment of the SPCA1 gene knockout rats is promising. It will provide a more profound understanding of the mechanism regarding SPCA1 in the neuroprotective effect of OM-MSCs on cerebral IRI.

In summary, our findings suggest that OM-MSCs may be a useful candidate of cell therapies for the treatment of ischemic stroke. OM-MSCs exert neuroprotective effects against cerebral IRI, probably *via* modulating SPCA1 and reducing the edema and dissolution of the GA in neurons. Further studies will be conducted to highlight the role of SPCA1 in the neuroprotection of OM-MSCs *in vivo* by constructing gene knockout animal models of ischemic stroke.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical Committee of Hunan Normal University. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Laboratory Animal Ethics Committee of the Second Affiliated Hospital of Hunan Normal University.

AUTHOR CONTRIBUTIONS

ZH acquired the funding. JH attended in research design, experimental performances except animal behavioral tests, data analysis, and drafting the manuscript. JL participated in cell culture and animal behavioral tests. YH participate in cell culture and data analysis. YZ participated in animal experiment and behavioral tests. WC took part in animal experiment. DD and XT discussed the results. ZH and ML took care of all aspects including research design, data analysis, and manuscript preparation. All authors read and approved the final manuscript.

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BRIEF RESEARCH REPORT
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Stem Cell Factor in Combination With Granulocyte Colony-Stimulating Factor Protects the Brain From Capillary Thrombosis-Induced Ischemic Neuron Loss in a Mouse Model of CADASIL

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Cerebral autosomal dominant arteriopathy with subcortical infarct and leukoencephalopathy (CADASIL) is a Notch3 mutation-induced cerebral small vessel disease, leading to recurrent ischemic stroke and vascular dementia. There is currently no treatment that can stop or delay CADASIL progression. We have demonstrated the efficacy of treatment with combined stem cell factor (SCF) and granulocyte colony-stimulating factor (G-CSF) (SCF+G-CSF) in reducing cerebral small vessel thrombosis in a TgNotch3R90C mouse model of CADASIL. However, it remains unknown whether SCF+G-CSF treatment protects neurons from microvascular thrombosis-induced ischemic damage. Using bone marrow transplantation to track thrombosis, we observed that capillary thrombosis was widely distributed in the cortex, striatum and hippocampus of 22-month-old TgNotch3R90C mice. However, the capillary thrombosis mainly occurred in the cortex. Neuron loss was seen in the area next to the thrombotic capillaries, and severe neuron loss was found in the areas adjacent to the thrombotic capillaries with bifurcations. SCF+G-CSF repeated treatment significantly attenuated neuron loss in the areas next to the thrombotic capillaries in the cortex of the 22-month-old TgNotch3R90C mice. Neuron loss caused by capillary thrombosis in the cerebral cortex may play a crucial role in the pathogenesis of CADASIL. SCF+G-CSF treatment ameliorates the capillary thrombosis-induced ischemic neuron loss in TgNotch3R90C mice. This study provides new insight into the understanding of CADASIL progression and therapeutic potential of SCF+G-CSF in neuroprotection under microvascular ischemia in CADASIL.

Keywords: CADASIL, SCF, G-CSF, capillary thrombosis, microinfarction

INTRODUCTION

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is the most common monogenic cause of stroke and vascular dementia in adults (Chabriat et al., 2009). Currently, the pathogenesis of CADASIL remains poorly understood, and there is no treatment that can stop or delay CADASIL progression.

CADASIL is caused by mutations in the NOTCH3 gene (Joutel et al., 1996). NOTCH3-encoded Notch3 receptor is predominantly expressed in vascular smooth muscle cells (VSMCs) of small arteries (Wang et al., 2012) and pericytes of capillaries (Wang et al., 2014). Due to the specific distribution of Notch3, the typical pathologies in the brain are mainly found in small arteries and capillaries in both CADASIL patients and mouse models (Joutel, 2011). Pathological changes in endothelial cells (ECs) have been observed in CADASIL patients and the TgNotch3R90C mouse model of CADASIL (Ruchoux and Maurage, 1998; Ping et al., 2018, 2019). It has also been revealed that disrupted blood-brain barrier (BBB) integrity (Ping et al., 2018), increased thrombosis in cerebral small vessels (Ping et al., 2018), reduced cerebral blood vessel density (Liu et al., 2015; Ping et al., 2019) and impaired endothelium-dependent vasodilation (Stenborg et al., 2007) occur in CADASIL patients and TgNotch3R90C mice. Endothelial dysfunction is crucially involved in vascular ischemia as ECs shift from an anti-thrombotic to a pro-thrombotic stage when their function is dysregulated (Yau et al., 2015).

Cerebral microcirculation plays a vital role in brain health. Cerebral capillaries with a total length of ∼400 miles in humans are the primary site of oxygen, nutrient and metabolic exchange (Cipolla, 2009). Cerebral microcirculation impairment is a central pathology in Alzheimer's dementia (Iadecola, 2015; van de Haar et al., 2015), while it remains unclear about the pathological role of cerebral microvascular impairment in CADASIL. There is a knowledge gap about the involvement of cerebral capillary thrombosis in ischemic neuronal death in CADASIL.

Stem cell factor (SCF) and granulocyte colony-stimulating factor (G-CSF) are the essential hematopoietic growth factors regulating blood cell production and bone marrow stem cell survival (Welte et al., 1985; Zsebo et al., 1990). Recently, we have demonstrated that SCF+G-CSF treatment ameliorates cerebrovascular endothelial cell (EC) damage and reduces cerebral capillary thrombotic formation in TgNotch3R90C mice (Ping et al., 2018, 2019). In addition, SCF+G-CSF treatment has been shown to reduce infarction size in acute ischemic stroke in a rat model of focal cerebral ischemia (Zhao et al., 2007b). However, whether SCF+G-CSF treatment could enhance neuronal survival in the area of thrombotic capillary remains elusive.

This study aims to determine the distribution of capillary thrombosis in the brain, the existence of cerebral capillary thrombosis-caused ischemic neuron loss, and the efficacy of SCF+G-CSF treatment in reducing microvascular ischemic damage in TgNotch3R90C mice.

MATERIALS AND METHODS

Animals and Treatments

All procedures in this study were approved by Institutional Animal Care and Use Committee at SUNY Upstate Medical University and LSUHSC.

Transgenic mice (TgNotch3R90C) carrying a full-length human NOTCH3 gene with the Arginine-to-Cysteine (Arg90Cys) mutation driven by the SM22α promoter were used as a mouse model of CADASIL (Ruchoux et al., 2003). The original breeders were generously provided as gifts from Dr. Anne Joutel (Faculté de Médecine, Paris, France). Eight-month-old male TgNotch3R90C mice were randomly divided into two groups: a vehicle control group and an SCF+G-CSF-treated group. All mice received a lethal dose of radiation (900 rad) to destroy their own bone marrow. Within 24 h after irradiation, bone marrow isolated from transgenic mice ubiquitously expressing enhanced green fluorescent protein (GFP) (UBC-GFP mice) was transplanted to the irradiated mice. One month after bone marrow transplantation, the first treatment of SCF+G-CSF was initiated at 9 months of age, which is 1 month before cerebrovascular dysfunction is shown in TgNotch3R90C mice (Ruchoux et al., 2003; Lacombe et al., 2005). Recombinant mouse SCF (100 μg/kg, diluted with saline) (PeproTech, Rocky Hill, NJ, United States) and recombinant human G-CSF (50 μg/kg, diluted with 5% dextrose) (Amgen, Thousand Oaks, CA, United States) were subcutaneously administered for 5 days. An equal volume of vehicle solution (50% saline and 50% of 5% dextrose) was injected into the control mice. The same treatment was then repeated an additional 4 times at the ages of 10, 12, 15, and 20 months. The final treatment was given at 200 μg/kg of SCF and 50 μg/kg of G-CSF to further enhance the effectiveness. Mice were euthanized at the age of 22 months (n = 5/group) (**Figure 1A**).

Bone Marrow Transplantation

The bone marrow of UBC-GFP mice (C57BL/6 background) was transplanted into the TgNotch3R90C mice (C57BL/6 background) to track blood clots (thrombosis) in the cerebral capillaries of TgNotch3R90C mice. UBC-GFP mice (male, 6 to 8 weeks old, Jackson Laboratory) were anesthetized with Avertin (0.4 g/kg body weight, intraperitoneally) (Sigma-Aldrich, St. Louis, MO, United States) and euthanized. The femur bones were dissected and placed into a cell culture dish with ice-cold sterile Hanks Balanced Salt Solution (HBSS) (ThermoFisher Scientific, Pittsburgh, PA, United States). Bone marrow cells were flushed out, gently triturated, and filtered through a 70 μm nylon mesh (Corning, Fisher Scientific, Pittsburgh, PA, United States). Harvested cells were centrifuged, re-suspended with HBSS into single cell suspension and then transplanted to the irradiated TgNotch3R90C mice by tail vein injection (1×10^7 bone marrow cells in 0.6 ml HBSS per mouse).

Brain Tissue Preparation and Immunohistochemistry

After being anesthetized with Avertin (0.4 g/kg, intraperitoneally), mice were euthanized by transcardiac

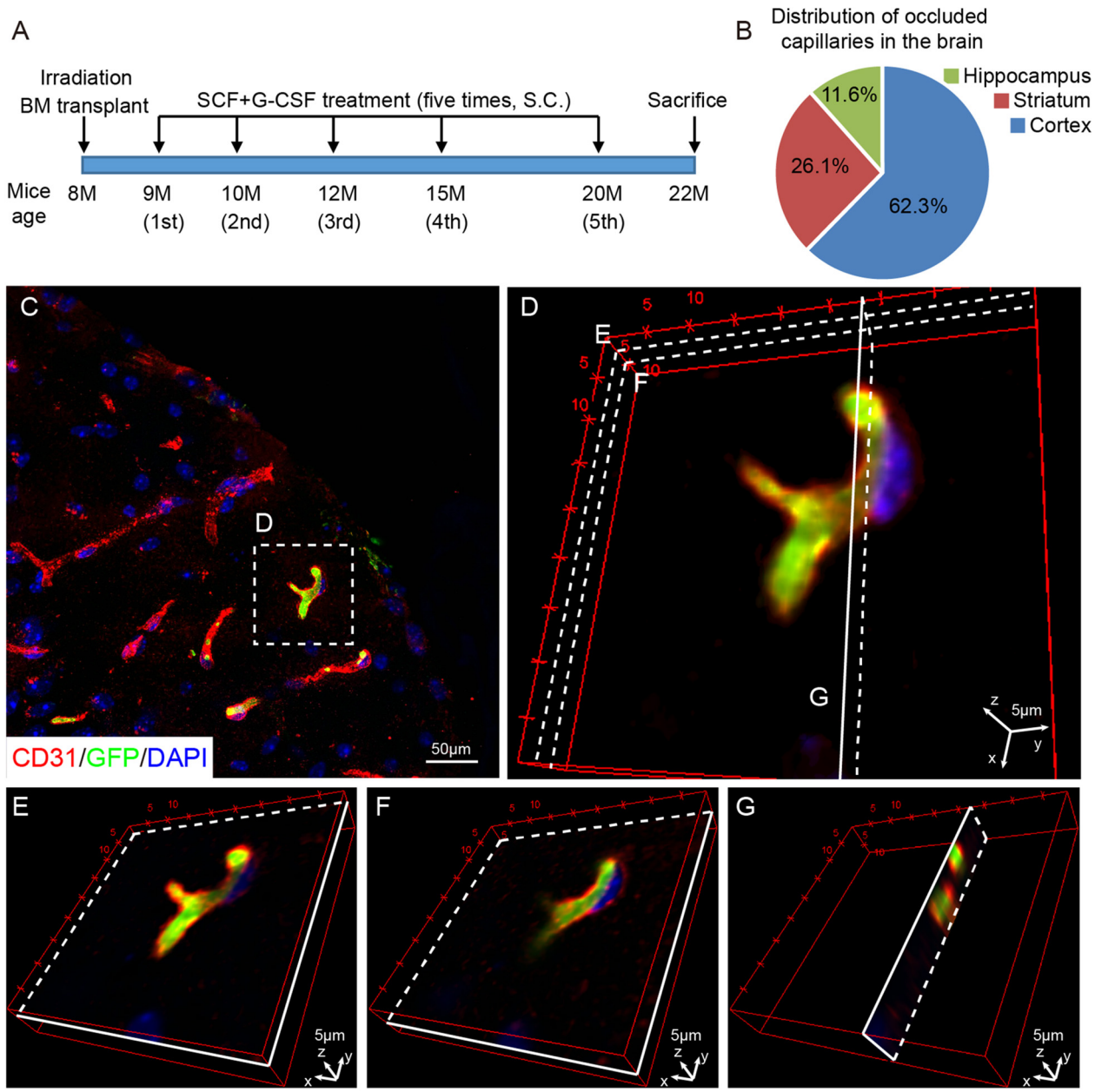

FIGURE 1 | Capillary thrombosis is mainly located in the cortex of TgNotch3 mice. **(A)** Schematic diagram of the experiment. Eight-month-old TgNotch3R90C mice received lethal dose irradiation and bone marrow transplantation. At 9 months of age, mice received subcutaneous (s.c.) injections of SCF+G-CSF or vehicle solution for 5 days. The same treatment was repeatedly administered at the age of 10, 12, 15, and 20 months. All the mice were euthanized at the age of 22 months (n = 5/group). **(B)** A pie chart displays the percentage of thrombotic capillaries in the brain of 22-month-old TgNotch3R90C mice. The thrombotic capillaries were detected by GFP expressing blood cells that occluded capillaries. **(C)** Representative confocal image shows that GFP expressing blood cells occlude capillaries (<10 mm in diameter) in the cerebral cortex of a 22-month-old TgNotch3R90C mouse. **(D)** Three-dimensional image shows the capillary occluded by GFP expressing blood cells. Three different layers were selected to show that the capillary was completely occluded by GFP expressing blood cells. **(E,F)** Three-dimensional images illustrate that GFP expressing blood cells occlude a cerebral capillary at two different longitudinal layers [see the areas labeled with **(E,F)** in panel **D**]. **(G)** Three-dimensional image illustrates that GFP expressing blood cells occlude a cerebral capillary in a transverse view [see the area labeled with **(G)** in panel **D**]. Scale bars, 50 μm in panel **(C)** and 5 μm in panels **(D–G)**.

perfusion of phosphate-buffered saline (PBS) (ThermoFisher Scientific, Pittsburgh, PA, United States) containing heparin (10 U/ml, Sagent Pharmaceuticals) followed by 10% neutral buffered formalin (Sigma-Aldrich, St. Louis, MO, United States). Brains were removed and post-fixed in the same fixative solution overnight at 4°C, and then dehydrated in 30% sucrose solution (Sigma-Aldrich, St. Louis, MO, United States) in PBS for 2 days at 4°C. Brains were sectioned at a thickness of 30 μm using

a Cryostat (Leica Biosystems, Wetzlar, Germany). All brain sections were stored at −20°C with anti-freeze buffer containing 30% ethylene glycol and 30% glycerol (Sigma-Aldrich, St. Louis, MO, United States) prepared in PBS until used for immunohistochemistry.

Two brain sections per mouse (bregma 0.02 mm to −1.82 mm) were used for immunohistochemistry. After brain sections were rinsed with PBS, nonspecific binding was blocked with 10% donkey serum prepared in 1% bovine serum albumin (BSA, IgG free, Jackson ImmunoResearch Laboratories, West Grove, PA, United States) and 0.3% Triton X-100 (Sigma-Aldrich, St. Louis, MO, United States) solution for 1 h at room temperature. For the primary antibodies from mouse origin, brain sections were further blocked with mouse-on-mouse blocking reagent (M.O.M^{TM}, Vectashield, Vector Laboratories, Burlingame, CA, United States). Brain sections were incubated with rat anti-mouse CD31 (1:50) (BD biosciences, San Jose, CA, United States), mouse anti-mouse NeuN (1:600) (ThermoFisher Scientific, Pittsburgh, PA, United States) and goat anti-mouse GFP (1:600) (Novus Biologicals, Littleton, CO, United States) primary antibodies at 4°C overnight. The next day, brain sections were washed with PBS and then incubated with the appropriate secondary antibody for 2 h at room temperature in the dark. The secondary antibodies used were Alexa Fluor 594-conjugated donkey anti-rat (1:500), Alexa Fluor 594-conjugated donkey anti-mouse (1:500) and Alexa Fluor 488-conjugated donkey anti-goat (1:500) (ThermoFisher Scientific, Pittsburgh, PA, United States). The antibodies were all diluted in PBS containing 1% BSA and 0.3% TritonX-100. Nuclei were stained with mounting medium (VECTASHIELD) containing DAPI (Vector Laboratories, Burlingame, CA, United States). Images were taken with a Zeiss 780 confocal microscope (Carl Zeiss Microscopy, LLC, Thornwood, NY, United States).

Statistical Analysis

Data analysis was performed in a blinded manner. Two-group comparisons were analyzed using a student *t*-test based on the distribution of sample data. Two-way analysis of variance (ANOVA) followed by LSD *post hoc* multiple comparison tests were used to analyze two factor comparisons. All the data are presented as mean ± standard error of mean (SEM), and results were considered significantly different when a *p*-value was less than 0.05. Analyses were performed, and data were displayed using Prism software (GraphPad Software, Inc., La Jolla, CA, United States).

RESULTS

Most Capillary Thrombosis in the Brains of TgNotch3R90C Mice Occurs in the Cortex

We first examined the distribution of the capillary thrombosis in the brains of TgNotch3R90C mice by detecting bone marrow-derived GFP positive blood cells that occluded the capillaries (capillaries: <10 μm in diameter) through immunofluorescence double staining of CD31 (EC marker) and GFP (bone marrow-derived blood cells). We observed that capillary thrombosis was randomly located in the brain. The most capillary thrombosis was seen in the cortex, which accounted for 62.3% of total occluded capillaries. The frequency of capillary thrombosis in the striatum and hippocampus was 26.1% and 11.6%, respectively, (**Figure 1B**). To further confirm that the capillaries were occluded by blood cells, three dimensional images were captured with a confocal microscope. We found that the occluded capillaries were filled with GFP positive bone marrow-derived blood cells (**Figures 1C–G**), indicating that capillary thrombosis occurs in the brains of TgNotch3R90C mice.

SCF+G-CSF Treatment Attenuates Neuron Loss in the Areas Next to Thrombotic Capillaries in the Cortex of TgNotch3R90C Mice

Next, we sought to determine the involvement of capillary thrombosis in neuronal damage and the effect of SCF+G-CSF treatment in neuroprotection in the brains of TgNotch3R90C mice. First, we quantified the number of neurons in the cortex, striatum and hippocampus through NeuN immunostaining. We observed that the number of NeuN positive neurons was not significantly different between the SCF+G-CSF-treated and non-treated TgNotch3R90C mice in the cortex, striatum and hippocampus (**Figures 2A–D**). We then quantified the number of NeuN positive neurons in the microareas next to the occluded capillaries (the microarea = 50 μm from the center of occluded capillary × the length of occluded capillary) in the cerebral cortex of TgNotch3R90C mice (**Figures 2E–I**). We selected the cortex for determining capillary thrombosis-induced neuron damage due to its high frequency of capillary thrombosis. We found that the number of NeuN positive neurons was significantly decreased in the areas next to the thrombotic capillaries with bifurcation as compared to areas adjacent to the thrombotic capillaries without bifurcation (**Figures 2E** and **H**, $p < 0.05$). In the areas next to the thrombotic capillaries with non-bifurcation, SCF+G-CSF treatment significantly increased the number of NeuN positive neurons (**Figures 2F** and **I**, $p < 0.01$). Moreover, the number of NeuN positive neurons was also significantly increased in the areas adjacent to the thrombotic capillaries with the bifurcation after SCF+G-CSF treatment (**Figures 2G** and **I**, $p < 0.05$). However, SCF+G-CSF treatment-enhanced neuron survival was more robust in the microareas next to thrombotic capillaries without bifurcation than in the microareas next to thrombotic capillaries with bifurcation (**Figure 2I**, $p < 0.05$). These data indicate that the structure of cortical capillaries may influence the microenvironment in the brains of the TgNotch3R90C mice, which affects the severity of capillary thrombosis-caused ischemic neuron damage. The findings also demonstrate that SCF+G-CSF treatment protects neurons from ischemic injury caused by capillary thrombosis in the cortex of TgNotch3R90C mice. The extent of SCF+G-CSF-enhanced neuron survival in the microareas next to the

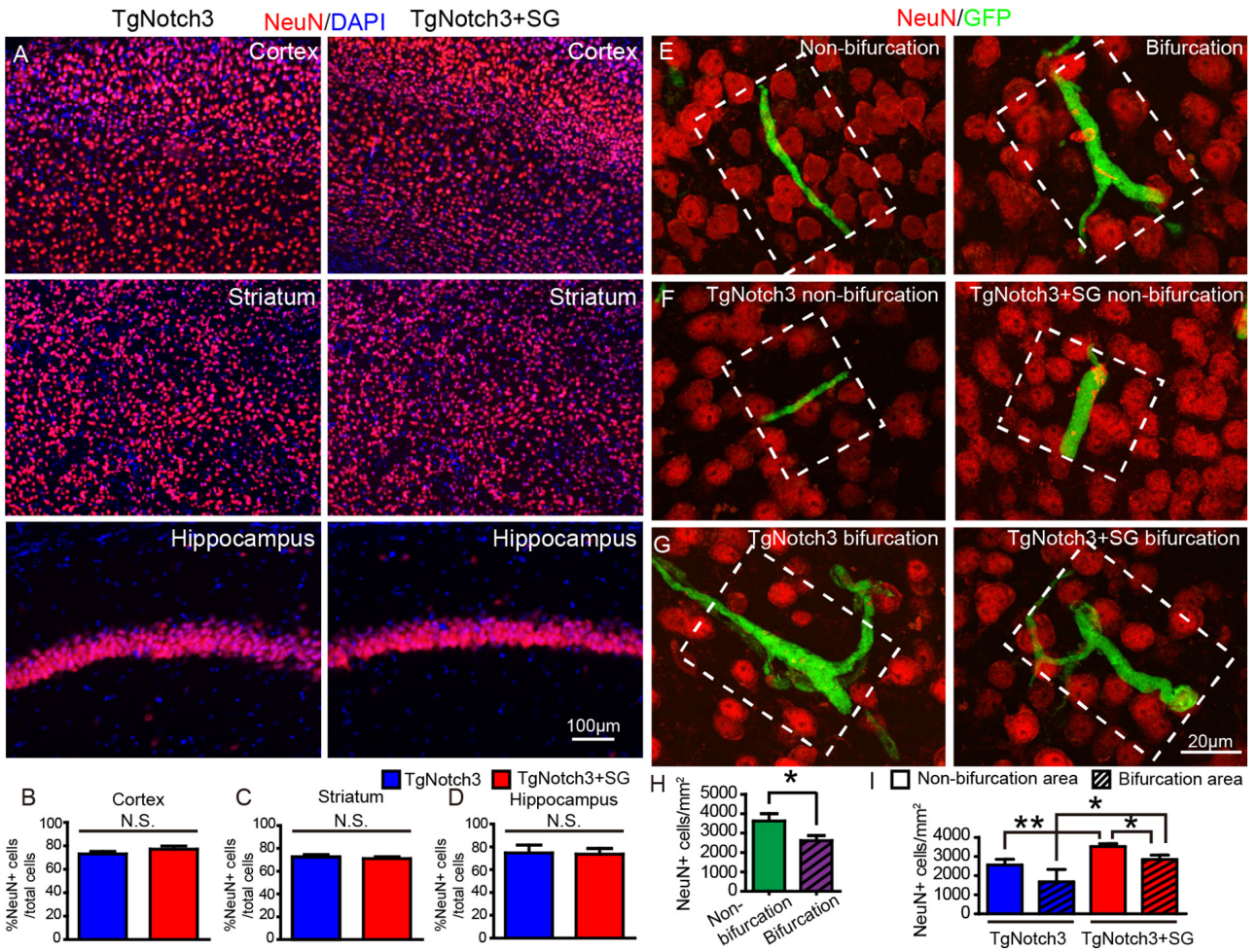

FIGURE 2 | SCF+G-CSF treatment attenuates neuron loss in the areas next to the thrombotic capillaries in the cerebral cortex of TgNotch3R90C mice. Detection of neuron loss: NeuN positive neurons were quantified within the white dotted square area which is 50 μm adjacent to the occluded capillaries. **(A)** Representative confocal images show NeuN positive neurons in the cerebral cortex, striatum and hippocampus of TgNotch3R90C mice with or without SCF+G-CSF treatment. Red: NeuN positive neurons. Blue: DAPI nuclear counterstain. **(B–D)** Statistical analysis data show that the number of NeuN positive neurons in the cortex, striatum and hippocampus of 22-month-old TgNotch3R90C mice has no difference between vehicle controls and SCF+G-CSF-treated mice. **(E)** Representative confocal images show NeuN positive neurons in the microareas adjacent to the thrombotic capillaries with or without bifurcation. Red: NeuN positive cells. Green: GFP expressing blood cells that occlude capillaries. **(F)** Representative confocal images show NeuN positive neurons adjacent to the thrombotic capillaries without bifurcation in the cortex of TgNotch3R90C mice with or without SCF+G-CSF treatment. **(G)** Representative confocal images illustrate NeuN positive neurons next to the thrombotic capillaries with bifurcations in the cortex of TgNotch3R90C mice with or without SCF+G-CSF treatment. **(H)** Statistical analysis data show that the number of NeuN positive neurons in the areas next to the thrombotic capillaries with bifurcation is significantly decreased as compared with the areas next to the thrombotic capillaries without bifurcation in the cortex of all 22-month-old TgNotch3R90C mice including both SCF+G-CSF treated and non-treated mice. **(I)** Two-way ANOVA statistical analysis data show that SCF+G-CSF treatment significantly enhances neuron survival in both the areas adjacent to the thrombotic capillaries with and without bifurcation. The SCF+G-CSF-enhanced neuron survival is more robust in the area of thrombotic capillaries without bifurcation than in the area of thrombotic capillaries with bifurcation. $N = 5$. $^{*}p < 0.05$, $^{**}p < 0.01$.

thrombotic capillaries is also influenced by the structure of the thrombotic capillary.

DISCUSSION

In the present study, we have identified that the majority of capillary thrombosis in the brain of TgNotch3R90C mice occurs in the cortex. We have also, for the first time, demonstrated that (1) microscopically detectable neuron loss exists in the area next to the thrombotic capillary, (2) the neuron loss becomes more severe in the areas adjacent to the thrombotic capillaries with bifurcations, and (3) SCF+G-CSF treatment ameliorates the neuron loss adjacent to the thrombotic capillaries in the cerebral cortex of 22-month-old TgNotch3R90C mice (**Figure 3**).

We employed the bone marrow transplantation in this study to track blood cells in the cerebral capillary thrombosis. Before the bone marrow transplantation, all the mice received a lethal dose (900 rad) of radiation to ablate their bone marrow. This bone marrow transplantation approach has been successfully

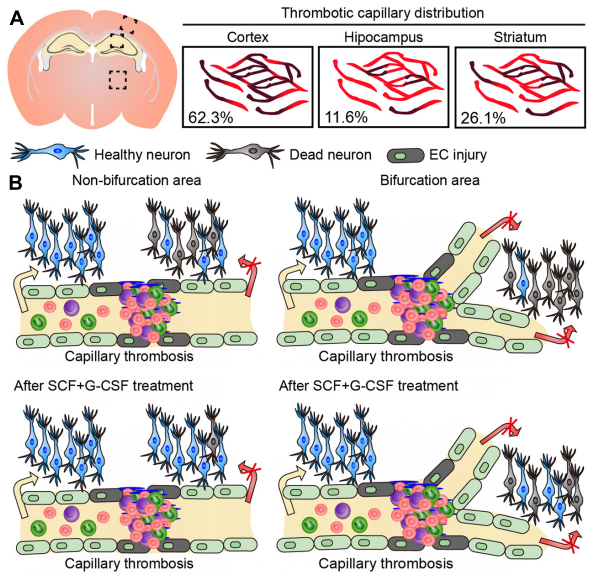

FIGURE 3 | Graphic summary of the findings in this study. **(A)** The majority of capillary thrombosis is seen in the cerebral cortex of 22-month-old TgNotch3R90C mice. **(B)** Neuron loss occurs in the area next to thrombotic capillaries. The neuron loss becomes more severe in the areas of thrombotic capillaries with bifurcations. SCF+G-CSF treatment attenuates the neuron loss adjacent to the thrombotic capillaries in the cerebral cortex of 22-month-old TgNotch3R90C mice.

used for tracking bone marrow-derived cells in our previous studies (Piao et al., 2009; Li et al., 2011; Liu et al., 2015). It has been well documented that transplantation of a genetically identical graft (syngeneic graft or autologous bone marrow) does not lead to rejection (Duran-Struuck and Dysko, 2009). The UBC-GFP mice (bone marrow donors) and TgNotch3R90C mice (bone marrow recipients) both have C57BL/6 genetic background. The bone marrow transplantation was performed within 24 h after irradiation. In the present study, we did not observe bone marrow failure or the graft-versus-host disease in TgNotch3R90C mice. The commonly used C57BL/6 mice have been shown to tolerate the radiation doses of 1,000 to 1,100 rad (Duran-Struuck et al., 2008). In the current study, we did not see irradiation-induced injury in TgNotch3R90C mice. Taken together, all the TgNotch3R90C mice used in this study were in good condition after irradiation and bone marrow transplantation.

Capillary Thrombosis Leads to Local Neuron Damage in the Brains of TgNotch3R90C Mice

Our data show that capillary thrombosis is widely distributed in the cerebral cortex, striatum and hippocampus of 22-month-old TgNotch3R90C mice. These findings are consistent with our earlier study (Ping et al., 2018) revealing EC degeneration/damage-induced cerebral capillary thrombosis in 22-month-old TgNotch3R90C mice. These observations suggest that cerebral capillary thrombosis is involved in the pathogenesis of CADASIL. It has been questioned whether thrombosis or hemorrhage in cerebral small vessels leads to CADASIL-caused lacunar infarcts because microbleeds are observed in CADASIL patient's brain but there is a lack of evidence that supports thrombotic genesis of ischemic events (Di Donato et al., 2017). Here we have provided key evidence revealing that capillary thrombosis occurs in the brains of mice carrying CADASIL-related Notch3 mutations and that the capillary thrombosis leads to ischemic neuron loss.

In TgNotch3R90C mice, degenerating ECs and impaired cerebrovascular function are found as early as 10 months of age (Ruchoux et al., 2003; Lacombe et al., 2005). Our previous study has shown EC damage and thrombosis in the cerebral capillaries and small vessels of 22-month-old TgNotch3R90C mice (Ping et al., 2018), suggesting that endothelial dysfunction-induced thrombosis occurs in the cerebral microvessels because ECs shift from an anti-thrombotic to a prothrombotic stage when their function is dysregulated (Yau et al., 2015). The underlying mechanism of EC degeneration/damage in CADASIL remains elusive. It is well documented that VSMCs and pericytes are required for maintaining vascular function and stabilization (Franco et al., 2011; Miyagawa et al., 2019). Pharmacological or genetic ablation of pericytes leads to reduced EC survival, BBB breakdown and vascular degeneration (Franco et al., 2011; Winkler et al., 2011). In our earlier studies, we have observed the autophagic degeneration of capillary pericytes and capillary EC degeneration/damage in the cerebral cortex of 22-month-old TgNotch3R90C mice (Gu et al., 2012; Ping et al., 2018), suggesting that capillary EC degeneration/damage in the brains of 22-month-old TgNotch3R90C mice is associated with capillary pericyte degeneration.

Our data have also revealed that the majority of blood-occluded capillaries are located in the cerebral cortex, and that neuron loss occurs in the areas next to the thrombotic capillaries in TgNotch3R90C mice. These observations are in line with clinical findings revealing microinfarcts in the cortex of CADASIL patients (Jouvent et al., 2011; De Guio et al., 2014). These findings renew the traditional notion that CADASIL only affects the subcortical white matters (Dichgans, 2002), and provide insightful evidence demonstrating that capillary thrombosis-caused ischemic damage in the cerebral cortex may play a crucial role in the pathogenesis of CADASIL.

It remains unclear why the capillary thrombosis is mainly located in the cortex, instead of the striatum and hippocampus. It has been documented that the topological distribution of the capillary network is different throughout the brain to match the regional metabolism (Shaw et al., 2019; Zhang et al., 2019). The vascular network shows distinct distribution and density in the cortex, hippocampus and striatum. The cortical parenchyma is full of abundant vessels, which are well arranged into a mesh-like network. The vascular density in the hippocampus is significantly lower than the cortical vessel density (Zhang et al., 2019). No obvious difference of vascular density is seen between the striatum and cortex (Di Giovanna et al., 2018). Based on these studies, we postulate that the different varieties of metabolic rates with the different distribution and density of vascular network in different brain regions may influence the formation of capillary thrombosis in the TgNotch3R90C mice.

Vascular occlusion and hemorrhage are the vascular events that lead to regional neuronal death (i.e., infarcts) (Nishimura and Schaffer, 2013). Here we have observed that blood clots completely occlude the capillaries, demonstrating the formation of capillary occlusion (i.e., capillary thrombosis). The occlusion of capillaries prevents blood flow from reaching a small territory of brain tissue leading to ischemic damage and local neuron death. Our findings have also revealed that neuron loss is increased in the microareas next to the occluded capillaries with bifurcations as compared to the microareas adjacent to the occluded capillaries without bifurcations, indicating that the severity of ischemia-induced neuron loss is capillary structure-related. In the cerebral vasculature, the number of vascular branches is dramatically increased in the capillaries as compared with other types of blood vessels (Reina-De La Torre et al., 1998). The bifurcation is the major junction in the capillary bed (Gould et al., 2017). Blood flow starts from the arterial inlet and makes random choices at each bifurcation, making the blood flow complex at the site of the bifurcation; as a result, it leads to the ECs in the bifurcation area being more vulnerable to damage in the presence of risk factors for vascular diseases (De Syo et al., 2005). It is possible that once the thrombotic occlusions occur at the bifurcation site of the capillary network, two or more downstream capillaries will be affected, which leads to an increased severity of ischemia and neuronal loss in the territory of affected capillaries.

SCF+G-CSF Treatment Ameliorates Ischemic Neuron Loss in the Area Next to the Thrombotic Capillary

In the present study, SCF+G-CSF treatment-reduced neuronal loss has been observed in the areas adjacent to the thrombotic capillaries, demonstrating the neuroprotective efficacy of SCF+G-CSF on capillary thrombosis-induced ischemic damage in the brains of TgNotch3R90C mice. The effects of SCF and G-CSF in neuroprotection have been reported in focal cerebral ischemia. Focal cerebral ischemia is produced by occlusion of a relatively large artery, the middle cerebral artery (MCA), leading to massive brain tissue loss (i.e., infarct) in the territory of the occluded MCA. In rodent models of focal cerebral ischemia, systemic administration of SCF (Zhao et al., 2007b), G-CSF (Schneider et al., 2005; Zhao et al., 2007b), and SCF+G-CSF (Kawada et al., 2006; Zhao et al., 2007b) initiated in the acute phase of ischemic stroke (<48 h after MCA occlusion) results in the reduction of infarction size. Strikingly, subcutaneous injection of SCF+G-CSF in the subacute phase (during 11–20 days post-ischemia) (Kawada et al., 2006) or in the chronic phase (14 weeks post-ischemia) (Zhao et al., 2007a) of ischemic stroke still shows significant reductions of infarct volume. These studies have demonstrated the effectiveness of SCF+G-CSF treatment in protecting neurons from both acute ischemic damage and the focal cerebral ischemia-induced long-term progressive neuron loss. In contrast to stroke that occurs as a sudden event, CADASIL causes progressive damage in cerebral small arteries and capillaries, leading to progressive thrombosis in these vessels. Most likely, when we give treatment to different ages of TgNotch3R90C mice, the capillary thrombosis-induced ischemic neuron loss at different locations may be under different post-ischemic stages/phases. SCF+G-CSF treatment may exert its universal protection against neuron death from the different stages/phases.

The mechanism underlying SCF+G-CSF-enhanced neuroprotection in different phases of cerebral ischemia remains unclear. Expression of receptors for SCF (Zhao et al., 2007b) and G-CSF (Schneider et al., 2005) on neurons reflects their direct efficacy in neuroprotection. In cultured primary cortical neurons, SCF protects neurons from excitotoxicity through MEK/ERK and PI3K/AKT/NF-kB pathways and from apoptosis through PI3K/AKT/NF-kB/Bcl-2 signaling, and the SCF-enhanced neuroprotection is dependent on its receptor (c-kit) expression (Dhandapani et al., 2005). G-CSF receptor, G-CSFR, is robustly expressed in the peri-infarct neurons of rat brain (Schneider et al., 2005) and human brain (Hasselblatt et al., 2007) in the acute phase of ischemic stroke. G-CSF counteracts programmed neuron death via PI3K mediation in cultured cortical neurons (Schneider et al., 2005).

In addition to the direct effects of SCF and G-CSF in neuroprotection demonstrated in *in vitro* studies, many *in vivo* studies have revealed that SCF and G-CSF may protect the brain from post-ischemic neuron loss through an indirect way. It has been shown that brain ischemia-triggered neuroinflammation in both the acute and subacute phases leads to secondary neuron loss including apoptotic neuron death (Duris et al., 2018; Jayaraj et al., 2019). G-CSF treatment in the acute phase of focal cerebral ischemia suppresses pro-inflammatory cytokines and inflammatory mediators in the peri-ischemic areas (Gibson et al., 2005; Sehara et al., 2007), reduces the disruption of the BBB (Lee et al., 2005), inhibits peripheral inflammatory cell infiltration to the ischemic hemisphere (Lee et al., 2005) and reduces neuronal apoptosis in the ipsilesional cortex (Solaroglu et al., 2006). SCF+G-CSF treatment in the subacute phase of focal cerebral ischemia upregulates IL-10, an anti-inflammatory cytokine, in the ipsilesional cortex (Morita et al., 2007). In our earlier study, the level of vascular endothelial growth factor (VEGF) is decreased in the brains of TgNotch3R90C mice, while SCF+G-CSF treatment increases cerebral VEGF in TgNotch3R90C mice (Ping et al., 2019). Intracerebral injection of VEGF reduces infarct volume in focal cerebral ischemia, and the direct effect of VEGF in neuroprotection is also demonstrated in *in vitro* hypoxia models (Greenberg and Jin, 2013). It would be possible that increased cerebral VEGF may also contribute to the SCF+G-CSF-induced neuroprotection in the area of capillary thrombosis in TgNotch3R90C mice.

Our previous studies have demonstrated that repeated treatments with SCF+G-CSF reduce cerebral capillary thrombosis, attenuate capillary thrombosis in the bifurcation regions (Ping et al., 2018), enhance angiogenesis and increase blood vessel density in the brains of TgNotch3R90C mice (Liu et al., 2015; Ping et al., 2019), which may lead to increasing collateral circulation and ameliorating ischemic damage in the areas next to the thrombotic capillaries. Our present findings reveal that SCF+G-CSF-reduced neuron loss is more robust in the areas with a single thrombotic capillary than the areas

with multiple thrombotic capillaries (i.e., thrombotic capillaries with bifurcations). These findings suggest that the increase of collateral circulation by SCF+G-CSF may be more sufficient to rescue neurons from the microareas without severe ischemic damage than from the microareas with severe ischemic damage due to having multiple thrombotic capillaries.

In conclusion, this study provides new and important evidence demonstrating that capillary thrombosis-caused microvascular ischemic damage exists in the brains of 22-month-old TgNotch3R90C mice. The thrombotic capillaries are mainly located in the cortex, and the capillary thrombosis leads to local neuron loss. Systematic administration of SCF+G-CSF attenuates neuron loss in the areas next to the thrombotic capillaries. These findings advance toward an understanding of the pathological role of cortical capillary thrombosis-caused microvascular ischemia in the pathogenesis of CADASIL and reveal a new therapeutic target for developing treatment to ameliorate microvascular ischemia in CADASIL.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, and further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee at SUNY Upstate Medical University and LSUHSC.

AUTHOR CONTRIBUTIONS

SP, MG-T, and XL performed the experiment. SP prepared the first draft of the manuscript. XQ provided assistance in the data analysis. LRZ conceived the study, supervised the experiment, and revised the manuscript. All authors reviewed and approved the submitted version of the manuscript.

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Potential Mechanisms and Perspectives in Ischemic Stroke Treatment Using Stem Cell Therapies

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Ischemic stroke (IS) remains one of the major causes of death and disability due to the limited ability of central nervous system cells to regenerate and differentiate. Although several advances have been made in stroke therapies in the last decades, there are only a few approaches available to improve IS outcome. In the acute phase of IS, mechanical thrombectomy and the administration of tissue plasminogen activator have been widely used, while aspirin or clopidogrel represents the main therapy used in the subacute or chronic phase. However, in most cases, stroke patients fail to achieve satisfactory functional recovery under the treatments mentioned above. Recently, cell therapy, especially stem cell therapy, has been considered as a novel and potential therapeutic strategy to improve stroke outcome through mechanisms, including cell differentiation, cell replacement, immunomodulation, neural circuit reconstruction, and protective factor release. Different stem cell types, such as mesenchymal stem cells, marrow mononuclear cells, and neural stem cells, have also been considered for stroke therapy. In recent years, many clinical and preclinical studies on cell therapy have been carried out, and numerous results have shown that cell therapy has bright prospects in the treatment of stroke. However, some cell therapy issues are not yet fully understood, such as its optimal parameters including cell type choice, cell doses, and injection routes; therefore, a closer relationship between basic and clinical research is needed. In this review, the role of cell therapy in stroke treatment and its mechanisms was summarized, as well as the function of different stem cell types in stroke treatment and the clinical trials using stem cell therapy to cure stroke, to reveal future insights on stroke-related cell therapy, and to guide further studies.

Keywords: stem cell, cell therapy, ischemic stroke, transplantation, clinical trial, regenerative medicine

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INTRODUCTION

Stroke is a common cerebrovascular disease with high rates of fatality and disability (Feigin et al., 2017) and is the second leading cause of death, with 5.5 million deaths every year (GBD 2016 Stroke Collaborators, 2019; Lindsay et al., 2019). In the acute phase of ischemic stroke (IS), mechanical thrombectomy (MT), and the administration of tissue plasminogen activator (tPA) can recanalize the occlusive lesion. However, these approaches are limited by the narrow time window for application of 3–4.5 h after IS onset in case of thrombolytic therapy (Roth, 2011;

Wardlaw et al., 2014) and up to 24 h after stroke onset in case of MT, depending on imaging criteria (Thomalla and Gerloff, 2019), and only 5–10% of patients are eligible to receive a reperfusion treatment (Mozaffarian et al., 2016). In the subacute to chronic phases, stroke treatment strategies may be changed to long-term antiplatelet or anticoagulation drugs depending on patients' condition (Bala et al., 2020) or intervention in patients' risk factors of stroke such as hypertension, diabetes, and hypercholesterolemia (Rundek and Sacco, 2008; Tziomalos et al., 2009). While these treatments show significant benefits in stroke patients, they are still not enough to ensure an acceptable quality of life. Recently, cell therapy has gained much attention as a potential IS treatment, since the results obtained so far suggest it has a bright future. Several types of cell therapy are available, including stem cell therapy (Suda et al., 2020), polarized cell therapy (Jiang et al., 2013), and genetic modification or pretreatment stem cell therapy (Kurozumi et al., 2005; Damasceno et al., 2020). In recent years, stem cell therapy has garnered more attention due to its satisfactory results in some preclinical and clinical studies. We believe that stem cell therapy will make an important contribution to the field of stroke treatment, and further research will compliment and add to the successful results seen when using other cell types. The stem cells used in cell therapy include mesenchymal stem cells (MSCs), neural stem cells (NSCs), induced pluripotent stem cells (iPSCs), hematopoietic stem cells (CD34-positive) (HSCs), dental pulp stem cells (DPSCs), embryonic stem (ES) cells (ESCs), and multilineage-differentiating stress-enduring cells (Muse cells) (Chen et al., 2001; Yang et al., 2016; Niizuma et al., 2018; Rikhtegar et al., 2019). The potential mechanisms underlying the protective role of stem cells have been extensively studied, such as the promotion of angiogenesis and endogenous neurogenesis, immunomodulatory functions, differentiation into cells that facilitate repair or replacement of a damaged tissue, secretion of cytokines helping to restore neural injury, and cell migration (Gutiérrez-Fernández et al., 2013; Shichinohe et al., 2015; Tan et al., 2018; Rikhtegar et al., 2019; Kikuchi-Taura et al., 2020). This review summarizes and discusses the pathophysiological changes after IS, which are also the treatment targets of stem cell therapy, the current types of cell therapy, the stem cell used, and the neuroprotective mechanisms. Clinical studies on stem cell stroke therapy are discussed along with the choice of stem cell types, time of transplantation, route of administration, and cell dose, as these are regarded as controversial and worrying aspects by clinicians and neuroscientists.

POTENTIAL THERAPEUTIC TARGETS OF STEM CELL THERAPY IN ISCHEMIC STROKE

Ischemic stroke accounts for 71% of strokes and is the treatment target in most stroke trials (Feigin et al., 2018). In the past decades, the pathophysiological changes after stroke have been extensively investigated. Understanding the changes in the microenvironment and cell activities that occur after IS can help to focus on the potential effective role of stem cell therapy, and the pathophysiological changes may also be potential stem cell therapy targets.

Blood–Brain Barrier Disruption

The BBB plays a vital role in the protection of the central nervous system (CNS) (Daneman and Prat, 2015). The basic structure of the BBB consists of endothelial cells (ECs), astrocytes, and pericytes, with the ECs fused together by intercellular junctions such as tight junctions (TJs) and adherent junctions (AJs). After the onset of stroke, the BBB can be disrupted, followed by the extravasation of blood components into the brain, thus compromising the normal neuronal function. BBB dysfunction is characterized by the structural disruption of the intercellular junctions and increased vascular permeability, which allows the components of the peripheral blood system to cross into the parenchyma more easily. After IS, BBB permeability is increased and cell adhesion molecules are upregulated in the activated endothelium, and peripheral immune cells can access the parenchyma. The infiltrating leukocytes can contribute to BBB dysfunction and injury progression (Shi et al., 2019), and neutrophils are another important peripheral immune cell type that can cross the damaged BBB. In addition to the release of chemokines (Nathan, 2006), activated neutrophils can increase the formation of neutrophil extracellular traps (NETs), which contain double-stranded DNA, histone, and granule proteins including neutrophil elastase, cathepsin G, and myeloperoxidase (MPO) (Urban et al., 2009). The latest research suggests that reduced neovascularization and increased BBB damage can be observed under the increased formation of NETs induced by the overexpression of peptidylarginine deiminase 4, while the inhibition of this process by DNase 1, genetic ablation, or pharmacologic administration improves BBB function (Kang et al., 2020).

Neuroinflammation and Immune Response

Neuroinflammation after stroke is a sterile inflammation defined as an inflammatory response by cells of the innate or adaptive immune systems within the CNS. In IS, neuronal and non-neuronal cell death can produce damage-associated molecular patterns (DAMPs) because of oxygen and glucose deprivation (OGD). During ischemia, DAMPs can activate astrocytes and microglia, increasing production of pro-inflammatory cytokines and chemokines. Peripheral blood then invades the infarcted area, leading to the exacerbation of tissue damage (Dabrowska et al., 2019a). After DAMPs are released into the brain parenchyma, microglia cells are the first line of defense. They secrete pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), NF-κB, interleukin (IL)-1β, IL-12, IL-23, and nitrogen monoxide (NO), which induce tissue damage (Benakis et al., 2014; Xiong et al., 2016). Recently the inflammasome, which is a multi-molecular protein complex and one of the intracellular typical pattern recognition receptors (PRRs) located in neurons, microglia, astrocytes after IS, has attracted a lot of attention. The members of the nucleotide-binding domain (NOD)-like receptor (NLR) family including the NODs,

the NLRPs, and the IPAF subfamilies exhibit inflammasome activity (Mohamed et al., 2015). The NLRP3 inflammasome can cleave pro-IL-1 and pro-IL-18, which become mature IL-1 and IL-18 (Alishahi et al., 2019). Neutrophils are among the first cells to arrive into the lesion within the first hour after IS (Garcia et al., 1994) and leukocytes invading the CNS release pro-inflammatory factors in the ischemic region. Neutrophils increase the recruitment of leukocytes by degranulation of their content rich in cytokines/chemokines, free radicals, proteolytic enzymes, and activated-complement system, which in turn exacerbates neuroinflammation (Mayadas et al., 2014). Recently, the detrimental role of neutrophils in IS has been demonstrated. The release of pro-inflammatory cytokines/chemokines, proteases, and oxygen radicals (Neumann et al., 2015), combined with the antagonistic effect of some chemokines such as C-X-C motif chemokine receptor 2 (CXCR-2) and (C-X-C motif) ligand 1 (CXCL-1), can prevent neutrophil recruitment into the infarcted area and reduce the experimental infarcted volume (Gelderblom et al., 2012; Herz et al., 2015).

Excitotoxicity

Excitotoxicity was first described in 1986 to describe the ability of glutamate and structurally related excitatory amino acids to destroy neurons (Olney, 1969). Glutamate is the main excitatory neurotransmitter in the CNS. The OGD resulting from the interruption of blood flow in IS disrupts ATP supply, which leads to perturbation of the transmembrane gradient, impairment of the neuronal signaling, and neurotransmitter release as a result of anoxic depolarization (Obrenovitch et al., 1993). Depolarization leads to a transient neurotransmitter release, and the reuptake of excitatory neurotransmitters from the synaptic cleft is an ATP-dependent process. Therefore, ischemia leads to an increase in extracellular neurotransmitter concentrations. *N*-Methyl-D-aspartate (NMDA) is a glutamate receptor that plays a key role in excitotoxicity, and in a normal physiological state, its function is controlled by extracellular magnesium and the resting membrane potential. However, after IS, neuronal depolarization removes magnesium, and the NMDA receptor is activated, leading to calcium influx. The primary influx then induces the secondary release of large amounts of calcium from the intracellular environment leading to cell death (Choi, 1992; Lai et al., 2014; Chamorro et al., 2016). To deal with the high concentration of intracellular calcium, mitochondria sequester the majority of the intracellular calcium after glutamate excitotoxicity (Wang and Thayer, 1996). The increase in the glutamate concentration can also activate the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, which in normal conditions are not calcium permeable (Peng et al., 2006), but after IS, permeability increases up to 18-fold, contributing to calcium-dependent cell death (Liu et al., 2006).

Oxidative Stress

After the onset of IS, the balance between pro-oxidants [such as reactive oxygen species (ROS)] and antioxidants is compromised, leading to oxidative stress and damage of the cell structures, including lipids, proteins, and DNA, finally leading to cell death (Zhao et al., 2016). ROS greatly contributes to lipid peroxidation after IS by increasing the production of conjugated dienic hydroperoxides (Adibhatla and Hatcher, 2010) and can decompose the ω-6 polyunsaturated fatty acids into 4-hydroxynonenal (4-HNE), a traditional marker of oxidative stress (Niki, 2009). 4-HNE can enhance apoptosis/autophagy and increase the cerebral infarct area (Lee et al., 2012). Phospholipase A2 (PLA2) is another contributor to lipid peroxidation that can lead to the hydrolysis of membrane phospholipids and the release of free fatty acids. At a high ROS concentration, lipid peroxides are formed and degraded into active aldehydes, which can bind to proteins or nucleic acids, causing neuronal damage (Muralikrishna Adibhatla and Hatcher, 2006). The products of lipid peroxidation such as malondialdehyde (MDA), 4-HNE, and acrolein participate in oxidative stress injury on proteins (Del Rio et al., 2005; Moghe et al., 2015). Oxidative stress can also cause cell death by damaging the DNA (Li et al., 2011). This damage activates the DNA repair signal, which includes the activation of poly(ADP-ribose) polymerase-1 (PARP-1). To repair the DNA strand, β-nicotinamide adenine dinucleotide (NAD^+) is consumed by PARP-1, which can result in impairment of NAD^+-dependent processes, including mitochondrial respiration, leading to ATP starvation and, eventually, neuronal death (Southan and Szabó, 2003).

CELL THERAPY

Because IS involves complex pathophysiological processes, one targeted therapeutic strategy is far from enough. The use of cell therapy, including stem cells, gene-modified stem cells, and polarized cell therapy, has been widely discussed in preclinical or clinically studies. After the onset of IS, CNS cells cannot completely replace the lost or compromised ones because of their limited regeneration and differentiation ability. Stem cells have emerged as a novel and promising option due to their capacity for self-renewal, homing, and multilineage differentiation (Birbrair, 2017; Huang et al., 2018). A variety of stem cell types derived from neural and non-neural tissues have been widely studied as potential donors for IS treatment.

Neurotrophic factor secretion is an important mechanism underlying the function of stem cell therapy in IS. Growth factors such as brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF), and erythropoietin (EPO) are involved in the restoration of the damaged tissue to prevent neuronal death (Schäbitz et al., 2000), angiogenesis, anti-inflammation, or brain repair after IS. Therefore, gene-modified stem cells, which can overexpress different growth factors or cytokines through gene transfection, will possess a unique superiority in terms of injury repair compared with unmodified stem cells (Geiseler and Morland, 2018).

The phenotypes of several CNS cells, such as microglia, macrophages, astrocytes, and neutrophils, are in a dynamic flux and can thus exhibit two different phenotypes: the classic pro-inflammatory type and alternative protective type. After the onset of IS, the pro-inflammatory phenotype

cells increase inflammatory mediators inducing brain tissue injury (Block et al., 2007; Kanazawa et al., 2017b; Liddelow and Barres, 2017), while the anti-inflammatory phenotype cells can secrete neurotrophic factors and protective cytokines (Imai et al., 2007; Crain et al., 2013), which have a neuroprotective function (Schäbitz et al., 2000; Hu et al., 2012; Dudvarski Stankovic et al., 2016; Geiseler and Morland, 2018). When considering classical or gene-modified stem cells, ethical issues and tumorigenicity must be taken into account, as highlighted in previous studies (Murry and Keller, 2008; Ratajczak et al., 2019). Targeting microglia or other polarized cells after IS may be an optional cell therapy strategy. Because of the lack of relevant research, the specific protective function should be a focus of future studies (Hatakeyama et al., 2020b).

DIFFERENT STEM CELL TYPES IN CELL THERAPY

Mesenchymal Stem Cells

Mesenchymal stem cells have the capacity for self-renewal and potential multidirectional differentiation into different cell types and are available from almost any tissue type. The bone marrow-derived MSCs (BM-MSCs) are the most widely studied and are capable of differentiating into neurons or glial cells and, thus, are able to replace damaged cells in brain tissues. The ability to differentiate into ECs and release trophic factors such as VEGF, BDNF, glial cell-derived neurotrophic factor (GDNF), and transforming growth factor (TGF), contribute to angiogenesis and neuroprotection (Wakabayashi et al., 2010; Wagenaar et al., 2018). Although MSCs have a high ability to differentiate into neuronal lineage cells and migrate into ischemia lesions, only a small proportion of stem cells enter the infarct core area (Modo et al., 2004). The paracrine action or "bystander effect," which relies on the secretion of trophic factors, is the main contributor to the therapeutic effect rather than direct cellular replacement. The trophic factors released from MSCs promote angiogenesis, induce the proliferation and recruitment of endogenous stem cells from the subventricular zone (SVZ) to the infarcted area, and modulate neuroinflammation. MSCs also promote the production of regulatory T cells (Wang et al., 2014), which increase the expression of IL-10, attenuate astrocytes and microglial reactivity, exert an anti-apoptotic effect, and reduce levels of the inflammatory cytokines IL-1β and IL-6 (Zhu et al., 2014; Sotomayor-Sobrino et al., 2019).

Multilineage-Differentiating Stress-Enduring Cells

Multilineage-differentiating stress-enduring cells are characterized by self-renewal and multipotency and can differentiate into all three germ layers (Wakao et al., 2011; Heneidi et al., 2013; Dezawa, 2016). MUSE cells are found in the bone marrow, adipose, dermis, and connective tissues (Heneidi et al., 2013; Gimeno et al., 2017) and can be purchased or collected from human tissues. After the first report on MUSE cells in 2010 (Kuroda et al., 2010), several other studies have investigated their therapeutic potential in diseases such as acute myocardial infarction (MI), liver disease, and chronic kidney disease (CKD) (Katagiri et al., 2016; Yamada et al., 2018). In normal conditions, MUSE cells are inactive. After IS, the ischemia insult stimulates MUSE cell mobilization from the bone marrow into the peripheral blood flow (Hori et al., 2016). MUSE cells effectively migrate into the infarcted area where they differentiate into neural cells to restore the damaged brain tissues. Their efficient migration has also been observed in other diseases including MI, CKD, and liver disease (Uchida N. et al., 2017; Yamada et al., 2018). After reaching the ischemic area, MUSE cells can replace the damaged cells by differentiating into neuronal-lineage cells (Uchida et al., 2016; Abe et al., 2020). The administration of diphtheria toxin (DT) in a rat IS model inhibits recovery, suggesting that the neural circuit reconstruction is a key mechanism underlying the therapeutic effects (Uchida H. et al., 2017; Abe et al., 2020). MUSE cells can also modulate neuroinflammation and immune response by downregulating the secretion of pro-inflammatory cytokines including TNF-α (Gimeno et al., 2017). While tumor formation and other adverse effects have not been reported, some issues remain to be solved, including the appropriate timing, dose, and safety, before their use in clinical practice can progress.

Neural Stem Cells

Neural stem cells are self-renewing cells and possess the multi-potential to differentiate into neurons, astrocytes, and oligodendrocytes (Boese et al., 2018; Obernier and Alvarez-Buylla, 2019; Ottoboni et al., 2020). Exogenous NSCs can be derived from ESCs, iPSCs, fetal tissue, and adult nervous systems. Their abilities to produce primary CNS cells make NSCs promising candidates for the replacement of the damaged or lost cells in the brain tissues after IS. Under normal conditions, neurogenesis persists throughout the whole life in rodents and humans (Boldrini et al., 2018), and endogenous NSCs, found mainly in the dentate gyrus of the hippocampus, the SVZ, and the olfactory bulb in mammals, remain inactive (Obernier and Alvarez-Buylla, 2019). After IS, cytokines or chemokines such as stromal cell-derived factor-1 (SDF-1), VEGF, and monocyte chemoattractant protein (MCP)-1 are released, increasing the proliferation and migration of NSCs from the SVZ into the ischemic area (Mine et al., 2013; Leal et al., 2017; Geiseler and Morland, 2018).Thanks to their strong ability to differentiate into neuronal cells, transplanted NSCs have been shown to be effective in promoting the neurological function recovery in preclinical studies. NSCs can migrate into the ischemic brain area and differentiate into mature neurons to reconstruct the neural circuit (Shetty and Hattiangady, 2016). Exogenous NSCs are also capable of stimulating neurogenesis in the SVZ. NSCs release neurotrophic factors such as BDNF, VEGF, and nerve growth factor (NGF), which can prevent neural apoptosis and improve functional recovery by directly or indirectly increasing the angiogenesis, endogenous neurogenesis, and plasticity of neuronal cells (Shetty and Hattiangady, 2016; Ottoboni et al., 2020). The activation

of microglia and infiltration of neutrophils also decrease after NSC transplantation, thus relieving neuroinflammation (Lee et al., 2008).

Induced Pluripotent Stem Cells

Induced pluripotent stem cells are somatic-derived stem cells characterized by an increased translational potential. iPSCs were first established in 2006 by introducing transcription factors into mouse fibroblasts during a pluripotent state (Takahashi and Yamanaka, 2006). This somatic cell-derived feature also gives iPSCs the following advantages over other stem cells: (i) they can be obtained from different sources, and isolation does not require invasive measures; (ii) iPSC culture provides an adequate cell number for transplantation in a shorter time; and (iii) autologous cells show almost no immunogenicity after transplantation (Ojeh et al., 2015; Vitrac and Cloëz-Tayarani, 2018). Furthermore, iPSCs have similar advantages to ESCs in that they are pluripotent, can undergo self-renewal, and can differentiate into cells from three germ layers (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). While their differentiation into neurons, ECs, or astrocytes may improve the overall effect of the treatment, there is also a risk of tumorigenicity (Suda et al., 2020).

Dental Pulp Stem Cells

Dental pulp stem cells are ectoderm-derived stem cells residing within the dental pulp (Nosrat et al., 2004), which can differentiate into multiple cell types including neural cells, muscle, cartilage, and adipocytes (Dailey et al., 2013). They have similar properties to both NSCs and MSCs in terms of surface markers and biological features (Gronthos et al., 2000; Martens et al., 2013). The expression of nestin, MAP2, the astrocytic marker GFAP, and NeuN by DPSCs has also been reported (Gronthos et al., 2002; Sakai et al., 2012; Foudah et al., 2014), suggesting that they have the potential ability to differentiate into neuron-like cells. In an animal model of spinal cord injury (SCI), transplanted DPSCs improved functional recovery by three mechanisms: (1) decreased CNS cell apoptosis; (2) their paracrine effect minimized the influence of multiple axon growth inhibitors such as chondroitin sulfate proteoglycan and myelin-associated glycoprotein; and (3) they differentiated into mature neural cells and replaced the lost brain tissues (Sakai et al., 2012). DPSCs can also differentiate into dopaminergic neurons and reconstruct immature neural networks (Chun et al., 2016). While they have properties similar to those of MSCs and NSCs, they confer more advantages, their proliferation is significantly faster than that of MSCs (Ponnaiyan and Jegadeesan, 2014), and they can also more effectively reduce the infarcted area and improve motor function compared with MSCs (Song et al., 2017). Moreover, DPSCs secrete specific neurotrophic factors such as GDNF, NGF, and BDNF; and the conditioned medium containing these factors is also a promising novel treatment for IS (Kichenbrand et al., 2019). Finally, DPSCs protect neural cells from oxidative stress by decreasing OGD-induced ROS production (Song et al., 2015) and contributing to immune system modulation by inhibiting T cell activation and increasing $CD3^+$ T cell apoptosis (Pierdomenico et al., 2005; Demircan et al., 2011).

Hematopoietic Stem Cells (CD34 Positive)

Hematopoietic stem cells are a rare cell population obtained from the bone marrow, mobilized peripheral blood (MPB), and umbilical cord blood (UCB) (Behzad-Behbahani et al., 2005; Sovalat et al., 2011). HSCs are able to self-renew and can differentiate into a full spectrum of blood cells (Derakhshani et al., 2019). $CD34^+$ HSCs were found to invade the peripheral blood after acute stroke (Hennemann et al., 2008). In published studies, HSCs are almost all CD34 positive (Uemura et al., 2012) and under normal conditions maintain a quiescent state via the coupling of CXCL12 with CXCR4. After IS, the increase in sympathetic tone and upregulation of granulocyte colony-stimulating factor (G-CSF) promote the mobilization of $CD34^+$ HSCs, which are recruited to the injured lesion where they can exert a protective effect (Borlongan et al., 2011). $CD34^+$ HSCs from bone marrow and UCB enhance the neuroprotective ability through angiogenesis, neurogenesis, and the regulation of inflammation (Hennemann et al., 2008). HSCs are also feasible, safe, and effective in the treatment of hematologic, cardiovascular, bone, and genetic defect diseases (Borlongan et al., 2011). This may be related to their inability to undergo neuronal differentiation and are thus unable to complete the complex restoration process necessary to repair the damage after IS. However, HSCs can improve neuronal structures through fusion with their residential components and adoption of their phenotype (Terada et al., 2002).

Embryonic Stem Cells

Embryonic stem cells are isolated from early embryonic or primitive gonads, are immortalized *in vitro*, and possess self-renewal and multilineage differentiation abilities (Murry and Keller, 2008). ESCs can be induced to differentiate into almost all somatic cell types when cultured with specific cytokines (Ratajczak et al., 2019). After being considered cells of unlimited potential for regenerative medicine, a variety of problems emerged, causing widespread concern around the world. The first is the ethical issue, which is constantly debated, but other concerns exist, such as tumorigenicity, immune rejection, induction of epigenetic changes, and genetic alterations, which have also been discussed (Murry and Keller, 2008; Ratajczak et al., 2019). It is worth pointing out that the tumorigenicity of ESCs can be reduced by modulating the anti-apoptotic Bcl-2 gene to regulate epigenetic stability (Allegrucci et al., 2007).

Very little research on ESC transplantation in stroke has been done. Striatal transplantation of mouse ESCs in a rat IS model was shown to alleviate neurological dysfunction by improving dopaminergic function (Yanagisawa et al., 2006). Other animal experiments have revealed that ESCs exert a protective effect on cerebral ischemia after generating secondary cells such as neural precursors, neural lineage cells, and NSCs after differential induction *in vitro*. These inductions can be caused by neurotrophic factors such as BDNF, small molecule inhibitors, co-culture with other cells like bone marrow stromal cells (BMSCs), or other means (Yang et al., 2009; Drury-Stewart et al., 2013; Rosenblum et al., 2015). Although clinical trials in the

United States have used ESCs to treat SCI (Ilic et al., 2015), their use is not widespread due to theoretical and technical difficulties, as well as public opinion.

While different stem cell types have similarities, or overlaps in their protective functions, they each have particular strengths and limitations. MSCs are one of the most studied stem cells; they have a rich research knowledge base, have strong differentiation ability, and are easy to collect but have limited ability to enter the infarct core area. Other stem cells such as MUSE cells and NSCs can efficiently enter the infarct area and reconstruct the neuronal structure. In current research examining the protective function of a specific stem cell, researchers pay greater attention to the role of the "bystander effect," exosomes, and paracrine effect and, thus, to some extent, ignore the basic characteristics of stem cells such as the ability of differentiating into mature cells, which can directly replace the damaged brain tissue. In MUSE cells, iPSCs, and ESCs, the focus has been less on bystander effects and more on their strong ability to differentiate and replace damaged cells and rebuild the neuronal circuit; however, ethical issues and tumorigenicity must be taken into consideration in further ESCs or iPSC research. The role of stem cells is not limited to one cell or mechanism, and there are more stem cell types and mechanisms for us to discover and explore. Further study on induced differentiation, gene-edited stem cells, or finding new stem cell types will greatly advance our current knowledge base.

PROTECTIVE MECHANISMS REGULATING STEM CELL THERAPY AFTER IS

Stem cell transplantation can improve the IS outcome from different aspects, exerting a variety of protective effects thanks to the pleiotropic mechanisms underlying stem cell therapy. Over the last decades, several stem cell types have been widely studied and even been used in clinical practice. Mechanisms to regulate their therapeutic effects include modulation of neuroinflammation and the immune response, angiogenesis, neurogenesis, secretion of protective factors, and cell migration.

Stem Cell Migration

The first step in restoring damaged cells following stroke is the migration of stem cells into the damaged brain regions (De Feo et al., 2012); however, it is not yet clear how the stem cells enter the infarct core or boundary area after IS; therefore, modulating their migration remains a challenge in translating stem cell therapy from the laboratory to clinical practice (Sullivan et al., 2015). Understanding this mechanism should be a critical part of future studies on the protective effects of stem cell therapy.

Although endogenous NSCs located in the SVC remain inactive in normal conditions (Obernier and Alvarez-Buylla, 2019), the onset of IS is associated with its activation and recruitment to the adjacent lesioned areas (Nakayama et al., 2010), and the ischemic insult also leads to increased migration, proliferation, and differentiation. During the ischemia condition, the hypoxia-inducible factor-1α (HIF-1) represents an important transcriptional factor induced by hypoxia itself, which can promote neuroprotection during a particular time window (Yamashita and Abe, 2016). HIF-1 can upregulate EPO release (Li et al., 2020a), which contributes to stem cell migration after IS, while the knockdown of EPO receptors significantly decreases post-stroke neurogenesis by impairing stem cell migration (Tsai et al., 2006). The upregulation of EPO increases BDNF expression, which is neuroprotective in IS. A recent study revealed that although BDNF administration has no effect on migration speed of stem cells, BDNF treatment of NSCs can result in significant chemotactic and directional migration in CXXL12 gradients compared with untreated NSCs (Xu and Heilshorn, 2013). Due to the role of BDNF in stem cell migration, recent research has shown that a certain level of BDNF can serve as a minimum predictive value for the recovery of motor function after IS (Luo et al., 2019). The mesencephalic astrocyte-derived neurotrophic factor (MANF) also plays a crucial role in regulating neural progenitor cell (NPC) migration and protects endogenous NSCs against OGD-induced injury (Tseng et al., 2018).

As discussed above, the BBB is a selective barrier composed of ECs, pericytes, and astrocytes; and the TJs and AJs between ECs help the BBB to separate the CNS from the peripheral blood flow. The entrance of transplanted stem cells from the peripheral blood circulation to the brain parenchyma through the BBB is therefore necessary for stem cells to exert an effective therapeutic effect. The expression of E- and P-selectin in the cerebral microvasculature increases after IS (Huang et al., 2000). A recent study observed increased adhesion of BMSCs in cerebral venules after the IS compared with sham mice, and the immunoneutralization of either E- or P-selectin blocks the recruitment of adherent BMSCs (Yilmaz et al., 2011). These results suggest that ECs exhibit a pro-adhesive phenotype that increases selectin-dependent rolling and adhesion after IS. VCAM-1 is also an adhesion molecule upregulated on EC membrane after IS. Transplanted NSCs can adhere to the VCAM-1 in a static adhesion assay, and CD49 may serve as the critical ligand for VCAM-1-mediated NSC adhesion (Guzman et al., 2008). Laminin signaling can increase the migration of neuroblasts along vascular scaffolds to injured areas via β1 integrin (Fujioka et al., 2017). Selectin-mediated rolling and integrin-associated adhesion play important roles in the transendothelial processes of transplanted stem cells, although they can also increase the recruitment and infiltration of neutrophils after the onset of IS, which can aggravate brain damage (Ao et al., 2018); however, its detailed mechanisms need to be further elucidated. After stem cell adhesion to the ECs, CXCL-11 is released, which can bind CXCR-3 and increase the permeability of BBB by opening the TJs under the activation of ERK1/2 signaling pathway (Feng et al., 2014). Stem cell therapy can also reduce BBB disruption by preventing the upregulation of MMP-9 and downregulating ICAM-1 in ECs (Cheng et al., 2018). The apparent opposite effect of stem cells on the BBB depends on the different stages of stem cells, an aspect that requires further exploration.

After IS, HIF-1 stimulates the transcription of SDF-1/CXCL12 (Ceradini and Gurtner, 2005); and the levels of CXC chemokine receptor 4 (CXCR4), an SDF-1 receptor, increase on the membrane of stem cells (Delcroix et al., 2010;

Moll and Ransohoff, 2010). SDF-1 binding to CXCR4 activates several signaling pathways such as the phosphatidylinositide-3-kinase (PI3K/Akt), ERK1/2, c-Jun N-terminal kinase (JNK), and p38 MAPK pathways, which enhance stem cell migration. The extent to which pathways are activated is influenced by several factors such as the differentiation stage (Chen Y. et al., 2014) and the concentration of SDF-1 (Yu et al., 2016). Despite the interaction between SDF-1 and CXCR4, a recent study found that osteopontin (OPN) is a phosphoglycoprotein constitutively expressed in the brain, which plays an important role in tissue homeostasis. OPN expression upregulates and stimulates the migration of NSCs via CXCR4, resulting in an effective therapeutic effect after IS (Rabenstein et al., 2015). During ischemia, C-C motif chemokine ligand 2 (CCL2), one of the most highly expressed chemokines, contributes to neurological repair; and the interaction between CCL2 and CCR2 (CCL2 receptor) enhances stem cell migration to the infarcted area, promoting subsequent brain repair (Huang et al., 2018; Lee et al., 2020). TLRs are important mediators that cause neuroinflammation but are also involved in brain repair after IS. The ischemic insult induces TLR4 activation, which is involved in the early expression of SDF-1 and faster recovery of BDNF expression, which promotes migration of endogenous neuroblasts (Palma-Tortosa et al., 2019). Neuroinflammation is a crucial aspect of IS pathophysiology; and inflammatory chemotactic agents and cytokines such as MCP-1, macrophage inflammatory protein-1alpha (MIP-1alpha), and IL-8 expressed in the injured brain can increase stem cell migration into the infarcted areas (Wang et al., 2002; **Figure 1**).

Modulation of Neuroinflammation and Immune Response

After the onset of IS, the DAMPs released from the damaged CNS cells bind receptors such as TLRs or nuclear receptors to activate the innate immune system (Benakis et al., 2014), and increase expression of pro-inflammatory cytokines. As previously summarized, the cascade is mediated by the activation of innate immune cells or leukocytes from the peripheral blood infiltrating through the damaged BBB. Transplanted stem cells can ameliorate the inflammatory process and exert a protective function.

To deal with the cascade, transplanted stem cells can decrease the expression of pro-inflammatory cytokines. MSC added to conditioned media can reduce the secretion of IL-6 and TNF-α by microglia (Dabrowska et al., 2019a; Tobin et al., 2020). The reduction of IL-1β, IL-6, and TNF-α can also be observed in MSC-treated mice following ischemia (Cheng et al., 2018). Other stem cell types, including NSCs, DPSCs, and iPSCs, can also protect the CNS from damage by pro-inflammatory cytokines (Eckert et al., 2015; Nito et al., 2018). Stem cells can also secrete anti-inflammatory cytokines including IL-4, IL-10, and TGF-β1 (Zhang et al., 2016).

In addition to the important role of ameliorating neuroinflammation, different cell types involved in neuroinflammation or the immune response such as macrophages, microglia, astrocytes, and even some antigen presenting cells can also be a stem cell target. DPSCs, for example, possess a potent immunoregulatory function by increasing $CD3^+$ T cell apoptosis and the expression of anti-inflammatory cytokines and by decreasing the expression of pro-inflammatory cytokines (Demircan et al., 2011). BMSCs can also decrease infiltration of gamma delta T (γδT) cells and increase infiltration of regulatory T cells (Tregs) after IS (Wang et al., 2014). Macrophages co-cultured with MSCs exhibit a phenotype with low secretion of IL-6 and TNF-α and high expression of IL-10, which is similar to an anti-inflammatory phenotype, indicating that stem cells can modulate the immunophenotype and functional characteristics of macrophages (Kim and Hematti, 2009). MSCs can reduce proliferation of NK cells, cytokine secretion, and the expression of receptors by similar regulatory mechanisms (Valencia et al., 2016). After the onset of IS, the activation of residual cells in the CNS plays an important role in neuroinflammation. NSC transplantation decreases the number of infiltrating microglia, macrophages, inducible nitric oxide synthase (iNOS), and cyclooxygenase (COX)-2 expressing cells (Watanabe et al., 2016) and also reduces cytokine levels (Lee et al., 2008). Furthermore, iPSC transplantation in an SCI model not only reduces the activated macrophages or microglia but also inhibits GFAP-positive cells and glial scar formation. The increasing expression of IL-10 after stem cell transplantation partly accounts for the attenuation of astrocyte reactivity.

In the acute phase of IS, leukocytes invade the brain parenchyma due to the increased BBB permeability and the upregulation of ICAM and VCAM. The results of histologic quantifications in the peri-stroke areas revealed that NSC transplantation decreases MPO^+ neutrophil infiltration (Lee et al., 2008). Another study revealed that NSC transplantation downregulates ICAM and VCAM, which can, to some extent, repair the BBB (Huang et al., 2014). However, some stem cells cannot directly improve inflammation and immune response by targeting inflammatory factors and immune cells, or maybe their exact mechanism has not yet been discovered. In the chronic phase of IS, some stem cells can indirectly improve neovascularization, neurogenesis, and restoration of BBB, thus exerting anti-inflammatory effects. The recent increase in interest in human pluripotent stem cells (hPSC) is due to the fact that they have the potential to differentiate into cells with BBB markers and characteristics (Lippmann et al., 2020); thus, their protective effect could be partly due to their ability to restore the BBB. In light of all these discoveries, stem cell therapy represents a potential treatment to protect the CNS through neuroinflammation activity (De Feo et al., 2012; **Figure 2**).

Secretion of Protective Factors

Some recent studies have found a lower density of transplanted stem cells in the stroke core compared with the penumbra or surrounding area, suggesting that functional improvements are more likely mediated by the release of trophic factors rather than cell replacement and differentiation, a phenomenon known as the "bystander effect."

The bystander effect is common in the protective mechanisms of stem cell used in the IS therapy. The use of GRID, a contrast agent that allows monitoring of NSCs, revealed that these stem

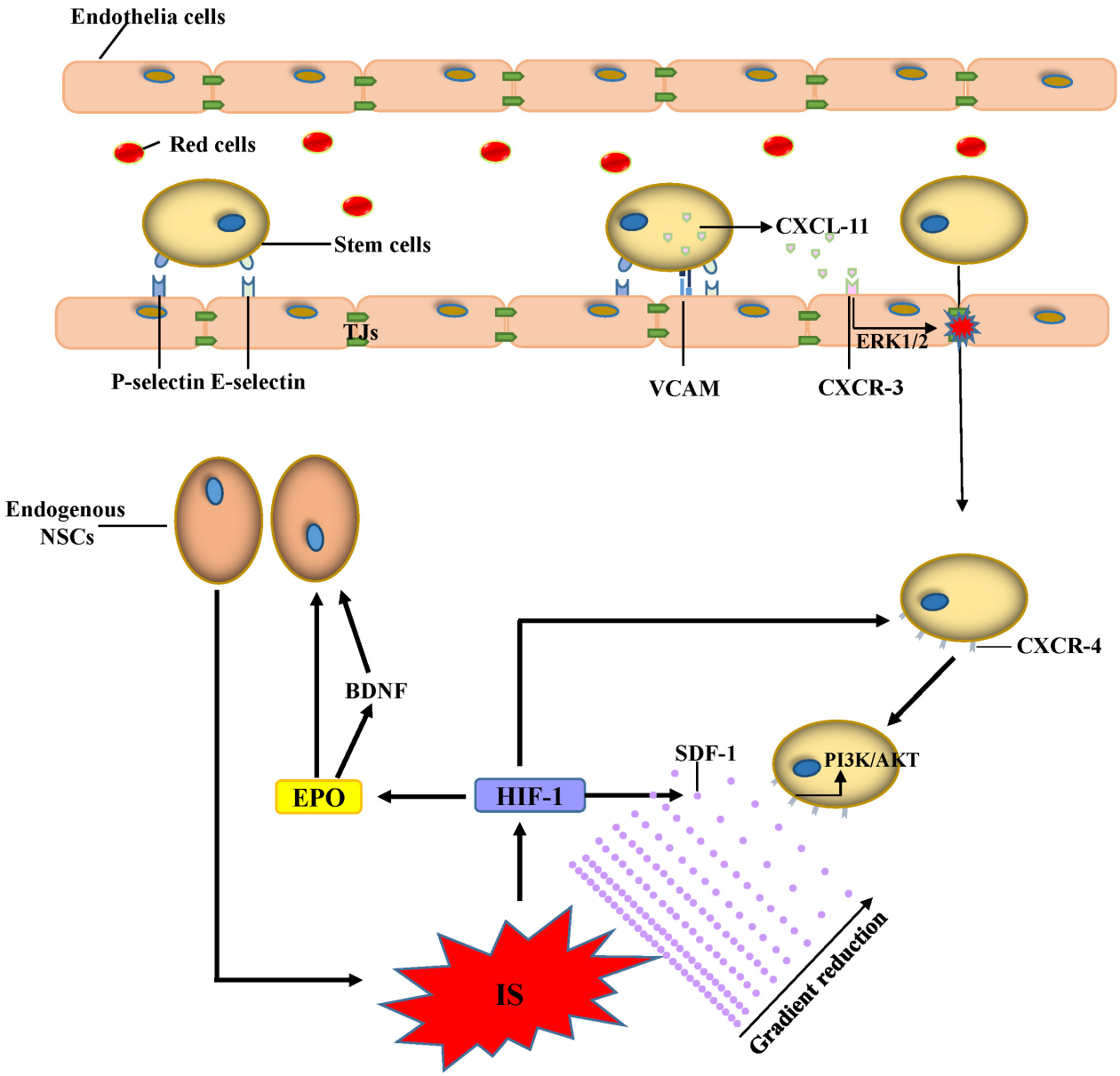

FIGURE 1 | Mechanisms of stem cell migration. Adhesion between stem cells and endothelial cells increases after IS due to the increased expression of E-selectin, P-selectin, and VACM. CXCL-11 released from stem cells can bind CXCR-3 and increase the permeability of the BBB by opening the TJs under the activation of the ERK1/2 signaling pathway. Administered stem cells can migrate to the ischemic lesion by the gradient of SDF-1 through the activation of the PI3K/AKT signaling pathway. The proliferation and migration of endogenous NSCs increase due to BDNF and EPO after IS. Abbreviations: IS, ischemic stroke; VCAM, vascular cell adhesion molecule; CXCL, C-X-C motif ligand; CXCR, C-X-C motif chemokine receptor; EPO, erythropoietin; HIF, hypoxia-inducible factor; SDF, stromal cell-derived factor; PI3K, phosphoinositide-3-kinase; NSCs, neural stem cells; TJs, tight junctions.

cells migrate to the area around the ischemic infarct instead of the core area in rats (Modo et al., 2004). The change of function and structure of the neuron network was non-invasively monitored using MRI for 3 months after stem cell implantation in rats. It is that revealed the functional network sharply decreased for the entire 3 months, while it was previously stabilized in mice with NSC transplantation, indicating the important paracrine role of stem cell treatment in IS therapy (Green et al., 2018). DPSCs can move into the boundary area of the ischemic lesion and mostly differentiate into glial cells instead of neurons (Song et al., 2017); however, trophic factors such as cytokines, chemokines, and exosomes released by the stem cells can interact with different cells in the CNS to improve the outcome after IS. These factors are released into the microenvironment via direct permeation and extracellular vesicles (EVs) such as exosomes or microvesicles and exert a paracrine effect. In recent years, many studies have shown that microglia, astrocyte, and macrophage activation can be effectively attenuated by neurotrophic factor

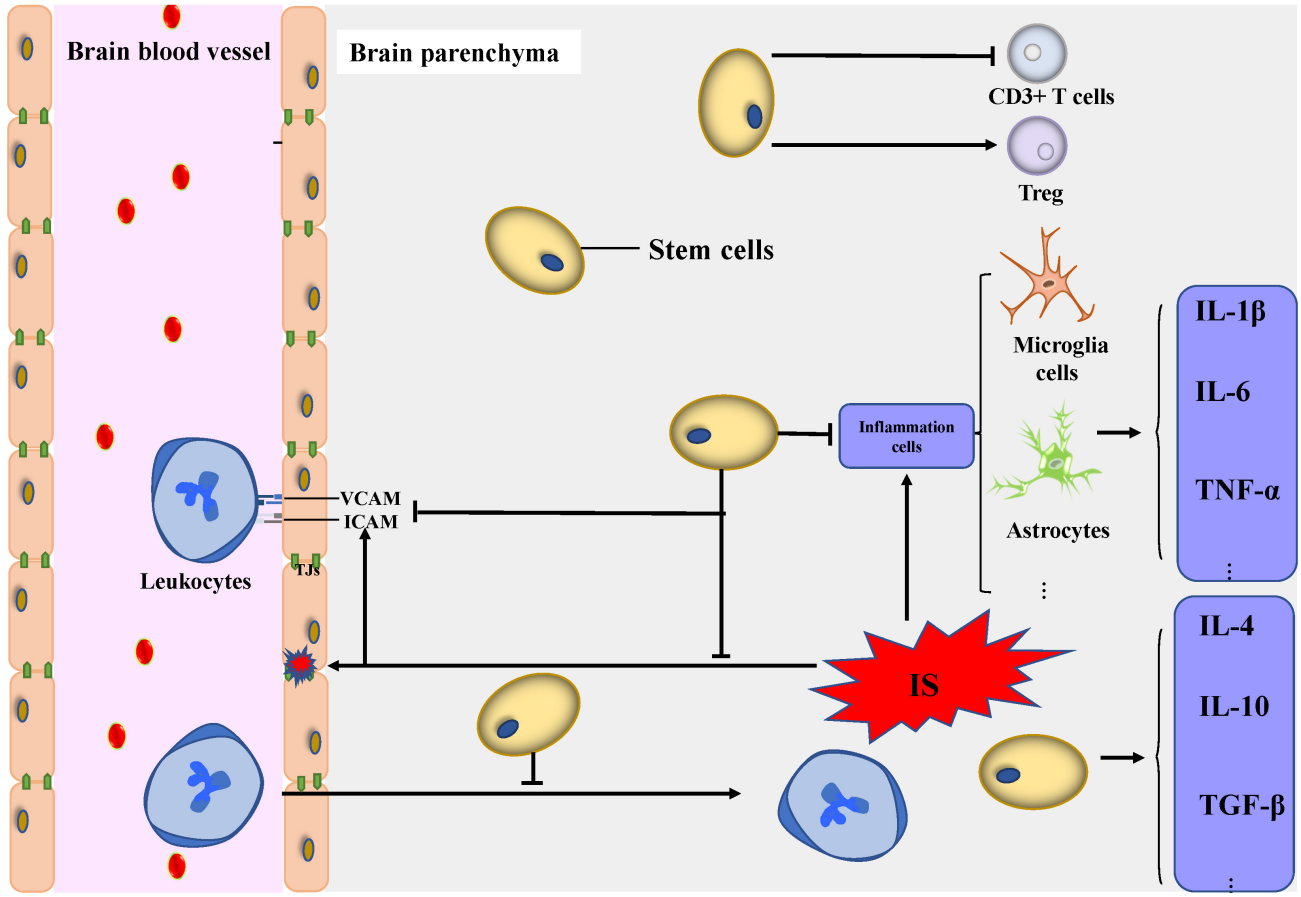

FIGURE 2 | Modulation of neuroinflammation and immune response. Transplanted stem cells can decrease the expression of pro-inflammatory cytokines including IL-1β, IL-6, and TNF-α and secrete anti-inflammatory cytokines including IL-4, IL-10, and transforming growth factor-β1. Transplanted stem cells can modulate the activity of inflammatory cells including microglial cells, astrocytes, and T cells. The infiltration and pro-inflammation effect of leukocytes from peripheral blood decreased under the administration of stem cells. Abbreviations: IS, ischemic stroke; VCAM, vascular cell adhesion molecule; ICAM, intercellular adhesion molecule; IL, interleukin; TNF, tumor necrosis factor; TGF, transforming growth factor; Treg, regulatory T cells; TJs, tight junctions.

secretion (Valencia et al., 2016; Watanabe et al., 2016) after stem cell transplantation. The secretion of high levels of protective cytokines and neurotrophic and angiogenic factors is involved in the paracrine effect after IS (Stonesifer et al., 2017; Geiseler and Morland, 2018), and this effect on stem cells and its role in IS have been recently investigated. After injection with a conditioned medium rich in MSCs, paracrine factors into the lateral ventricle of the middle cerebral occluded artery of rats, the infarct volume and cerebral edema were significantly reduced, and the neurological deficits were improved (Asgari Taei et al., 2020). In rats receiving transplanted stem cells, human insulin-like growth factor 1 (IGF-1) was present in the peri-infarcted area at 3 days, and they had a higher level of neurotrophic factors such as VEGF, epidermal growth factor (EGF), and basic fibroblast growth factor (bFGF) than the control group (Wakabayashi et al., 2010).

Extracellular vesicles have recently received a great deal of recent attention. They are membrane vesicles containing proteins, nucleic acids, and bioactive lipids that can be secreted by different types of cells (Raposo and Stoorvogel, 2013). After being released from the original cells, EVs enter the neuronal microenvironment; and the content of the original cells, such as membrane and cytosolic lipid, protein, and RNA, is transferred. EVs serve as a mediator, participating in intercellular communication and finally leading to epigenetic changes and functional modifications of neighboring or distant cells. The expression of VEGF and HIF-1α is upregulated in the core area and ischemic border zone (IBZ) after MSC transplantation, which can increase angiogenesis. When the human microglia cell line (HMO6) and MSCs are co-cultured *in vitro*, VEGF mRNA increases in both lines, indicating that MSCs increase angiogenesis by a paracrine effect (Sheikh et al., 2019). EVs may be the main intercellular mediator, and those secreted by MSCs can enhance angiogenesis by activating the signal transducer and activator of transcription 3 (STAT3), the suppression of which can abolish the paracrine effect (Xia et al., 2020). EVs from stem cells are rich in various microRNAs (miRNAs), which can target the 3'-untranslated region (UTR) to upregulate VEGF, bFGF, and HIF and activate the PI3K/AKT, Ras/Raf/MEK/ERK/MAPK, and Notch signaling pathways, all of which are associated with

angiogenesis (Zhu et al., 2015; Guo et al., 2018; Potz et al., 2018; Icli et al., 2019). Due to the lower migration of the transplanted adipose-derived stem cells (ADSCs) into the ischemic core area, a study aiming to discuss the potential paracrine effect of stem cell therapy after IS found that EVs mediate the transfer of miRNA-126, which significantly increases the expression of von Willebrand factor (an EC marker), thus promoting the neurological recovery after stroke (Geng et al., 2019). EVs from stem cells possess the ability to mimic the beneficial effects of the original cells through the transfer of miRNA and functional proteins (Qiu et al., 2018). Stem cells can participate in the inflammatory response and immune modulation after IS and modulate these pathophysiological changes in an indirect manner by the paracrine effect.

After IS, stem cell-derived EVs can reduce neuroinflammation by turning the microglia into an anti-inflammatory phenotype in rhesus monkeys (Go et al., 2020), which modulates neuroinflammation through interaction with immune recipient cells (Spellicy and Stice, 2020). In addition to microglia inhibition, EVs isolated from MSCs attenuate the activation of astrocytes and T cytotoxic cells, resulting in the inhibition of the expression of pro-inflammatory cytokines such as IL-1, IL-6, and TNF-β (Dabrowska et al., 2019b). After lipopolysaccharide (LPS) stimulation, the administration of stem cell-derived exosomes results in a decrease in astrocyte activation as well as a decrease in expression of TNF-α, IL-1β, and IL-6. These effects suggest that the paracrine effect ameliorates inflammation-induced astrocyte alterations (Xian et al., 2019). The immunomodulatory mechanisms are also established between transplanted stem cells and T cells by paracrine interaction. The paracrine factors released from MSCs suppress $CD4^+$–$Tbet^+$ (Th1) and $CD4^+$–$Gata3^+$ (Th2) cells but stimulate $CD4^+$–$Stat3^+$ (Th17) and $CD4^+$–$CD25^+$–$FoxP3^+$ (Treg) cells (Özdemir et al., 2016). The paracrine effect also has strong immunoregulatory functions contributing to the apoptosis of CD3 (+) T cells when co-cultured with DPSCs (Demircan et al., 2011). The beneficial paracrine effect of stem cell support and protect neurons in ischemic conditions, which is largely attributed to the release of neurotrophic factors such as SDF, NGF, BDNF, GDNF, and FGF (Wakabayashi et al., 2010). The infarct volume in IS rats significantly decreases after MSC transplantation into the infarcted area, accompanied by an increase in bFGF and SDF expression near the infarcted cortex compared with the control group rats (Toyoshima et al., 2015). NSC transplantation can also enhance neurogenesis and activate Akt and Erk1/2 signaling through the release of GDNF after IS (Yuan et al., 2013).

Although the neurotrophic factors mentioned above can protect neurons, some miRNAs also exhibit a beneficial function as intercellular messengers. Exosomes from stem cells containing miR-17-92 have significantly robust beneficial effects in the improvement of the neurological function, and they can rescue the boundary area of the infarcted lesion by enhancing neuronal dendrite plasticity and neurite remodeling (Xin et al., 2017). After IS, ADSC-derived exosomes containing miR-126 increase von Willebrand factor and doublecortin (a neuroblast marker) expression, decrease neuronal death, and increase neuron proliferation, improving neuronal functional in IS rats (Geng et al., 2019). Endogenous NSCs and NPCs are located mainly in the dentate gyrus of the hippocampus, the SVZ, and the olfactory bulb and are normally inactive (Obernier and Alvarez-Buylla, 2019); however, the paracrine effect of transplanted cells can promote the proliferation and migration of endogenous NPCs to the injured brain areas (Leal et al., 2017; Geiseler and Morland, 2018). In recent years, interest in the benefits of the paracrine effect of stem cells transplantation has increased the attention on EVs and exosomes due to their low immunogenicity and high BBB permeability and therefore are a potential IS treatment (**Figure 3**).

Angiogenesis and Blood–Brain Barrier Repair

Temporary or permanent blood flow deficiency is considered to be the initial cause of a series of pathophysiological changes during IS, and the destruction of BBB directly related to vascular injury is a vital pathological change leading to hemorrhage and poor prognosis (Jiang et al., 2018). Increasing microvessel density and the repair of the damaged BBB to reconstruct the blood flow to the core ischemic area to re-establish the supply of oxygen and nutrients may represent another potentially relevant treatment. Several animal experiments have shown that a variety of stem cells can induce angiogenesis and BBB repair after IS (Uemura et al., 2012; Wang J. et al., 2013; Wang et al., 2015; Zhu et al., 2015; Muir et al., 2020).

Angiogenesis is the formation of new microvessels that branch off from preexisting capillaries (Hatakeyama et al., 2020a). The increased microvessel density and TJ protein (occludin) expression in the border of the animal ischemic core suggest that angiogenesis and BBB repair occur after stem cell treatment (Zacharek et al., 2007). These effects can be divided into two types: cell differentiation and neurotrophic factor secretion. These stem cells directly replace the damaged vascular cells through migration and differentiation after crossing the BBB. Several types of mononuclear cells (MNCs), including $CD34^+$, promote angiogenesis through their direct differentiation into smooth muscle cells (SMCs) and ECs in MCAO rats (Terry et al., 2011; Wang J. et al., 2013). In addition to the promotion of angiogenesis, the administration of endothelial progenitor cells (EPCs) seems to be effective in BBB renovation due to their vascular phenotype (Moubarik et al., 2011). The extensive vascular engraftment of human bone marrow EPCs (hBMEPCs) pre-labeled with β-gal suggests a necessary mechanism of direct differentiation for BBB repair (Garbuzova-Davis et al., 2017). MSC differentiation into ECs mediated by the Notch signaling pathway and mitochondrial nanotube transportation are other vital mechanisms of angiogenesis (Zhu et al., 2015). However, the differentiation of grafted stem cells into cells such SMCs or ECs is limited. The existence of direct differentiation has not been confirmed in either HSCs or other stem cell types (Uemura et al., 2012).

The bystander effect exerts a more comprehensive and effective influence in promoting angiogenesis and BBB preservation. Coordinated remodeling of ECs, basal matrix, and pericytes induces angiogenesis to produce new blood vessels

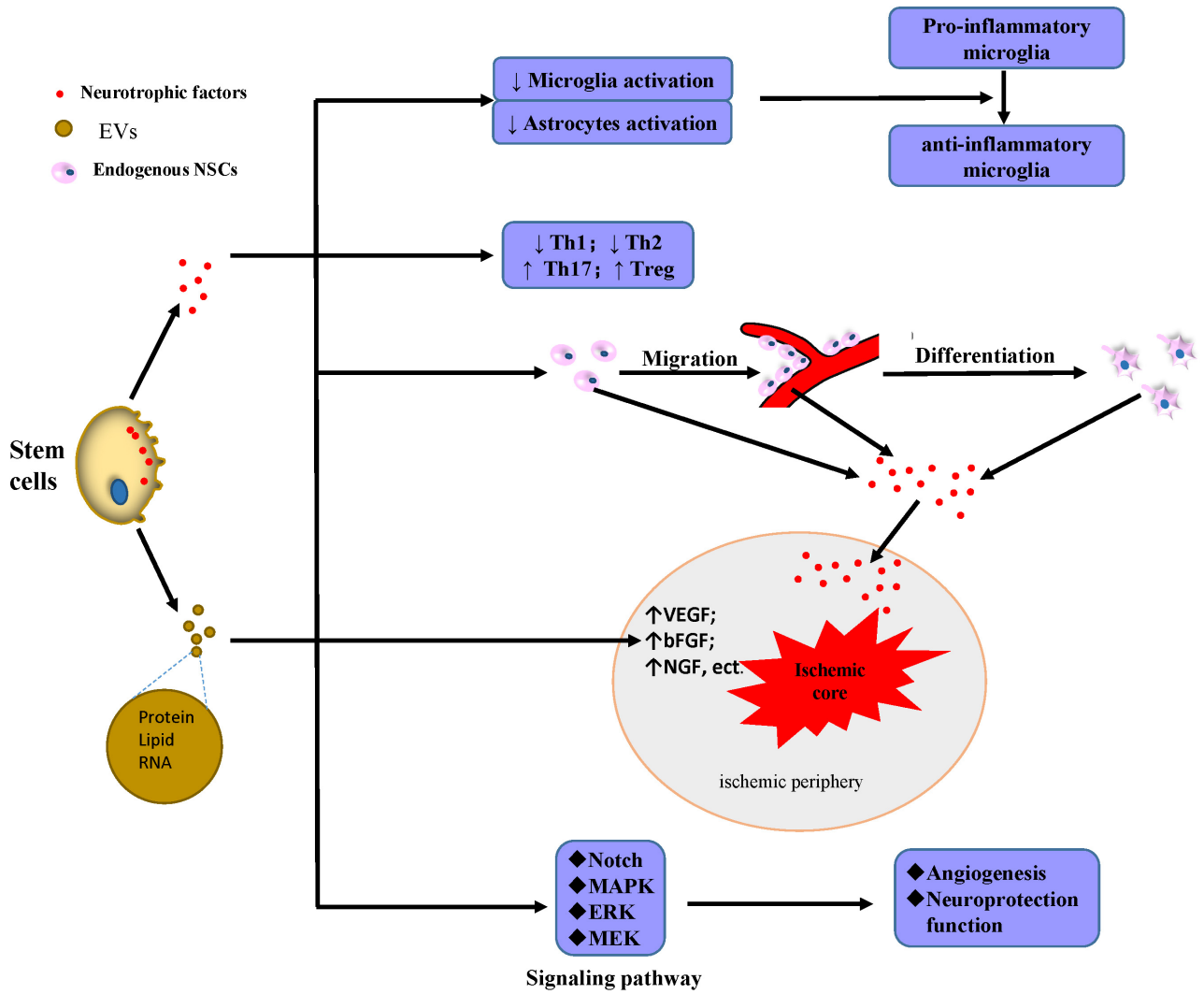

FIGURE 3 | Mechanisms of secretion of protective factors. Stem cells secrete neurotrophic factors or generate EVs mainly containing proteins, lipids, and RNA with multiple neuroprotective effects: inhibiting the activation of microglia and astrocyte, and also promoting microglia shift to the anti-inflammatory phenotype instead of the pro-inflammatory; downregulating Th1 and Th2 and upregulating Th17 and Treg; promoting the migration, differentiation, and secretion of neurotrophic factors to the ischemic periphery; upregulating the content of many factors including VEGF, bFGF, and NGF in the ischemic periphery; and mediating angiogenesis and neuroprotection through the induction of MEK/ERK/MAPK and Notch signaling pathway. Abbreviations: ↑, upregulation; ↓, downregulation; EVs, extracellular vesicles; NSCs, neural stem cells; Th, T helper cells; Treg, regulatory T cells; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; NGF, nerve growth factor; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, MAP kinase.

(Li and Carmeliet, 2018). This complex process is regulated by a variety of cytokines and receptors; and some mediators such as VEGF and TGF-β modulate angiogenesis during the natural pathophysiological process (Kanazawa et al., 2019). VEGF, which has been widely studied in the transplantation of MNCs and MSCs, is able to promote angiogenesis by the bystander effect. The expression of other trophic factors such as BDNF, GDNF, Angiotensin 1 (Ang-1), Angiotensin 2 (Ang-2), IGF-1, and bFGF is upregulated after MNC treatment, contributing to the formation of immature vessels (Ikegame et al., 2011; Li et al., 2020b). Most of these cytokines promote angiogenesis by binding to specific receptors. For example, VEGF binding to VEGF receptor-2 (VEGFR-2) with high tyrosine-kinase activity improves angiogenesis, while binding to another receptor such as VEGFR-1 results in no protective effect. Some studies have shown that VEGFR-2 increases with VEGF upregulation after stem cell therapy in ischemic rats and that the same pattern was also observed in Ang-1 and its receptor Tie2 (Zacharek et al., 2007; Toyama et al., 2009). The angiogenic effect of VEGF in IS also varies according to the time window, source, and administration mode. Early administration of recombinant human VEGF in ischemic rats can significantly aggravate BBB leakage and hemorrhagic transformation (Zhang et al., 2000). The regulation of nutritional factors in the molecular mechanism of angiogenesis is multifaceted and often associated with neurogenesis (Hatakeyama et al., 2020a). A recent study

found that autophagy may be a cause of vascular injury and that EVs secreted from MSCs can activate signal transduction and the activation of the transcription factor STAT3 to inhibit the autophagy-related pathway (Xia et al., 2020).

There has been a recent increase in miRNA research, and EVs containing certain miRNAs produce protective effects in stem cells through the activation of signaling pathways related to angiogenesis and neurogenesis (Bang and Kim, 2019; Moon et al., 2019). ADSCs *in vitro* secrete miRNA-181b, which promotes the angiogenesis in brain microvascular ECs through the transient receptor potential melastatin 7 (TRPM7) axis (Yang et al., 2018). MiRNAs can also regulate stem cell function; for example, miR-126 overexpression can promote the proliferation, migration, and tube formation abilities of EPCs and also the increase NO production of EPCs via activation of the PI3K/Akt/eNOS pathway, thus increasing cerebral microvascular density and angiogenesis (Pan et al., 2018). Angiogenesis is considered to be helpful for neurogenesis and the formation of neurovascular units and is favorable for regeneration and functional recovery of neurons.

Neural Circuit Reconstruction

Cells with different properties and functions in the brain form neural circuits and neural networks at different levels through various complex connections; and positive and negative feedback regulates the complex functions of the brain (Helmstaedter, 2013). IS results in the loss of brain neurons and the destruction of nerve connections, leading to severe neurological dysfunctions. Adult mammals have limited brain regeneration abilities (Li et al., 2020b), meaning that stem cell therapy can play an important role in the repair and functional reconstruction of damaged neural circuits.

Neurogenesis involves the generation of new functional neurons from NSCs, which includes their proliferation, migration, and differentiation into mature neurons. In the adult brain, endogenous neurogenesis continuously persists in two areas, the SVZ of the lateral ventricle and the subgranular zone (SGZ) of the hippocampus; and it increases after IS (Hatakeyama et al., 2020b). Several signaling pathways and extracellular factors are involved in the regulation of neurogenesis, which is further enhanced by stem cell treatment (Hatakeyama et al., 2020a,b). The activation of NSCs proliferation is regulated by Notch-mediated signaling and other transcription factors such as Wnt and the Sonic Hedgehog signaling pathway (Faigle and Song, 2013). The proliferation of endogenous NSCs can be enhanced by several extracellular factors such as FGF-2, IGF-1, and platelet-derived growth factor (PDGF) (Hatakeyama et al., 2020a), all of which are abundantly secreted by transplanted stem cells. Following NSC proliferation, several mechanisms mediated by the administered stem cells promote neuroblast migration from the SVZ to the peri-infarcted region, especially where angiogenesis occurs (Ruan et al., 2015). BDNF secreted by ECs promotes vascular-guided NSC migration (Grade et al., 2013). In newborn capillaries working as a scaffold, β1 integrin expressed in neuroblasts can adhere to laminin expressed in the vessels to achieve migration (Grade et al., 2013). MSC treatment promotes NSC migration after the release of neurotrophic factors beneficial to angiogenesis. After proliferation and migration, NSCs differentiate into different mature neurons according to the local microenvironment in which they are located to repair damaged brain areas (Kawabori et al., 2020a). Angiogenesis plays a crucial role in the differentiation of NSCs into mature neurons in the ischemic area, as observed in *in vitro* experiments (Shen et al., 2004) and also in a recent animal study reporting that angiogenesis in the cortex can induce NSC transition from a proliferative form to a differentiated one (Shen et al., 2004). Transplanted stem cells can also perform neurogenesis by directly differentiating into neuronal cells. More than 50% of transplanted stem cells in the ischemic brain express a neuronal phenotype at 2 months after cell transplantation (Kawabori et al., 2012, 2013).

The sprouting of axons increases the connections between different areas of the brain; however, in the adult brain after IS, axonal sprouting is limited by the failure of neurogenesis to be fully activated and by inhibitory cytokines (Carmichael et al., 2017). Treatment with stem cells such as MSCs, NSCs, and bone marrow-derived stem cells (BMCs) ameliorates this defect by promoting angiogenesis and bystander effects (Neuhuber et al., 2005; Fujioka et al., 2017; Huang et al., 2019). VEGF released from vessels, and laminin/β1 integrin signal in ECs induced by microtubule assembly and stabilization are both crucial aspects in promoting axonal outgrowth (Jin et al., 2006; Fujioka et al., 2017). Since axonal sprouting is limited before angiogenesis, this suggests a close connection between axonal sprouting and angiogenesis (Kanazawa et al., 2017a). Treatment with BDNF, gene-transfected MSCs, and axonal outgrowth can also be promoted (Hira et al., 2018).

Synaptogenesis is a long back-and-forth process involving synapse formation, synapse stabilization, and activity-dependent synapse refinement and elimination, all important aspects to maintain the stability and precision of neural circuits (Cepeda et al., 2002). Research on synaptogenesis after stem cell therapy is currently focused on the changes in characteristics. Following treatment with reprogrammed human neural precursor cells derived from iPSCs, the increased expression of the presynaptic vesicle protein synaptophysin enhances synaptogenesis after IS (Vonderwalde et al., 2020). Another study demonstrated that stem cell factor and G-CSF may promote synaptogenesis through VEGF-α (Ping et al., 2019).

Stem cell therapy mechanisms can be categorized into two distinct forms: direct action characterized by cell migrating to the infarct area and bystander effort, and indirect action characterized by improving local microenvironment through angiogenesis, neurogenesis, and the regulation of inflammation. Both mechanisms can combine to improve neuronal function after IS; however, the different mechanisms do not receive the same level of focus in the current research. There has been a lot of fundamental research into exosomes and their related secreted factors, as a medium for information exchange between cells, in stem cell therapy. However, there is limited research on direct differentiation of stem cells, which may be related to the current lack of effective detection methods. The examination of new key molecules or regulatory pathways

that regulate indirect action should also be encouraged, as these mechanisms are part of a closely connected network. For example, neuroinflammation reduction can be assisted by the alleviation of inflammatory factor infiltration when the BBB is repaired, and the regulation of inflammation provides a favorable microenvironment for angiogenesis or neurogenesis, which in turn supplies oxygen and nutrients to ischemic tissue and neurotrophic factor necessary for axonal outgrowth. Stem cell therapy, through the regulation of multiple mechanisms, is an innovative treatment with outstanding advantages (**Figure 4**).

CLINICAL STEM CELL THERAPY TRIALS

Over the last decades, a large number of preclinical studies have been carried out on the role of stem cell therapy in IS treatment. Thanks to the promising results of the preclinical studies, an increasing number of clinical trials have investigated the safety and efficacy of different stem cells in IS treatment. Some issues still need to be resolved, such as the choice of the cell type, cell dose, time of transplantation, and injection routes, all of which benefit from a close link between basic and clinical research (**Table 1**).

Choice of Stem Cell Types

Several types of stem cells have been widely studied in IS animal models, and some of them have been examined in clinical trials. All the stem cells used play important roles in the treatment of IS through different mechanisms. Stem cells can be obtained from different tissues of the same IS patient, including bone marrow, adipose tissue, dental pulp, and connective tissue; or they can be commercially purchased. Although several clinical studies have investigated the safety and effectiveness of some stem cells, more attention has focused on BMCs such as MSCs (Steinberg et al., 2018). Tissue-derived MSCs remain the main cells used in stem cell therapy to treat IS because of their easy access and different protective mechanisms; however, the choice of a proper stem cell type in clinical IS treatment should take into account not only the advantages mentioned above but also the accessibility, ethical issues, risk of posttransplant rejection, allergies, and even tumorigenicity. When there is a high risk of posttransplant rejection, autologous stem cells are more suitable (Bang et al., 2005a; Chen D.C. et al., 2014; Trounson and McDonald, 2015). However, since IS is an acute disease, the time required to culture some autologous stem cells, such as BM-MSCs to reach the number required for transplantation, limits their application in acute phase of IS, and allogeneic stem

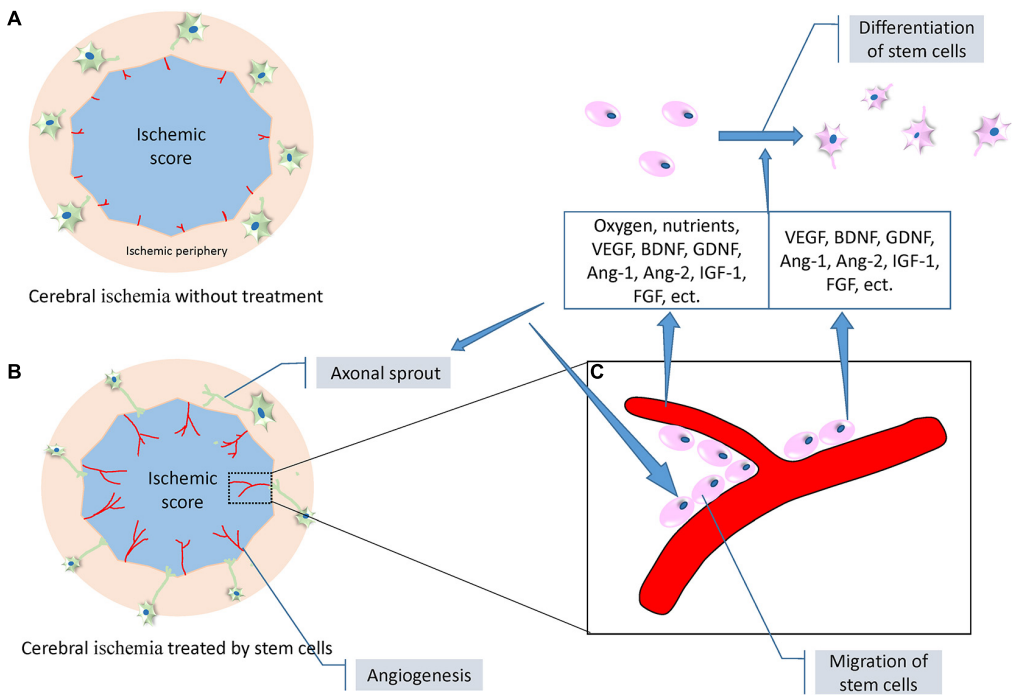

FIGURE 4 | A set of diagrams of angiogenesis, neurogenesis, and axonal outgrowth under the treatment of stem cells following ischemic stroke (modified from the reference of Masato Kanazawa et al., 2019). **(A)** A proposed diagram depicting the location of angiogenesis and axonal outgrowth after ischemic stroke without intervention. Weak angiogenesis (red lines) occurs at the border of the ischemic core (blue) and peripheral areas, and limited axonal sprouting occurs in the neurons (green cells) of the peripheral areas. **(B)** A proposed diagram depicting enhanced angiogenesis and evident axonal sprouting and outgrowth under the treatment of stem cells. **(C)** A representative diagram expanded from a certain area of the diagram in (**B**). Stem cells (pink) adhering to the newly formed blood vessels to achieve migration. The blood vessels and stem cells themselves secrete a variety of neurotrophic factors including VEGF, BDNF, GDNF, Ang-1, Ang-2, IGF-1, and FGF. In addition, blood vessels release additional oxygen and nutrients to promote the proliferation, migration, and differentiation of stem cells, and axonal outgrowth. Abbreviations: VEGF, vascular endothelial growth factor; BDNF, brain-derived neurotrophic factor; GDNF, glial cell-derived neurotrophic factor; Ang-1, angiotensin 1; Ang-2, angiotensin 2; IGF-1, insulin-like growth factor 1; FGF, fibroblast growth factor.

TABLE 1 | Summary of stem cells administered in clinical trials.

Stem cell types	Routes	Timing	Dose	Outcome	References
MSCs	Intracerebral	Subacute phase	$2.5–10 \times 10^6$	No adverse events, and dose-limiting toxicity or death and improved clinical outcomes.	Steinberg et al., 2018
MSCs	Intravenous	Subacute phase	$0.6–1.5 \times 10^8$	Reduced lesion volume as demonstrated by MRI and functional recovery with no tumor or abnormal cell growth.	Honmou et al., 2011
MSCs	Intravenous	Chronic phase	1.5×10^6/kg	Safety and improvements were seen in the National Institutes of Health Stroke Scale, Barthel Index, Mini-Mental Status Exam, and Geriatric Depression Scale scores during the follow-up study.	Levy et al., 2019
MSCs	Intravenous	Acute phase	1×10^6/kg	No adverse events, neurologic and systemic complications, and tumor development and improved neurologic recovery with decreased infarct size.	Díez-Tejedor et al., 2014
MSCs	Intravenous	Subacute phase	2.875×10^9	No significant difference between the BMSC arm and control arm in the Barthel Index score, Modified Rankin Scale shift analysis, National Institutes of Health Stroke Scale, and infarct volume.	Prasad et al., 2014
HSCs	Intra-arterial	Acute phase	1×10^8	Improvements in the Modified Rankin Scale score and National Institutes of Health Stroke Scale score and reductions in lesion volume during a 6-month follow-up period.	Banerjee et al., 2014
HSCs	Intrathecal	Chronic phase	$0.8–3.3 \times 10^7$	Improved muscle tone, rigidity, and motor power.	Wang L. et al., 2013
NSCs	Intracerebral	Chronic phase	$2 \times 10^6/5 \times 10^6$	No immunological or cell-related adverse events, decreased infarct lesions observed by MRI.	Kalladka et al., 2016
NSCs	Intracerebral	Subacute to chronic phases	2×10^7	4 of 23 participants improved by the pre-specified Action Research Arm Test subtest level; transient procedural adverse effects were observed, but no stem cell-related adverse events.	Muir et al., 2020
DPSCs	Intracerebral	Chronic phase	1×10^7	Safe, feasible, and observed function recovery.	Nagpal et al., 2016
PBSCs	Intracerebral	Chronic phase	$3–8 \times 10^6$	No serious adverse events; improvements in the National Institutes of Health Stroke Scale, Modified Rankin Scale, European Stroke Scale (ESS), and ESS Motor Subscale from the baseline to the end of the 12-month follow-up period.	Chen D.C. et al., 2014
MSCs	Intravenous	Subacute phase	1×10^8	No adverse cell-related, serological, or imaging-defined effects, and the Barthel index and Modified Rankin Scale score consistently improved during the follow-up period with less prominent infarcted areas.	Bang et al., 2005a
BMMNCs	Intra-arterial	Subacute phase	5×10^8	No procedure-related mortality, complications, new infarct, or symptomatic intracranial hemorrhage and good clinical outcome observed by Modified Rankin Scale score.	Bhatia et al., 2018
BMMNCs	Intravenous	Acute phase	$2.5 \times 10^8/3.4 \times 10^8$	Improved cerebral blood flow and metabolic rate of oxygen consumption 6 months after treatment with significantly better neurologic outcomes.	Taguchi et al., 2015
BMMNCs	Intravenous	Acute phase	1×10^7/kg	No severe adverse events and diffusion tensor tractography illustrates an increase in cortical spinal tracts fiber volume.	Vahidy et al., 2019
Fetus neuronal cell	Intracerebral	Chronic phase	5×10^6	Safe and feasible but no evidence of significant benefit in motor function were observed.	Kondziolka et al., 2005
Fetal porcine cells	Intracerebral	Subacute to chronic phase	1×10^6	Temporary worsening of motor deficits and seizure; 2 of 5 patients showed improvement in speech, language, and/or motor impairments over several months and persisted at 4 years. The study was terminated by the Food and Drug Administration.	Savitz et al., 2005

Abbreviations: *PBSCs, peripheral blood stem cells; MMCs, marrow-derived mononuclear cells; BMMNCs, bone marrow mononuclear cells; MSCs, mesenchymal stem cells; HSCs, hematopoietic stem cells; DPSCs, dental pulp stem cells; NSCs, neural stem cells.*

cells are therefore usually selected. In a phase I safety study, the fourth injection of allogeneic human UCB cells proved to be safe in adults with IS, and patient recovery was improved (Laskowitz et al., 2018). Although allogeneic stem cells have promising prospects, some issues still need to be solved, including the lack of systems guaranteeing the standard quality of stem cells administered in clinical trials or treatments, and the expense of producing, preserving, and transferring these cells. Although most methods used for stem cell therapy appear to be safe, some studies have reported on the continuous limitations in the administration of some stem cells such as ESC and iPSCs because of the risk of tumorigenicity, immunogenicity, and heterogeneity (Yamanaka, 2020). The transplantation of iPSCs can increase the risk of teratoma, especially in IS models (Yamashita et al., 2011), suggesting that the safety of stem cells should be evaluated. On the other hand, several studies have obtained encouraging results using ESC and iPSC; thus, the confidence of using them in clinical treatments is increased. The organ-on-a-chip model,

which is a sensitive *in vitro* system, is being developed to effectively predict the possibility of tumor formation (Sato et al., 2019). There are many mechanisms characterizing the underlying therapeutic effect of stem cells, meaning that one of them may be making the main contribution. For example, the bystander effect in MSCs after IS can effectively improve functional recovery, NSCs possess a strong ability to differentiate into neurons and reconstruct the neural circuit, and HSCs can significantly stimulate angiogenesis, which ameliorates OGD damage. The self-renewal and differentiation abilities of stem cells greatly differ. During IS, the pathophysiological changes after stroke are due to a dynamic process, which in turn requires a flexibility in stem cell choice dependent on individual patient's conditions. In the acute phase of IS, the main danger can be the acute cascade of neuroinflammatory factors, damage of the BBB, and the apoptosis of CNS cells, while sustained neuroinflammation, the formation of glial scar, and the destruction of the neural circuit influence the functional recovery in the subacute to chronic phase. MSC administration can significantly reduce the volume of the ischemia lesion and increase the functional recovery with the improvement of the outcome of the Modified Rankin Scale (mRS) and National Institutes of Health Stroke Scale (NIHSS) (Honmou et al., 2011; Díez-Tejedor et al., 2014). The paracrine effect of MSCs contributes, at least in part, to this neuroprotective effect. The safety of HSCs therapy is proven (Wang L. et al., 2013), and it can significantly improve the functional outcome from the baseline to the end of the 12-month follow-up period (Chen D.C. et al., 2014). Based on the different features of stem cell types and the pathophysiological change in each phase of IS, it is advisable that the stem cell therapeutic strategy should be adjusted to the pathophysiology features according to further clinical studies.

Stem Cell Administration Routes

Different administration routes including intravenous, intra-arterial, intrathecal, intracerebroventricular, intracerebral, subarachnoid, intranasal, and intraperitoneal routes have been used in preclinical trials, and many clinical studies have reported on the safety of these administration routes. The intravenous and intracerebral routes are widely used in published clinical studies; however, it is not clear which of them represents the optimal route.

The intra-arterial route is less invasive than the others. Like the intravenous route, the intra-arterial route can be used with a large number of cells (Bang et al., 2005b; Savitz et al., 2011b); however, the advantage of the intra-arterial route is that stem cells can bypass the peripheral filtering organs such as the liver, the spleen, and the lungs, leading to a higher cell engraftment into the brain and biological distribution (Berkhemer et al., 2015). A study using stem cells transduced with a firefly luciferase reporter gene for bioluminescence imaging (BLI) revealed that the BLI signal in the brain of the rats treated with the intra-arterial route is significantly higher than that in the lungs and liver and is higher than the signal in the brain after intravenous injection (Pendharkar et al., 2010). The MT is widely used at 3–4.5 h after IS with the help of a catheter (Wardlaw et al., 2014), and it is worth considering the combination of this and the intra-arterial route for the delivery of stem cells into the ischemic lesion. However, there is still the risk that the stem cells can stick together and form microemboli, blocking the vasculature and leading to occlusions and local hypoperfusion (Walczak et al., 2008). The intravenous route and intra-arterial route show the same protective features or feasibility, but the intravenous route is the one mainly used in recently published studies because of repeatability, lower invasiveness, easy access, and inessentiality of special equipment such as catheters. The intravenous route greatly contributes to the amelioration of neuroinflammation, activation of immune cells, and neuron apoptosis thanks to the stem cells' secretion of neurotrophic factors to modulate neuroinflammation or immune response. The transplanted stem cells administered by intravenous injection go over the pulmonary vascular system arriving in the infarcted area in IS (Misra et al., 2012); however, a large proportion of transplanted stem cells are trapped in the peripheral organs (Kawabori et al., 2012; Boltze et al., 2015). Interestingly, the stem cells trapped in the spleen improve the splenic inflammatory response, reduce neuron apoptosis in the brain, and increase the functional recovery (Vasconcelos-dos-Santos et al., 2012; Huang et al., 2013; Acosta et al., 2015). Like the stem cells transplanted by the intra-arterial routes, stem cells transplanted by the intravenous one can also lead to the formation of microemboli causing fatal pulmonary embolism. Nevertheless, the intravascular routes (intravenous and intra-arterial route) mainly contribute to the functional recovery that may mainly rely on the bystander effect, while the intrathecal, intracerebroventricular, intracerebral, and subarachnoid routes are invasive routes that can directly deliver the stem cell into the CNS, thus facilitating the reconstruction of the neural circuit and the replacement of the damaged brain tissues in IS (Kelly et al., 2004). After IS, stem cell transplantation through the intracerebral route resulted in more transplanted stem cells appearing near the infarcted lesions because of the direct injection of millions of stem cells under the help of invasive surgery. Approximately 1/3 can migrate to the ischemic area (Darsalia et al., 2007; De Feo et al., 2012), thus achieving the highest stem cell engraftment of all administration routes. However, invasive surgery can bring a second attack to the patient, and intracerebral routes are predominantly used in the chronic phase of IS, according to published clinical studies (Kondziolka et al., 2005; Chen et al., 2013; Steinberg et al., 2016; Steinberg et al., 2018). The amount of transplanted stem cells is limited to avoid the mass effect, and the harsh microenvironment containing inflammatory factors can influence the grafted stem cells survival, differentiation, proliferation, and migration (Bühnemann et al., 2006). The risk of additional brain damage caused by injection needles and invasive surgery-related complications including death, seizure, and infection should be taken into consideration (Jeong et al., 2014; Kawabori et al., 2020b). The intracerebroventricular route may be less invasive than the intracerebral route, but complications such as hydrocephalus and liquorrhea may occur after administration. Recently, a novel delivery route for stem cells for IS therapy has been reported and gained attention because of its effectiveness and minimal invasiveness. Indeed, the

intranasal administration of cells allows the bypass of the BBB and increases the migration into the lesions (Wei et al., 2013, 2015), and MSCs administered through this route show great potential to enhance neurovascular regeneration and improve functional recovery (Wei et al., 2015). However, the intranasal and intraperitoneal routes are still at the stage of preclinical studies and need further safety and efficacy evaluation before use in large-scale clinical trials. So far, the strong connection between routes of administration and the target of stem cell therapy is quite evident. In the acute phase of IS, the neuroinflammation and neural cell death greatly contribute to the ischemia damage to the CNS, and at present, the intravascular routes are preferable in order to use the bystander effect. However, in the chronic phase of IS, when the acute pathophysiology changes are stabilized, the replacement of the damaged tissue can be more important, and intracerebral or other invasive routes are more proper to directly enhance cellular intercommunication. Nevertheless, many issues still exist, which should be taken into consideration when deciding the optimal choice of administration routes.

Time of Administration

Nowadays, not all patients are eligible for reperfusion treatment because of the narrow time window, although MT and the administration of tPA are widely used in clinical practice. In the different phases of IS such as acute, subacute, and chronic phases, many pathophysiological changes occur. However, some particular pathophysiological changes play major roles in different phases, enabling us to consider specific treatments. Based on the pathophysiology features of different IS phases, treatment strategies regarding the administration time have been discussed in clinical and preclinical studies. In acute or subacute phases of IS, a series of cytokines, chemokines, and reactive oxygen species are released, which change the microenvironment, finally damaging the neural cells (Choi, 1992; Adibhatla and Hatcher, 2010; Dabrowska et al., 2019a; Jayaraj et al., 2019), although some of these factors have positive effects. For example, the release of some chemokines including SDF after the onset of IS can induce the migration of exogenous transplanted stem cells (Chen Y. et al., 2014). Administration of stem cells in the early phases of IS can reduce neuroinflammation, modulate the immune system, regulate the microenvironment, and rescue neurons and glial cells in the peri-ischemia areas (Bang, 2016), suggesting that the focus of cell therapy in the acute and subacute phases should be on the rescue of ischemia-damaged cells (Savitz et al., 2011a). In the rat IS model, the administration of NSCs shortly after stroke (48 h) results in a better stem cell survival compared with stem cells administered 6 weeks after stroke. The same study also reported that the activation of microglia after the onset of IS reaches the maximum level at 1 to 6 weeks after stroke (Darsalia et al., 2011), suggesting that the administration of stem cells before the total establishment of neuroinflammation increases the survival of stem cells and improves their therapeutic effect. Thus, according to the instruction of preclinical studies, some clinical trials reported the safety and efficacy of stem cell administration in an early phase of IS (Kim et al., 2013; Shichinohe et al., 2017; Bhatia et al., 2018), resulting in a good clinical outcome, since 80% of the patients who received stem cell administration in the early phase of IS show good functional recovery (Bhatia et al., 2018). The early phase of IS is the appropriate period for traditional reperfusion treatment and stem cell therapy as well, but functional recovery in this phase is influenced by many factors including the spontaneous recovery of endogenous angiogenesis or neurogenesis. It is therefore important to take these aspects into consideration when assessing the effect of stem cell therapy in future clinical studies. The purpose of stem cell therapy in the chronic phase is totally different because it focuses mainly on neurogenesis, angiogenesis, and synaptic plasticity in peri-ischemic areas to restore neuronal functions. Trials in this phase mainly transplant the stem cells into peri-infarcted areas, promoting intercommunication between exogenous stem cells and cells that survived in the CNS, which increases the reconstruction of the neural circuit (Kalladka et al., 2016). Some clinical trials have reported the efficacy of stem cell therapy in the chronic phase of IS by intracerebral and intravascular routes (Chen D.C. et al., 2014; Levy et al., 2019; Muir et al., 2020). However, the study was interrupted because of a temporary worsening of the motor deficits and seizures when stem cells were administered in the chronic phase (Savitz et al., 2005). Therefore, the limited efficiency to replace the damaged tissue in the chronic phase of IS should be taken into consideration because of the formation of glial scar, irremediable loss of amount of brain parenchyma, and lack of blood supply to the transplanted stem cells (Colton, 1995; Diotel et al., 2020). Despite these negative aspects, tissue engineering hopes to solve the problems of administering stem cells in the chronic phase of IS. Some artificial scaffolds and matrix can serve as a platform to provide niches that can increase survival, proliferation, and functions of the transplanted stem cells (Murphy et al., 2010; Gopalakrishnan et al., 2019), thus solving the problems mentioned above, although the effect of stem cell therapy in the chronic phase of stroke still needs more research.

Cell Dose

The stem cell dose is flexible but should be adapted to the particular cell type or transplantation route used. The cell dose applied in clinical trials can widely differ from 10^6 to 10^9. Considering the easy access and the lowest invasiveness of the intravenous or intra-arterial routes, the cell dose can reach more than 10^8 (Taguchi et al., 2015; Hess et al., 2017; Bhatia et al., 2018; Levy et al., 2019; Vahidy et al., 2019). For example, the number of transplanted stem cells by intravenous route can reach 1,200 million, with no dose-limiting toxicity events, allergic reactions, and treatment-emergent adverse events, revealing the safety of such cell dose (Hess et al., 2017). A recent study used two different cell doses (10^5 and 10^6) of MSCs that were intravenously administered. Although both doses improved functional recovery, the result revealed higher stem cell engraftment in the peri-infarct area at the higher dose, suggesting that the therapeutic effect of stem cells is positively correlated with the cell dose in a proper range (Kawabori et al., 2013). A meta-analysis regarding the relationship between the therapeutic effect and dose of transplanted stem cells did not

show a significant linear regression relationship between them (Wang et al., 2016). Since a higher stem cell number can lead to the mass effect when administered by intracerebral route, the transplantation dose should be limited to no more than 10^7 (Kalladka et al., 2016, 2019; Nagpal et al., 2016; Muir et al., 2020). A heterogeneous functional outcome is still observed under the same cell dose administered by the intracerebral route, probably because of patients in different conditions and the influence of a particular microenvironment. Some studies on material technology give hope on the use of several engineered matrix such as a hyaluronic acid (HA)-based self-polymerizing hydrogel that can serve as a platform for transplanted stem cells, miming the native neural tissue environment, and minimizing the influence from the environment of the peri-ischemia area after transplantation into the stroke core. This improves the survival and migration of stem cells, finally differentially modulating the transplanted cell fate (Moshayedi et al., 2016; Gopalakrishnan et al., 2019). Administration of the engineered matrix can significantly increase the implantation efficiency and can influence the transplantation dose as well. Only few studies are available discussing the appropriate cell dose in stem cell therapy and considering the risk of formation of tumors and thrombosis, and thus, further studies should investigate these issues before the widespread use of stem cell therapy in clinical practice.

CONCLUSION

Our review summarizes the mechanisms of stem cell therapy in detail, as well as several key parameters elucidated by the current clinical research, providing a reference and a guide in the direction of further research. Stem cell therapy is a promising treatment strategy to cure IS, with multi-mechanisms that can effectively cope with the pathophysiological changes after stroke. In addition, many preclinical and clinical trials have revealed promising prospects in the administration of stem cell therapy to cure IS. However, much research still needs to be performed, and new discoveries may help develop a better use or modification of stem cell therapy or the treatment methods derived from it. Current clinical trials are still mainly in phase I of safety. The next step may require exploration of more clinically suited issues, such as the choice of stem cell types, the route of stem cell administration, the administration time, and the cell dose. These still unclear aspects require results from double-blind studies with larger cohorts.

AUTHOR CONTRIBUTIONS

All the authors participated in analyzing and discussing the literature, commenting on and approving the manuscript. AS and LW supervised the research, led the discussion, and wrote and revised the manuscript. All authors read and approved the final manuscript.

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Adipose-Derived Mesenchymal Stem Cells From a Hypoxic Culture Improve Neuronal Differentiation and Nerve Repair

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Hypoxic expansion has been demonstrated to enhance *in vitro* neuronal differentiation of bone-marrow derived mesenchymal stem cells (BMSCs). Whether adipose-derived mesenchymal stem cells (ADSCs) increase their neuronal differentiation potential following hypoxic expansion has been examined in the study. Real-time quantitative reverse transcription-polymerase chain reaction and immunofluorescence staining were employed to detect the expression of neuronal markers and compare the differentiation efficiency of hypoxic and normoxic ADSCs. A sciatic nerve injury animal model was used to analyze the gastrocnemius muscle weights as the outcomes of hypoxic and normoxic ADSC treatments, and sections of the regenerated nerve fibers taken from the conduits were analyzed by histological staining and immunohistochemical staining. Comparisons of the treatment effects of ADSCs and BMSCs following hypoxic expansion were also conducted *in vitro* and *in vivo*. Hypoxic expansion prior to the differentiation procedure promoted the expression of the neuronal markers in ADSC differentiated neuron-like cells. Moreover, the conduit connecting the sciatic nerve gap injected with hypoxic ADSCs showed the highest recovery rate of the gastrocnemius muscle weights in the animal model, suggesting a conceivable treatment for hypoxic ADSCs. The percentages of the regenerated myelinated fibers from the hypoxic ADSCs detected by toluidine blue staining and myelin basic protein (MBP) immunostaining were higher than those of the normoxic ones. On the other hand, hypoxic expansion increased the neuronal differentiation potential of ADSCs compared with that of the hypoxic BMSCs *in vitro*. The outcomes of animals treated with hypoxic ADSCs and hypoxic BMSCs showed similar results, confirming that hypoxic expansion enhances the neuronal differentiation potential of ADSCs *in vitro* and improves *in vivo* therapeutic potential.

Keywords: ADSC, BMSC, hypoxic culture, neuronal differentiation, nerve repair

INTRODUCTION

Nerve grafts, which are composed of natural or artificial tubes, are considered ideal treatments to guide nerve regeneration across the injury gaps (Faroni et al., 2013, 2014). Artificial conduits with bone marrow-derived mesenchymal stem cells (BMSCs) have been found to benefit peripheral nerve regeneration (Frattini et al., 2012; Wang et al., 2020). The advantages of mesenchymal stem cells in facilitating neurogenesis might be due to varied neurotrophic factor secretion (Uccelli et al., 2011). As adipose-derived mesenchymal stem cells (ADSCs) can be easily collected from subcutaneous fat tissue by liposuction without great pain, more cells can be obtained (Gimble et al., 2007). Furthermore, ADSCs show no significant differences in differentiation capacity, cell senescence, and efficiency of gene transduction compared with BMSCs (De Ugarte et al., 2003; Fernandes et al., 2018).

Utilization of ADSCs to treat and improve nerve regeneration in animal models with peripheral nerve defects has been well reported (Di Summa et al., 2010; Erba et al., 2010; Liu et al., 2011; Scholz et al., 2011; Marconi et al., 2012; Orbay et al., 2012; Sowa et al., 2016). ADSCs can differentiate into Schwann cells (SCs)-like (Kingham et al., 2007; Di Summa et al., 2014) and neuron-like (Scholz et al., 2011; New et al., 2015) phenotypes *in vitro* and *in vivo* to promote nerve regeneration. In addition, ADSCs secrete neurotrophic factors such as vascular endothelial growth factors (VEGFs), brain-derived growth factors (BDNFs), nerve growth factors (NGFs), and glial line-derived neurotrophic factors (GDNFs) to facilitate intrinsic healing utilizing host inhabited SCs (Widgerow et al., 2013; Faroni et al., 2014). The neuroregenerative effect of ADSCs has been found to be comparable to transplantation of SCs (Sowa et al., 2016). SCs are difficult to harvest and expand (Kocsis et al., 2002), and the harvesting procedure might cause donor-site morbidity. In addition, the de-differentiation, macrophage recruitment and myelin clearance functions of SCs are impaired in elderly hosts (Painter et al., 2014). Therefore, ADSCs can serve as an alternative clinical application. Intravenous injections of ADSCs have been used to treat multiple nerve injury sites (Marconi et al., 2012). Besides, there is no difference in terms of transplantation with undifferentiated ADSCs or differentiated ones in nerve regeneration (Orbay et al., 2012), suggesting the treatment time can be shortened by direct ADSC transplantation.

It is known that hypoxic expansion decreases in senescence, increases in proliferation, and enhances multilineage differentiation potential in BMSCs (Grayson et al., 2006; Adesida et al., 2012). The purpose of this study was to examine whether hypoxic expansion could increase the neuronal differentiation potential *in vitro* and the treatment effect *in vivo* in ADSCs and compare the neuronal differentiation potential between ADSCs and BMSCs following hypoxic expansions.

Abbreviation:ADSCs, Adipose-derived mesenchymal stem cells; BMSCs, Bone marrow-derived mesenchymal stem cells; SCs, Schwann cells; VEGFs, Vascular endothelial growth factors; BDNFs, Brain-derived growth factors; NGFs, Nerve growth factors; GDNFs, Glial line-derived neurotrophic factors; IRB, Institutional Review Board; SVF, Stromal-vascular fraction; PBS, Phosphate-buffered saline; HBSS, Hank's Balanced Salt Solution; DMEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; β-ME, 2-Mercaptoethanol; RT-qPCR, quantitative reverse transcription polymerase chain reaction; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; IF, Immunofluorescence; TUBB3, Class III β-tubulin; MAP2, Microtubule-associated protein 2; NEFM, neurofilament medium polypeptide; NEFL, neurofilament light polypeptide; NEFH, neurofilament heavy polypeptide; cDNA, complementary DNA; MBP, myelin basic protein; IOD, integrated optical density; DAPI, 4,6-diamidino-2-phenylindole; SPIO, Superparamagnetic iron oxides; HE, Hematoxylin and eosin; ANOVA, One-way analysis of variance; HIF-1α, Hypoxia-inducible factor-1α; NCV, Nerve conduction velocity.

MATERIALS AND METHODS

Isolation of Human BMSCs and ADSCs

The human BMSC cell line was obtained from Professor Shih-Chieh Hung's Lab (Lin et al., 2015). Fat tissues were aspirated from healthy female donors undergoing liposuction of the abdomen. The procedures were approved by the Institutional Review Board (IRB) of the Taipei Veterans General Hospital. The fat tissue needed in this study was then stored at 4°C and the stromal-vascular fraction (SVF), which contained human ADSCs, was separated within 24 h through SVF isolation as previously described (Yoshimura et al., 2006; Wu et al., 2018). The excised fat tissue was carefully cut into small uniform pieces to yield similar digestion efficiency. The oil layer on the top and the aqueous layer were removed by centrifugation, and the collected fat volume was recorded. Excess blood was removed from the SVF after having been washed two times with cold PBS or the Hank's Balanced Salt Solution (HBSS; Gibco, Carlsbad, CA, United States). 0.075% (w/v) collagenase (Sigma-Aldrich, St. Louis, MO) in a prewarmed HBSS buffer was prepared for one to twofold fat volume and filtered with a 0.22-μm filter. The mix ratio of the collagenase buffer and fat was 1:1 or 2:1. After having been mixed, the fat mixture was incubated in a shaking water bath at 37°C for 30 min. Following digestion, a half volume of the Dulbecco's Modified Eagle Medium (DMEM; Gibco) and 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) were thoroughly mixed with the fat mixture for neutralization, which then underwent centrifugation and was washed with PBS three times to remove the collagenase. The pellets contained SVF and some fibrotic tissue suspended with an appropriate amount of the medium for cell counting afterward. Series of filtrations were performed with 100, 70 (optional), and 40-μm filters and filtered. Following isolation, 4–7 $\times$ 10^5 living cells in 1 ml of the normal human fat tissue could be extracted.

Cell Expansion

For the cell culture, approximately 2–3 $\times$ 10^6 cells at passages 3–6 were seeded in one 10-cm dish and cultured in a DMEM containing 10% FBS and Antibiotic-Antimitotic solution (Corning Life Science, New York, United States). For hypoxic expansion, the cells were cultured in 94% N_2, 5% CO_2 and 1% O_2 (Yew et al., 2012). For the normoxic culture, the cells were cultured in 5% CO_2 and 20% O_2 (a Forma Series II Water Jacketed CO_2 incubator, Thermo Fisher Scientific Inc., Waltham, MA, United States).

Neuronal Differentiation

The neuronal differentiation procedure was performed according to a previous study (Hung et al., 2002). Briefly, the cells with

80% confluence were induced by a DMEM supplemented with 50 μM All-trans retinoic acid (RA; Sigma-Aldrich) and 1 mM 2-Mercaptoethanol (β-ME; Sigma-Aldrich), which were placed in a 37°C, 5% CO_2 and 20% O_2 incubator for 24 hr. Then the cells were maintained with DMEM solution containing 1% FBS for 7 days.

Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR) Analysis

Total RNA was extracted from 1×10^6 cells using the Trizol reagent (Invitrogen, Carlsbad, CA) and adjusted to 1,000 ng. Random sequence primers were used to prime reverse transcription reactions, and the first strand complementary DNA (cDNA) synthesis was achieved by employing the iScript™ cDNA Synthesis Kit (Bio-Rad®, Hercules, CA). cDNA, which was synthesized by the M-MuLV reverse transcriptase, was applied as the template to perform the PCR reaction in a 20-μl reaction mixture with specific primers. The sequences of the primers in this study were listed in **Table 1**. A Fast SYBR® Green Master Mix (Applied Biosystems, Foster City, CA) was used to perform Real-time qPCR, with 40 cycles consisting of repeating the following steps: the holding stage for 10 min at 95°C, denaturation for 3 s at 95°C, and annealing stage for 30 s at 60°C. The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was detected as an internal control to confirm the efficiency of the PCR and cDNA synthesis. The results were produced using the comparative C_T ($\Delta\Delta C_T$) method.

Immunofluorescence (IF) Staining and Quantification

Primary antibodies for IF staining against Class III β-tubulin (TUBB3; Santa Cruz, CA, United States), microtubule-associated protein 2 (MAP2; GeneTex Inc., Irvine, CA), neurofilament medium polypeptide (NEFM; OriGene Technologies, Rockville, MD), and neurofilament light polypeptide (NEFL; OriGene Technologies, Rockville, MD) were added on 7 days differentiated cell slides at suitable dilution. Secondary antibodies with green fluorescence protein against the primary antibodies were used for fluorescence analysis. 4,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) for nuclear staining (blue) was used to counterstain the slides. The samples incubated with the secondary antibodies but without the first antibodies were employed as a negative control. Green fluorescence intensity was measured by the Image-Pro Plus (v4.5.0.29, Media Cybernetics, Silver Spring, MD). Total florescence intensity from 120 to 140 cells in six fields on each slide was measured, and the average intensity of individual cells was also calculated.

Animal Model

The procedure of the collection of rat ADSCs was the same as that of the human's. Each Sprague-Dawley rat (250–300 g in weight) was anesthetized with the Zoletil® 50 (Virbac, Carros, France) for 1 ml/kg. A 5-mm segmental nerve defect in the right sciatic nerve of each of all the rats was exposed by a dorsal gluteal-splitting approach, with the contralateral leg similarly exposed, but not the nerve defect, used as a sham control (n = 24). Each defect area was connected with a 5 mm long (∼1.53 mm inner diameter) microporous polylactide conduit (Hsu et al., 2013) and sutured with four 8–0 nylon sutures through the epineurium. Before injections, the ADSCs were pre-incubated with superparamagnetic iron oxides (SPIOs) for histological iron staining. The matrigel matrix (Corning life science) was mixed with 1×10^6 normoxic ADSC (n = 6), hypoxic ADSC (n = 6) or hypoxic BMSC (n = 6) cells or without cells (n = 3) in 100 μl volumes and injected into the conduits for nerve reconstruction, and followed by wound closures with postsurgical care. The sciatic nerve injury with a conduit connection but without a Matrigel injection was specified as the non-treatment group (n = 3). The experimental protocols and follow-up animal care were compliant with the institutional animal welfare guidelines of the Taipei Veterans General Hospital. The rats were anesthetized with 5 ml/kg Zoletil® 50 (Virbac) and then transcardially perfused with 4% paraformaldehyde in 0.1M PBS (pH 7.4) after treatments for 6 weeks. The regenerated sciatic nerve was removed from each conduit and prepared for the follow-up analysis. All the procedures involving the animals were approved by the Institutional Animal Care and Use Committee of the Taipei Veterans General Hospital.

Gastrocnemius Muscle Weight Analysis

The gastrocnemius muscles from the limbs of those without treatment, Matrigel, hypoxic, and normoxic ADSC treatments were dissected and immediately weighed, and then compared with the limbs of the sham group. The weights of the gastrocnemius muscles in each group that had been normalized with those of the sham group were converted to percentages as recovery rates, and the sham group was defined as 100% (Hsueh et al., 2014).

Histological Stain and Myelinated Fiber Quantification

The middle parts of the regenerated sciatic nerves in the conduits were cross sectioned for histological analysis; the paraffin-embedded nerve tissue sections were deparaffinized in xylene. Rehydration was performed with 100 to 70% graded alcohol, and the organic solution was removed by rinsing in ddH_2O. Hematoxylin and eosin (HE) staining (Sigma-Aldrich), toluidine blue staining (Sigma-Aldrich) (Ebrahimi et al., 2018), myelin basic protein (MBP) immunostaining (Merolli et al., 2019) and iron staining (Sigma-Aldrich) (Liu and Ho, 2017) were used to stain the sections, with the results being examined by a light microscope AX80 (Olympus, Tokyo, Japan).

Photographs of the toluidine blue staining sections taken under light microscopy at a 400 × magnification were used for quantification. The total numbers of the myelinated fibers in each section from three regenerated nerves were counted and normalized with the whole background of each section using the Image-Pro Plus (v4.5.0.29, Media Cybernetics) (Chen et al., 2020). The results showed the mean values calculated from three sections in each group.

TABLE 1 | Primer sequences of the neuron markers used for RT-qPCR analysis.

Gene	Accession number	Forward primer (5′–3′)	Reverse primer (5′–3′)
TUBB3	NM_139254	GCCAAGTTCTGGGAGGTCATC	GTAGTAGACACTGATGCGTTCCA
MAP2	X54100.1	CAAACGTCATTACTTTACAACTTGA	CAGCTGCCTCTGTGAGTGGAG
NEFH	NM_012607	AGTGGTTCCGAGTGAGATTG	CTGCTGAATTGCATCCTGGT
NEFM	NM_017029	AGTGGTTCAAATGCCGCTAC	TTTTCCAGCTGCTGGATGGT
CD90	NM_006288	ATCGCTCTCCTGCTAACAGTC	CTCGTACTGGATGGGTGAACT
CD105	NM_001114753	TGCACTTGGCCTACAATTCCA	AGCTGCCCACTCAAGGATCT
GAPDH	NM_017008	CAACTCCCTCAAGATTGTCAGCAA	GGCATGGACTGTGGTCATGA

Statistical Analysis

A quantitative analysis of the results was undertaken using the Prism (version 5.03, GraphPad, La Jolla, CA, United States). The Mann-Whitney *U*-test was used for comparing the two groups, and one-way analysis of variance (ANOVA) was for multi-group comparison. The results were presented as means ± standard errors; a *p*-value less than 0.05 was considered significant.

RESULTS

Neuronal Differentiation of Normoxic and Hypoxic ADSCs *in vitro*

After 2 weeks of expansion under normoxic (normoxic ADSCs) or hypoxic (hypoxic ADSCs) conditions, the ADSCs proceeded to neuronal differentiation in normoxia for 7 days. The process was showed in **Figure 1**. The undifferentiated ADSCs on day 0 were imaged using phase-contrast microscopy (**Figures 2A–D**). The neuron-like morphologies showing stretches on differentiated normoxic (**Figures 2E,F**) and hypoxic ADSCs (**Figures 2G,H**) with β-ME induction were examined on day 7, which confirmed that both normoxic and hypoxic ADSCs could be induced to experience neuronal differentiation. RT-qPCR was used to confirm the neuronal markers of the normoxic and hypoxic ADSC differentiated neuron-like cells by monitoring the gene expressions of *TUBB3* (**Figure 2I**), *MAP2* (**Figure 2J**), *NEFM* (**Figure 2K**), and *NEFH* (**Figure 2L**) through the time course. The *TUBB3* expressions were significantly increased in the hypoxic ADSCs compared with those of the normoxic ones after 3 days of induction ($P < 0.05$). The expressions of *MAP2* were highly induced in the hypoxic ADSC group on days 1 and 7 of differentiation ($p < 0.05$). The *NEFM* gene were highly expressed in the hypoxic ADSC differentiated neuron-like cells on day 1 ($P < 0.05$), and *NEFH* was also significantly enhanced on days 3 and 7 ($P < 0.05$). In addition, the differentiation efficiency was determined by detecting the cell marker genes of ADSCs, such as CD90 and CD105, after differentiation. RT-qPCR detection of marker gene expressions in the normoxic or hypoxic ADSCs on day 0 and differentiated neuron-like cells on day 7 was measured and compared. The result showed that there were no significant differences in the ADSC markers of the normoxic and hypoxic ADSC differentiated neuron-like cells on day 7 (**Supplementary Figure 1**).

IF staining was used to confirm the neuronal markers of the normoxic ADSC or hypoxic ADSC differentiated neuron-like cells on day 7 by examining the expressions of the TUBB3 (**Figures 3A–D**), MAP2 (**Figures 3E–H**), NEFM (**Figures 3I–L**), and NEFL (**Figures 3M–P**) proteins. The total fluorescence intensity and average fluorescence intensity of the TUBB3, MAP2, and NEFM protein expressions in the hypoxic ADSCs were significantly higher than those of the normoxic ADSCs ($p < 0.01$, **Figures 3Q,R**). NEFL protein was also higher in the hypoxic ADSC group although the result was not significantly different. The aforementioned results implied hypoxic expansion prior to differentiation might preserve neuronal differentiation potential in ADSCs.

ADSC Transplantation in the Animal Model

The surgical conduit connecting the distal stump of the rat sciatic nerve defect was imaged in **Figure 4A** and the middle of **Figure 4B**. The normoxic or hypoxic ADSCs mixed with Matrigel were injected into the conduit. The intact sciatic nerve was dissected and exhibited in the upper part of **Figure 4B**, and the regenerated nerve fibers in the conduits filled with the normoxic or hypoxic ADSCs injections were shown at the bottom of **Figure 4B**. The ankle of the rat limb with the sciatic nerve incision without treatment was not straightened while the feet were pulled, which was called an ankle contracture, and the gastrocnemius muscle would shrink. Nevertheless, the atrophied gastrocnemius muscle would repair as long as the nerves were regenerated (**Figure 4C**). The recovery rates which were calculated according to the weights of the gastrocnemius muscle in the hypoxic ADSCs (mean = 46% ± 1.39) were significantly higher than those of the normoxic group (mean = 36% ± 1.79, $p < 0.01$), non-treatment group (mean = 25% ± 4.2, $p < 0.001$), and the Matrigel group (mean = 28% ± 1.17, $p < 0.001$) (**Figure 4D**).

The section structure of the normal sciatic nerve and regenerated nerves in the conduits from the Matrigel, and with normoxic or hypoxic ADSC treatments were stained by HE staining (**Figures 5A–D**) to present the structures of the nerve section. Toluidine blue staining and MBP immunostaining were used to detect and quantify the regenerated myelinated nerve fibers (**Figures 5E–L**). The integrated optical density (IOD) of the stained fibers were normalized with those of the sham group and converted to percentages. The mean percentage value of the sham group was defined as 100%. The mean percentage

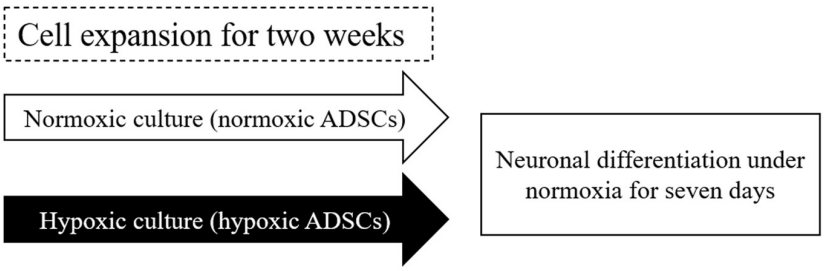

FIGURE 1 | Scheme of normoxic and hypoxic expansion and neuronal differentiation. Freeze-thaw or continuous-culturing human adipose-derived stem cells (ADSCs) at passages 3–5 were cultured under normoxic (normoxic ADSCs) or hypoxic condition (hypoxic ADSCs) for 2 weeks. After expansion, both the normoxic and hypoxic ADSCs were exposed to 2-Mercaptoethanol (β-ME) to induce neuronal differentiation for 7 days. The neuron-related genes and proteins of the normoxic and hypoxic ADSC differentiated cells were monitored from days 0 to 7.

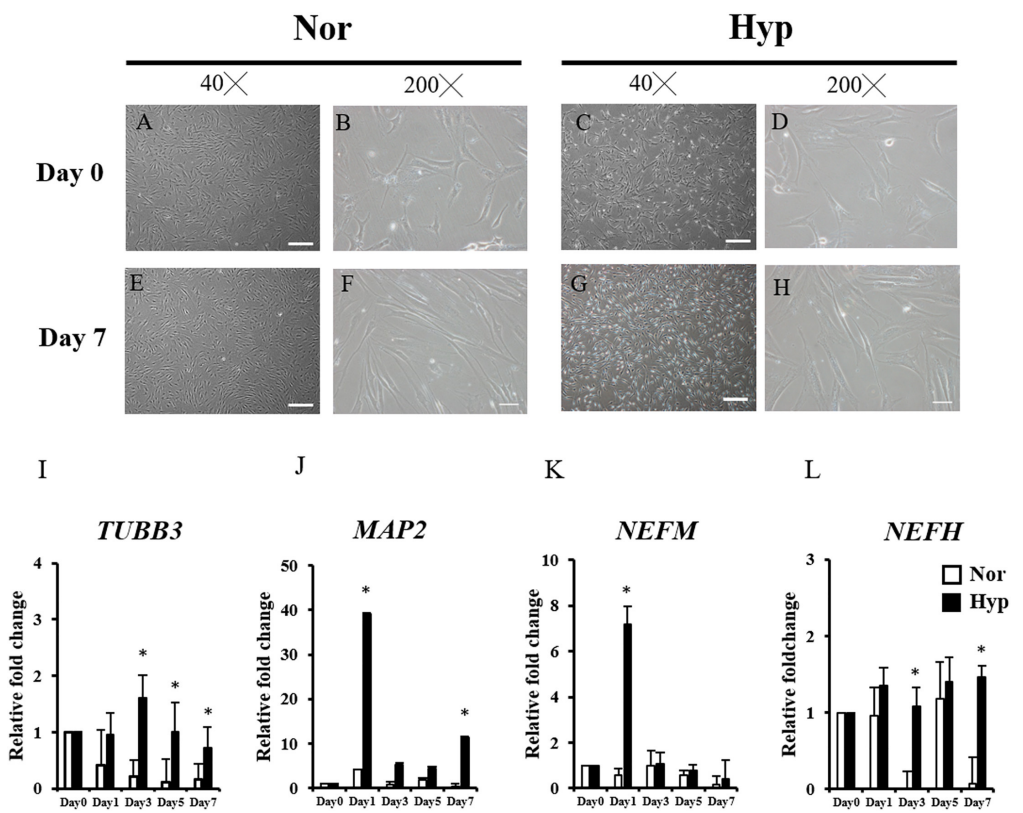

FIGURE 2 | Morphology and neuron-associated gene expressions of ADSC differentiated neuron-like cells. The morphologies of undifferentiated ADSCs expanded in normoxia **(A,B)** and hypoxia **(C,D)** before induction (Day 0) were examined using phase-contrast microscopy. The neuron-like morphologies differentiated from the normoxic **(E,F)** hypoxic ADSCs **(G,H)** under normoxia were observed on day 7 by β-ME induction. (Magnification × 40; the scale bar = 200 μm; magnification × 200; the scale bar = 50 μm). Genes including Class III β-tubulin (*TUBB3*) **(I)**, microtubule-associated protein 2 (*MAP2*) **(J)**, neurofilament medium polypeptide (*NEFM*) **(K)**, and neurofilament heavy polypeptide (*NEFH*) **(L)** of the normoxic and hypoxic ADSC differentiated cells were detected by RT-qPCR from days 0 to 7, and all the gene expression values were normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The values from day 1 to day 7 were compared with those of day 0 to show the relative fold change. The values were presented as mean ± SD with three replicates of the experiments. Statistical significances between the two groups were analyzed using the Mann-Whitney *U*-test. "*" represented $P < 0.05$.

of the nerve fiber detected by toluidine blue staining in the hypoxic ADSC group was 34%, which was significantly enhanced compared with that of the Matrigel group (19%, $p < 0.05$), and also higher than that of the normoxic ADSCs group (25%) (**Figure 5Q**). In addition, the mean percentage of the myelinated nerve fiber detected by MBP immunostaining in the hypoxic ADSC group was 55.9%, which was also significantly higher than that of the Matrigel group (35.12%, $p < 0.01$), and also higher than that of the normoxic ADSC group (43.6%) (**Figure 5R**).

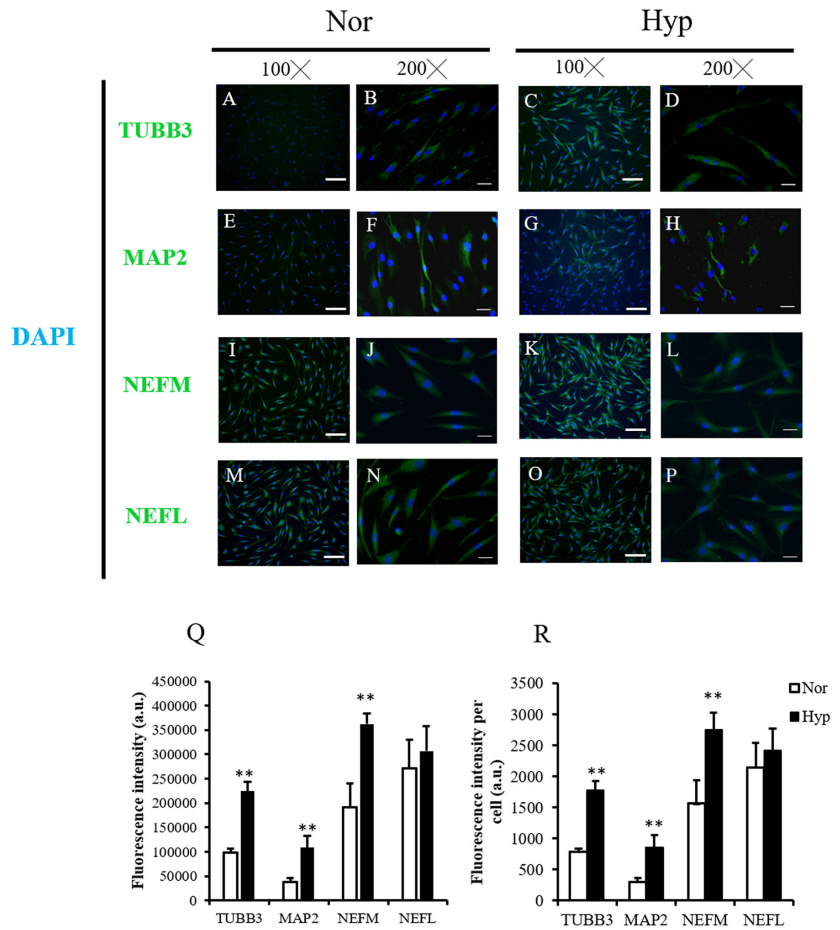

FIGURE 3 | Neuron marker detection of the normoxic and hypoxic ADSC differentiated neuron-like cells. **(A–D)** Class III β-tubulin (TUBB3), **(E–H)** microtubule-associated protein 2 (MAP2), **(I–L)** neurofilament medium polypeptide (NEFM), and **(M–P)** neurofilament Light polypeptide (NEFL) of the normoxic and hypoxic ADSC differentiated neural-like cells on day 7 of differentiation were stained by immunofluorescence staining (Magnification × 100; the scale bar = 100 μm; magnification × 200; the scale bar = 50 μm). **(Q)** Total immunofluorescence intensities of 120–150 cells from six fields were quantified by the Image-Pro Plus v4.5.0.29. The intensity of each cell was also calculated **(R)**. Values are presented as mean ± SD with three replicates of the experiments. Statistical significances between the two groups were analyzed using the Mann-Whitney *U*-test. "**" represented $P < 0.01$.

To detect the SPIO marked ADSCs in the regenerated nerve fibers, the nerve section of the sham group was referred to as a control (**Figure 5M**). There was no detection of iron marks in the regenerated nerves of the Matrigel group grown from the host stump nerve (**Figure 5N**). The SPIO previously seeded in the ADSCs was tracked by iron staining (**Figures 5O,P**).

Comparisons of the Neuronal Differentiation of Hypoxic ADSCs and Hypoxic BMSCs

To compare the potential of neuronal differentiation in the hypoxic ADSCs and BMSCs following hypoxic expansion (hypoxic BMSCs), neuron-associated genes, including *TUBB3* and *MAP2* (**Supplementary Figures 2A,B**), were monitored through the time course by RT-qPCR. The expressions of *MAP2* in the hypoxic ADSC differentiated neuron-like cells were significantly higher than those of the hypoxic BMSCs on days 1 and 7. The sciatic nerve injury rat model was also used to compare the outcomes of the hypoxic ADSC and hypoxic BMSC treatment. The recovery of the atrophied gastrocnemius muscle in these two groups was similar since the mean value of the hypoxic ADSCs was 49%, and that of the hypoxic BMSCs was 45% (**Supplementary Figures 2C,D**).

DISCUSSION

A comparison of ADSCs and BMSCs in terms of peripheral nerve regeneration on sciatic nerve injury was examined, and the study showed no significant difference in the results of ADSC and BMSC treatments for sciatic nerve function recovery (Fernandes et al., 2018). In this study, the neuronal differentiation potential of ADSCs and BMSCs expanded under hypoxia was also compared. The results showed the neuron markers were enhanced in the hypoxic ADSCs while neuronal differentiation proceeded *in vitro*. However, the results of the recovered gastrocnemius muscles of both cell

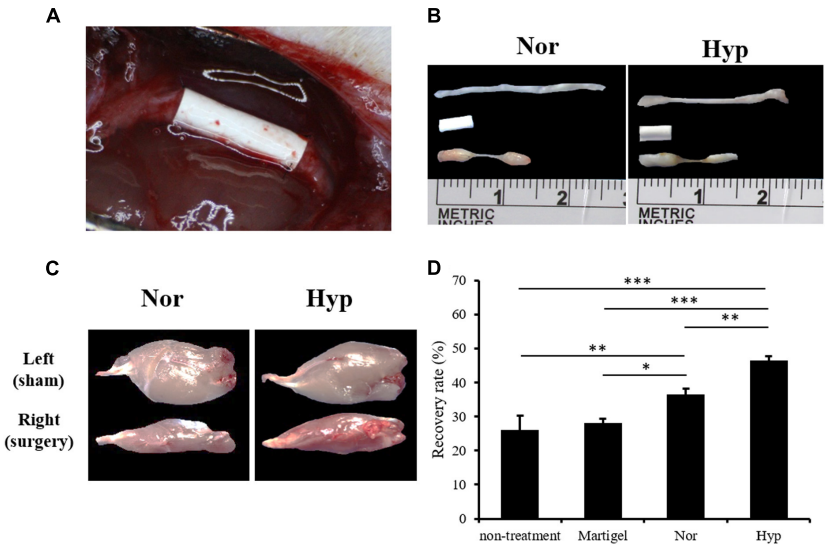

FIGURE 4 | The animal model of the sciatic nerve defect and outcome analysis of the ADSC treatment. **(A)** A polylactide conduit connecting a 5-mm sciatic nerve defect by surgical implantation in the Sprague-Dawley rats. The animals were sacrificed after 6 weeks. **(B)** The dissected normal sciatic nerve, implanted conduit, and regenerated nerve from the normoxic, or hypoxic ADSCs were exhibited from the top to the bottom, respectively. **(C)** The dissected gastrocnemius muscles from the left limbs of the rats as the sham group were showed in the upper panel. The lower panel displayed the atrophied muscles from the right limb with sciatica receiving a treatment of the normoxic or hypoxic ADSCs in the conduits. **(D)** The weights of the gastrocnemius muscles were measured and normalized with those of the sham group to calculate the recovery rate. The values were presented as mean ± SD with three replicates of the experiments. Statistical significance between the multiple groups was analyzed using the one-way analysis of variance (ANOVA) analysis. "*" represented $p < 0.05$, "**" represented $p < 0.01$, and "***" represented $p < 0.001$.

treatments were similar, which demonstrated the neuron repair function of ADSCs and BMSCs were comparable. Moreover, the application of ADSCs was more convenient compared with that of BMSCs due to the fact that the collection of ADSCs was more advantageous, and there was a greater gain in cell numbers, better proliferation, and less effects of age (Chen et al., 2012).

The physiological oxygen tension in the original niches of BMSCs and ADSCs is relatively lower than the ambient air. The hypoxic condition having oxygen tension of around 1–7% in bone marrow (Eliasson and Jonsson, 2010; Spencer et al., 2014) supports BMSCs in preserving their biological characters (Mohyeldin et al., 2010; Abdollahi et al., 2011), enhances proliferation (Adesida et al., 2012), and reduces senescence (Tsai et al., 2011). The oxygen concentration in adipose tissue varies from 3 to 11%, depending on the blood flow (Goossens and Blaak, 2012). A hypoxic culture is also found to reduce ADSC senescence, increase proliferation, and retain the differentiation properties (Weijers et al., 2011; Valorani et al., 2012). Hypoxia is considered an important condition to preserve the function of stem cells (Mohyeldin et al., 2010). The surgical nerve conduits without blood vessels are also a hypoxic microenvironment that would make macrophages activate the hypoxia-inducible factor (HIF-1α) to secrete VEGF-A and vascularization. The newborn blood vessels resolve the hypoxia in the conduit and lead the Schwann cell to pass the lesion (Cattin et al., 2015). Thus, BMSCs or ADSCs that continue culturing under hypoxia *in vitro* would survive in the hypoxic environment of the conduit before new blood vessels are formed. Furthermore, ADSCs would secrete VEGF, BDNF, NGF, and GDNF that enhance host SCs to undergo intrinsic healing (Faroni et al., 2014).

BMSCs continue culturing under hypoxia and are proven to reduce cellular senescence (Tsai et al., 2011). Since an ADSC culture in hypoxic conditions has been demonstrated to enhance proliferation and preserve stemness (Fotia et al., 2015), the hypoxia precondition for 24 h before transplantation was also found to benefit ADSCs in improving angiogenesis and neuroprotection and further enhance the therapeutic potential for erectile dysfunction (Wang et al., 2015). In this study, the ADSCs that were expanded and kept culturing under hypoxia for three to five passages before the normoxic neuronal differentiation *in vitro* or transplantation *in vivo* could preserve the potential of neuronal differentiation.

The limitations of this study are as follows. The process of ADSC neuronal differentiation *in vitro* was examined for a maximum of 7 days, and the mature ADSC neuron cells were not observed. The differentiation efficiency which had been determined by detecting the ADSC marker genes also pointed out the limitation of the induction time. The ADSC markers could still be detected after the 7 days induction. Although the neuron-like cells differentiated from the ADSCs expressed a neuron marker, this did not guarantee the cells would grow into functional neurons *in vitro*. In addition, differentiated or undifferentiated ADSC transplantation displayed no difference in nerve regeneration (Orbay et al., 2012). In this study, the ADSCs were transplanted directly without neuronal differentiation, revealing the beneficial therapeutic effects of ADSCs might not be due to the neuronal differentiation function but the secretion of

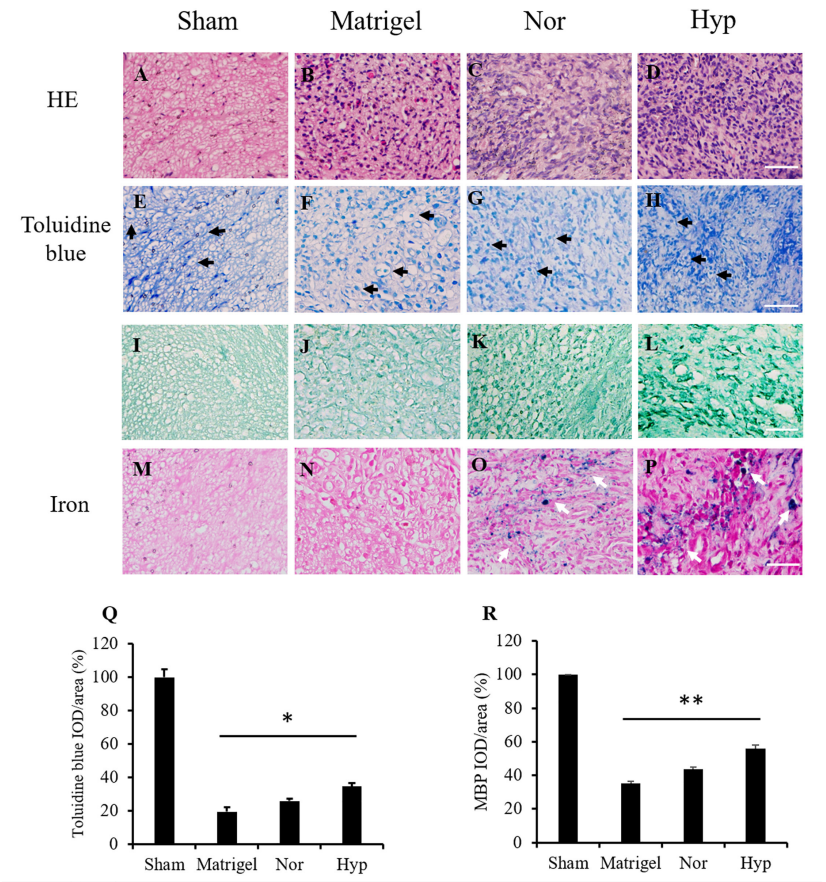

FIGURE 5 | Histologic staining of the nerve sections. The sections in the middle part of the sciatic nerve from the sham group **(A)** and the regenerated nerve from the Matrigel **(B)**, normoxic **(C)**, and hypoxic rat ADSCs (rADSCs) **(D)** were stained by hematoxylin and eosin (HE) staining. Toluidine blue was applied to detect nerve fibers (black arrows) from the sham **(E)**, Matrigel **(F)**, normoxic, **(G)** and hypoxic rADSCs **(H)**, the results of which were showed in the second row. Myelin basic protein (MBP) immunostaining was applied to detect myelin in the sham **(I)**, Matrigel **(J)**, normoxic **(K)**, and hypoxic rADSCs **(L)**, the results of which were showed in the third row. Iron particles (white arrows) in the injected rADSCs were also tracked in the sham **(M)**, Matrigel **(N)**, normoxic **(O)** and hypoxic rADSCs **(P)** (Magnification × 400; the scale bar = 100 μm). The nerve fibers from **(E–H)** and **(I–L)** were quantified by the Image-Pro Plus (v4.5.0.29, Media Cybernetics), respectively. **(Q,R)** The coverage percentages of the myelinated nerve fibers from three sections of each group **(E–H)** or **(I–L)** were calculated and normalized with those of the sham group. The values were converted to percentages and expressed as mean ± SD with three experimental replicates. Statistical significance between the multiple groups was analyzed using the one-way analysis of variance (ANOVA) analysis. "*" represented $P < 0.05$, "**" represented $P < 0.01$.

growth factors (Sowa et al., 2012). The neuron in the regenerated nerve fiber could not be confirmed to be developed by ADSCs. As to the limitations of the animal model experiment, animal behavior tests such as the rotarod test, functional assessment, and nerve conduction velocity (NCV) were not conducted in this study. Moreover, a nerve defect of humans is much longer than that of an experimental rat, which might be the reason the result did not show many differences.

CONCLUSION

ADSCs expanded under both normoxic or hypoxic conditions all make neuronal differentiation occur in normoxia. This study suggested that hypoxic expansion enhanced the neuronal differentiation potential of ADSCs *in vitro* and *in vivo*. ADSC expansion under hypoxia before transplantation may be a beneficial step to improve the clinical application of ADSCs in nerve regeneration and nerve repair, which could be administered to treat other peripheral neuropathy conditions.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board (IRB) of the Taipei Veterans General Hospital. The patients/participants provided

their written informed consent to participate in this study. The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of the Taipei Veterans General Hospital.

AUTHOR CONTRIBUTIONS

S-HW, Y-TL, and J-PW contributed to conception and design, acquisition of data, analysis, and interpretation of data. All authors participated in drafting the article or revising it critically for important intellectual content and gave final approval of the version to be submitted and any revised version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.658099/full#supplementary-material

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Cyclic Strain and Electrical Co-stimulation Improve Neural Differentiation of Marrow-Derived Mesenchymal Stem Cells

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The current study investigated the combinatorial effect of cyclic strain and electrical stimulation on neural differentiation potential of rat bone marrow-derived mesenchymal stem cells (BMSCs) under epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF2) inductions *in vitro*. We developed a prototype device which can provide cyclic strain and electrical signal synchronously. Using this system, we demonstrated that cyclic strain and electrical co-stimulation promote the differentiation of BMCSs into neural cells with more branches and longer neurites than strain or electrical stimulation alone. Strain and electrical co-stimulation can also induce a higher expression of neural markers in terms of transcription and protein level. Neurotrophic factors and the intracellular cyclic AMP (cAMP) are also upregulated with co-stimulation. Importantly, the co-stimulation further enhances the calcium influx of neural differentiated BMSCs when responding to acetylcholine and potassium chloride (KCl). Finally, the phosphorylation of extracellular-signal-regulated kinase (ERK) 1 and 2 and protein kinase B (AKT) was elevated under co-stimulation treatment. The present work suggests a synergistic effect of the combination of cyclic strain and electrical stimulation on BMSC neuronal differentiation and provides an alternative approach to physically manipulate stem cell differentiation into mature and functional neural cells *in vitro*.

Keywords: mesenchymal stem cells, strain, electrical stimulation, neuron, differentiation

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INTRODUCTION

Traumatic nervous system injuries, stroke, and many neurological disorders are characterized by the loss of neuronal functions. The damaged neural tissue rarely recovers spontaneously due to extremely low endogenous regenerative capacity and poor migrating ability of the neural stem cells. Stem-cell-mediated therapy has shown a great preclinical potential for neural injury and

Abbreviations: BMSC, bone marrow-derived mesenchymal stem cells; EF, electrical field; EGF, epidermal growth factor; FGF2, fibroblast growth factor 2; cAMP, cyclic AMP; KCl, potassium chloride; ERK, extracellular-signal-regulated kinase; AKT, protein kinase B; DMEM-LG, Dulbecco's modified Eagle medium-low glucose; NSE, neuron-specific enolase; MAP2, microtubule-associated protein 2; NT-3, neurotrophin 3; NT-4, neurotrophin 4; BDNF, brain-derived neurotrophic factorl; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

degenerative diseases. Mesenchymal stem cells (MSCs) have been widely used as a cell therapy to treat various diseases including bone diseases, cardiovascular diseases, autoimmune diseases, and inflammatory diseases (Shafei et al., 2017; Molendijk et al., 2018; Su et al., 2018; Yan et al., 2018). It is well established that MSCs have the capability to differentiate into several cell types, such as osteoblasts, chondrocytes, neural cells, hepatocytes, lung cells, and vascular endothelial cells (Chen et al., 2004; Tropel et al., 2006; Aurich et al., 2009; Jang et al., 2010). Previous work has demonstrated that MSCs can differentiate into neural-like cells under various conditions *in vitro* and *in vivo* (Deng et al., 2001; Cho et al., 2005; Yang et al., 2008). Furthermore, animal experiments showed that MSC-differentiated neuronal cells are beneficial for neuronal regeneration (Brazelton et al., 2000; Takizawa, 2003; Mimura et al., 2005; Bahat-Stroomza et al., 2009; Hayase et al., 2009).

Many treatments, including chemical compounds, growth factors, and genetic manipulation, have been adopted to improve BMSC neural differentiation (Deng et al., 2001; Cho et al., 2005; Yang et al., 2008). However, it suggested that morphological changes and a modest increase of gene expression levels for neural markers promoted by chemical induction were not real neurogenesis but merely cellular toxicity or cytoskeletal changes (Bertani et al., 2005). A growing number of bioengineering strategies such as cell culture biomaterials, mechanical force, and electrical field have been explored to evaluate the potential cues on the differentiation of MSCs into neural lineages. Studies have demonstrated that electrical stimulation plays a key part in broad biological activities, including proliferation, differentiation, and activation of intracellular pathways of various cell types (Schmidt et al., 1997; Sheikh et al., 2013; Yuan et al., 2014; Taghian et al., 2015). Specifically, electric field has been reported to be able to direct neural cell migration and neurite growth as well as promote neural stem cell proliferation and differentiation (Pan and Borgens, 2012; Babona-Pilipos et al., 2015; Pires et al., 2015; Petrella et al., 2018). In addition, electric field stimulation could repair the injury of neurons by increasing Netrin-1 and its receptor expression (Liu et al., 2018). Clinical applications of low-frequency electrical stimulation showed benefits of improved nerve regeneration and functional recovery (Gordon et al., 2009). On the other hand, native stem cells respond to dynamic local mechanical forces which show important regulatory roles in cell proliferation, metabolism, differentiation fates, and survival (Vining and Mooney, 2017; Romani et al., 2019). Accruing evidence showed that mechanical and physical cues, such as fluid shear stress, static stretch, and magnetic forces, can also contribute to stem cell fate determination (Clause et al., 2010; Marycz et al., 2016; Vining and Mooney, 2017). A recent study has revealed that extracellular physical cues could transduce into intracellular force to control the intestinal organoid growth and development through Wnt/β-catenin signaling (Li et al., 2020). Particularly, stretch could stimulate neuron growth (Loverde and Pfister, 2015; Breau and Schneider-Maunoury, 2017), axon growth (De Vincentiis et al., 2020), and neurite outgrowth (Higgins et al., 2013; Kampanis et al., 2020). Moreover, we have reported that fluid shear stimulation could boost BMSC differentiation into endothelial cells and cardiomyocyte-like cells (Bai et al., 2010; Huang et al., 2010).

In the present study, we examined the effect of the association of mechanical strain with electrical stimulation on BMSC neural differentiation, which was not observed under each individual stimulation. Cells were seeded on elastic silicone membranes and subjected to cyclic uniaxial stretching and/or electrical stimulation. Morphological characters, neuronal biomarker expression level, and calcium influx were evaluated under different treatments. Besides, transcriptome analysis was applied to elucidate the potential biological processes and signaling pathways of electric fields and strain co-stimulation-directed neuron differentiation. We proposed that the combined mechanical and electrical stimulation will potentially improve BMSC differentiation into neural cells.

MATERIALS AND METHODS

BMSC Culture

Primary BMSCs were isolated from the femurs and tibias from 4-week-old male Sprague-Dawley rats (Beijing Vital River Laboratory Animal Technology Co., Ltd, Beijing, China) by Percoll technique (Pharmacia, Uppsala, Sweden) as previously described (Huang et al., 2010). Isolated cells were seeded in 10 cm plastic culture dish and cultured in Dulbecco's modified Eagle medium-low glucose (DMEM-LG; Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS, Gibco). Non-adherent cells were removed after seeding for 3 days, and the medium was refreshed every 3 days. Cells were passaged when the cells reached 90% confluency by trypsin digestion, and cells used for all experiments were between passages 2–4. Isolated cells were confirmed by our lab that they expressed mesenchymal cell markers CD29, CD44, CD90, CD105, CD106, and CD166 and negative for CD34, CD45, and HLA-DR by flow cytometry analysis (Huang et al., 2010). Isolated cells also showed the multipotency to differentiate into osteoblasts (Li et al., 2014), endothelial cell (Bai et al., 2010), and cardiomyocyte-like lineage (Huang et al., 2012) in our previous studies.

Device

A self-designed device which could provide cyclic strain and pulsed biphasic electrical field (EF) stimulation was developed as shown in **Figures 1A,B**. The apparatus consisted of a step motor controlled by a motor driver and a signal amplifier, an alternating current signal generator, and a culture chamber with a transparent lid. Inside the culture chamber, there were two quadrate plastic culture plates, two fixed ends, and two mobile ends which can move forward and back under the control of the step motor driver. There were three struts on each end. BMSCs were seeded at the density of $2 \times 10e4/cm^2$ on pieces of elastic silicone membrane (USP class VI silicone, durometer 40, elastic modulus 7.7 GPa) with two handles. The strain was created by the stretching and shrinking of the elastic silicone membrane after putting the handles of the membrane onto the struts on fixed and mobile ends. To

generate the bidirectional pulse current, two platinic wires were placed in the plate and connected to the alternating current signal generator. The electrical field was 1 V/cm, 0.5 Hz (**Figure 1D**). The system was kept inside an incubator and sterilized by UV light for 30 min. Parallel static control cells were cultured on the silicone membrane without electrical or strain stimulation.

Neural Differentiation and Treatment

Cells were pretreated with a preinduction medium [DMEM-LG supplemented with 2% B27 (Gibco), 20 ng/ml fibroblast growth factor 2 (FGF2, A sigma), and 20 ng/ml epidermal growth factor (EGF, sigma)] for 7 days and then seeded on the elastic silicone membrane which was precoated with 0.01% poly-L-lysine (sigma). Then, the membranes were cultured under static and dynamic conditions with or without ES for 24 h. The membranes were then put in a plastic dish, and the medium was changed into differentiation medium (DMEM-LG medium supplemented with 10% FBS, 2% B27, 10 ng/ml FGF2, 10 ng/ml EGF, 100 U/ml penicillin, and 100 mg/ml streptomycin). Cells were differentiated for another 5 days and then harvested for qPCR, immunocytochemistry, and other assays (**Figure 1C**).

RNA Extraction and Quantitative RT-PCR

Total RNA isolation from cells under different treatments was performed with the Qiagen RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). cDNA was synthesized from total RNA samples using a Reverse Transcription Kit (TaKaRa, Kyoto, Japan). The forward and reverse primers used for quantitative RT-PCR were synthesized by Sangon Biotech (Shanghai, China); the sequences are listed in **Table 1**. Also, qPCR was performed on an Applied Biosystems ViiA^{TM} 7 Real-Time PCR System (Thermo Fisher Scientific, United States). Three replicas were performed in the qPCR analysis and the relative gene expression compared to the housekeeping gene GAPDH. Data from at least three independent experiments were collected.

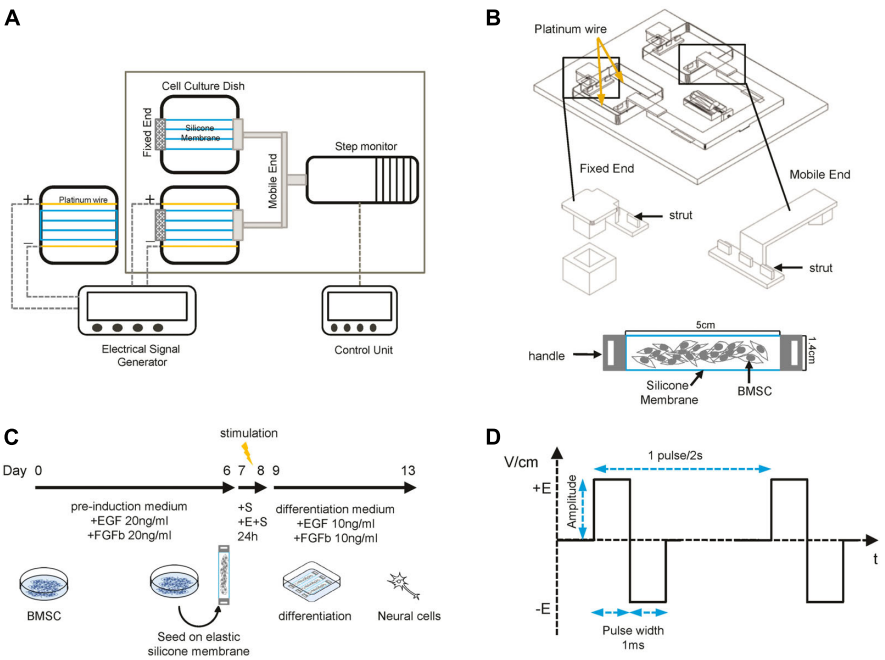

FIGURE 1 | Sketch of the device and differentiation process. **(A)** Schematic representation of the stretching and electrical field (EF) stimulation device. **(B)** Detailed structure of fixed and mobile ends and the elastic silicone membrane. **(C)** Schematic overview of the BMSC neural differentiation process. **(D)** The pulsed biphasic electrical signal.

TABLE 1 | The primers for RT-PCR.

Gene	Sense 5→3	Antisense 5→3	Size (bp)
NSE	CCGGGTCAAGACGCTAGAAGA	CTCCAGCTCTTCCGCAAGGTTGT	196
β-Tubulin III	GTCCGCCTGCCTCTTCGTCTCTA	GGCCCCTATCTGGTTGCCGCACT	93
MAP2	CAAACGTCATTACTTTACAACTTGA	CAGCTGCCTCTGTGAGTGAG	122
NT-3	CTTCTGCCACGATCTTAC	AACATCTACCATCTGCTTG	197
NT-4	CTAATGTGTGACTCTGCTAAC	GATACGGTGCTCAGGATAG	180
BDNF	GCGGCAGATAAAAAGACTGC	GCCAGCCAATTCTCTTTTTG	238
GAPDH	GGTGTGAACGGATTTGGCCGTAT	CTCAGCACCAGCGTCACCCCATT	262

RNA Sequencing Analysis

Total RNA sequencing was performed at Novogene Bioinformatics Technology Co. Ltd. (Beijing, China). HISAT2-2.1.0, StringTie-1.3.5, and DEseq were used to select the differentially expressed genes. Genes with adjusted $p < 0.05$ and log2(Fold Change) > 1 were screened out as significantly differentially expressed. Gene Ontology enrichment analysis was performed using the DAVID online tool. GO terms with corrected $p < 0.05$ and a fold change > 1.5 were considered to be significantly enriched by differentially expressed genes. The pathway enrichment analysis was based on the latest Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The Benjamini and Bonferroni approaches were used to control the false discovery rate.

Immunocytochemistry and Image Analysis

Cells were fixed with 4% paraformaldehyde (PFA; Sigma-Aldrich) for 10 min at room temperature (RT), triple rinsed with phosphate-buffered saline (PBS), and then permeabilized with 0.1% Triton X-100 for 10 min, followed by blocking with 5% BSA for 1 h at RT. Samples were incubated with primary antibodies anti-Nestin antibody (Abcam, cat# ab134017, diluted at 1:10,000) and anti-neuron-specific class III beta-tubulin (Abcam, cat#ab52623 diluted at 1:1,000), then washed three times with PBS, stained with secondary antibodies for 1 h at RT. Secondary antibodies included rabbit anti-chicken IgY H&L FITC (Abcam, cat#ab6749, diluted at 1:1,000) and R-Phycoerythrin AffiniPure F(ab′)$_2$ Fragment Goat Anti-Rat IgG (H + L) (Jackson ImmunoResearch, cat#112-116-143, diluted at 1: 200). 4′,6-Diamidino-2-phenylindole (DAPI, Dojindo, cat#28718-90-3) was used for nuclear staining. Rhodamine phalloidin (Thermo Fisher Scientific, cat#R415, 1: 200) was used for staining actin filaments. Confocal images were photographed using Leica DMI4000B.

The morphologic parameters were measured from images captured by the Olympus inverted microscope equipped with the Olympus digital camera DXM-1200 (Nikon Canada) and confocal microscope (Leica, TCS SPE). All images were analyzed by ImageJ package, Fiji. The neurite length was analyzed by Fiji with NeuronJ plugin (Pemberton et al., 2018), and lengths of the longest neurite for 44 cells per condition were used for statistical analysis.

Flow Cytometry Analysis

Cells were harvested and fixed with fixation/permeabilization solution (BD Pharmingen™) for 10 min at RT, washed with 1 × Perm/Wash Buffer (BD Pharmingen™), and then resuspended in 1 × Perm/Wash Buffer (2% BSA in PBS). 1 × 10e5 cells/well were incubated with first antibodies (anti-Nestin antibody, anti-III beta-tubulin) for 30 min at RT followed by twice washing steps with PBS. Cells were resuspended in 1 × Perm/Wash Buffer and incubated with relative fluorochrome-labeled second antibodies [rabbit anti-chicken IgY H&L FITC, R-Phycoerythrin AffiniPure F(ab′)$_2$ Fragment Goat Anti-Rat IgG (H + L)] for 30 min at RT. Cells were analyzed by flow cytometry using a BD FACSCelesta and FlowJo software (BD Biosciences, Heidelberg, Germany).

Measurement of cAMP and Phosphorylation of ERK

Quantification of cAMP in BMSC-derived neural cells after stimulation was carried out using a commercial kit (LANCE® Ultra cAMP Kit). After the strain and/or electrical stimulation, the differentiated cells were collected and seeding at 1,000 cells per well in a white OptiPlate™-384 microplate and then followed the manufacturer's guidance. The time-resolved fluorescence resonance energy transfer (TR-FRET) signal was measured on an EnVision® Multilabel reader (PerkinElmer, United States). The cAMP level was calculated according to the standard curve.

The phosphorylation of ERK and AKT was detected by AlphaLISA® SureFire® Ultra™ p-ERK 1/2 (Thr202/Tyr204) assay kit and AlphaLISA SureFire Ultra p-AKT1/2/3 (Thr308) Assay Kit, respectively (PerkinElmer, United States).

Live Cell Calcium Test

After differentiation, BMSC-derived neural cells were collected for calcium test using the fluorometric imaging plate reader (FLIPR Tetra, Molecular Devices, United Kingdom). Cells were seeded into 384-well plates with the density of 10,000 cells/well (25 μL) and cultured overnight before incubating with an equal volume of FILIPR Calcium 6 indicator (FLIPR Calcium 6 Assay Kits, Molecular Devices) in Hank's balanced salt solution (HBSS with 20 mM HEPES, pH 7.4) for 2 h at 37°C. Response signals (relative fluorescence units, RFU) were traced during 190 s when the stimuli acetylcholine (final concentration 0.1 mM) and KCl (final concentration 45 mM) were added automatically using the FLIPR instrument. To enable comparison, baseline was subtracted from response signals. Moreover, the peak amplitude was calculated by maximal–minimal signal.

Statistical Analysis

Cells for all experiments were isolated from at least three donors of rats, and all data were collected from independent isolations. Statistical analysis was performed using GraphPad Prism v.8.0 software (GraphPad Inc., San Diego, CA, United States). Graphed data were presented as mean ± standard deviation from at least three independent biological replicates. Groups were compared using Mann–Whitney Test *t*-tests and one-way analysis of variance (ANOVA) as appropriate. $^{*}p < 0.05$ and $^{**}p < 0.01$ were considered statistically significant.

RESULTS

Cell Alignment Under Cyclic Strain and Electrical Stimulation

The rat BMSCs were preinduced for 7 days, and then pyramidal-shaped cell bodies and extended short neurites, reminiscent of dendrites, could be identified. To test the combinatorial effect of strain and EF, cells were subjected to cyclic strain (5% elongation, 0.5 Hz, + S), EF (1 V/cm, 0.5 Hz, + E), and co-stimulation

(+ E + S) for 24 h before changing to a differentiation medium. Under strain and electrical stimulation, cells showed orientation change and alignment (**Figure 2A**). The cells in static control culture showed a random orientation. Cells under strain became oriented away from (perpendicular to) the direction of cyclic stretch, and cells under electrical stimulation aligned themselves with the direction of electrical stimulation. Some cells detached from the membranes during strain or electrical stimulation, and a few more cells detached and died under co-stimulation, but the remaining cells were still in good condition (**Supplementary Figure 1**). To quantify the cell orientation (**Figure 2B**), angles of 52 cells for each treatment were measured. The cell orientation distribution was analyzed by the cell frequency in each direction (**Figure 2C**). Cells under strain, electrical stimulation, and co-stimulation showed an increase in the frequency of cells oriented at angles near 90°.

Cyclic Strain and Electrical Co-stimulation Enhanced Neural Morphological Change

After another 5 days of differentiation, cells had typical morphological features of neurons, such as extending and branching processes. Morphology of cells was evaluated by the following parameters: the longest length of neurites and the number of the root and extremity of neurites (**Figure 3B**). Cells under strain alone and co-stimulation induced significantly longer neurites compared with electrical stimulation and static control (**Figure 3A**). The cyclic strain plus electrical stimulation could further increase the length than electrical treatment alone, indicating the enhanced impact of strain on neurite growth. Although co-stimulation induced additional increase in neurite length compared with strain alone, there was no significant difference. In contrast to neurite length, there were few neurite roots from cells under co-stimulation than under static control (**Figure 3C**); however, the extremity index was similar under different conditions except for the lower-extremity index under strain stimulation compared with co-stimulation (**Figure 3D**). Thin, hair-like filopodia can be seen along the neurites (**Figure 3E**). Compared with the strain and control groups, the filopodia density (the number of filopodia per 10 μm neurite length) was significantly higher in electrical stimulation and co-stimulation conditions (**Figure 3F**).

Cyclic Strain and Electrical Co-stimulation Increase Neural Cell Marker Expression

The influence of cyclic strain and electrical co-stimulation on gene expression of neural cell markers and neurotrophins involved in neural development was analyzed by qPCR. Compared to BMSC or electrical stimulation alone, co-stimulation induced a significant upregulation of Microtubule Associated Protein 2 (MAP2), β tubulin III, neuron-specific enolase (NSE) as well as neurotrophins, NT-3, NT-4, and brain-derived neurotrophic factor (BDNF) (**Figure 4A**). BMSCs differentiated into neural cells were further confirmed by positive staining of the immature neuron marker Nestin, and the immature and mature neuron marker β tubulin III (**Figure 4B**). The flow cytometry data confirmed that under strain or co-stimulation, the nestin and β tubulin III protein expression levels were significantly increased compared to static control (**Figures 4C,D**).

Cyclic Strain and Electrical Co-stimulation Enhanced the Neural Differentiation

It is well established that cyclic AMP (cAMP) signaling cascade plays an important role in neuronal differentiation, axonal guidance, neurite outgrowth, and neuron maturation (Cai et al., 2002; Fujioka et al., 2004; Aglah et al., 2008). As shown in **Figure 5A**, the cAMP levels under all the treatments increased after being differentiated from BMSCs. Specifically, for the co-stimulation, the level of intracellular cAMP was doubled compared to that of electrical or strain simulation alone.

Calcium signals are known to be important regulators of neurite outgrowth as well as a charge carrier. The calcium

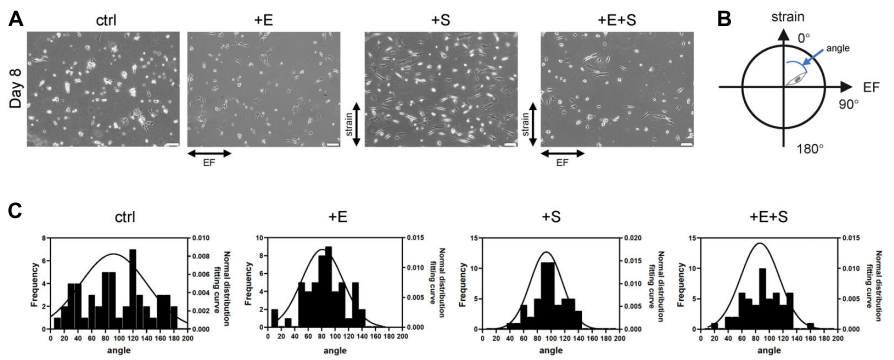

FIGURE 2 | BMSC reorientation under cyclical strain and electrical field stimulation. **(A)** The change of cellular orientation under static control (ctrl), electrical stimulation (+E), strain (+S), and co-stimulation (+E + S). Scale bar, 100 μm. The directions of strain and electrical field were indicated by arrows. **(B)** Schematic illustration indicates cell angle. The vertical upward direction was defined as 0°, and the horizontal right direction was defined as 90°. **(C)** Distribution of cellular orientation. The line was the normal distribution fitting curve.

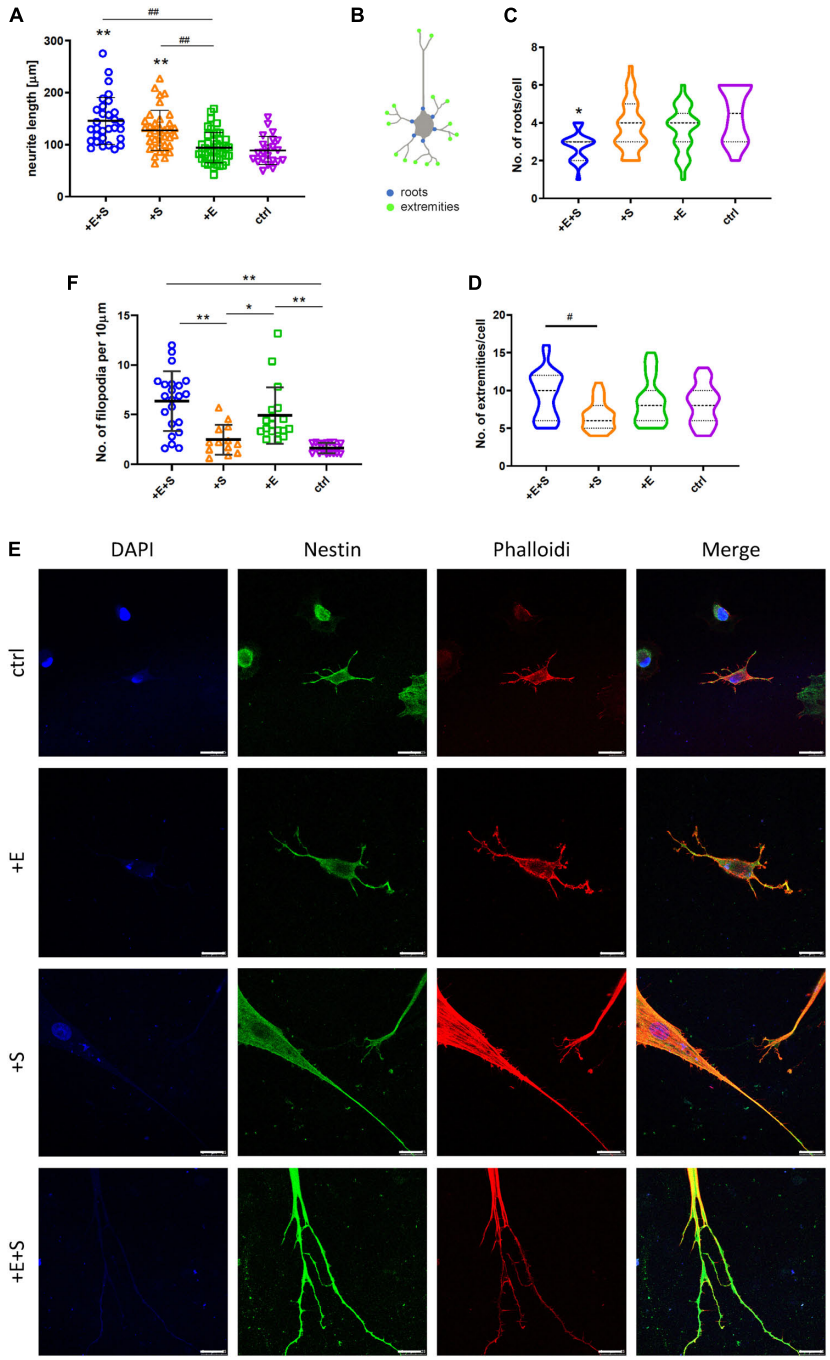

FIGURE 3 | BMSCs' morphologic change under cyclical strain and electrical field stimulation. **(A)** Co-stimulation (+E +S) and strain (+S) significantly elongated neurites compared with static control (ctrl) (**$p < 0.01$) and electrical stimulation (+ E) ($^{\#\#}p < 0.01$, ANOVA). **(B)** Diagram of the roots and extremities of neurites. The numbers of roots **(C)** and extremities **(D)** of neurites under each treatment were counted manually from four independent experiments. Values are mean ± SD. **(E)** Immunocytochemistry detecting actin filament (red), nestin (green), and nucleus (blue) expression in rBMSCs under treatments (scale bar = 25 μm). **(F)** Density quantification of filopodia under each treatment. The number of filopodia per 10 μm of neurite was used to calculate the filopodia density (*$p < 0.05$, **$p < 0.01$, ANOVA). $^{\#}p < 0.05$.

change was detected by the FLIPR system. **Figures 5C,E** show a representative calcium tracing signal when differentiating BMSCs treated with 0.1 mM acetylcholine and 45 mM KCl. Electrical stimulation and co-stimulation triggered higher calcium influx induced by acetylcholine (**Figure 5D**) and KCl (**Figure 5F**) than static control. Moreover, cells produced a significant higher calcium signal under co-stimulation than strain or electrical treatment alone (**Figures 5D,F**).

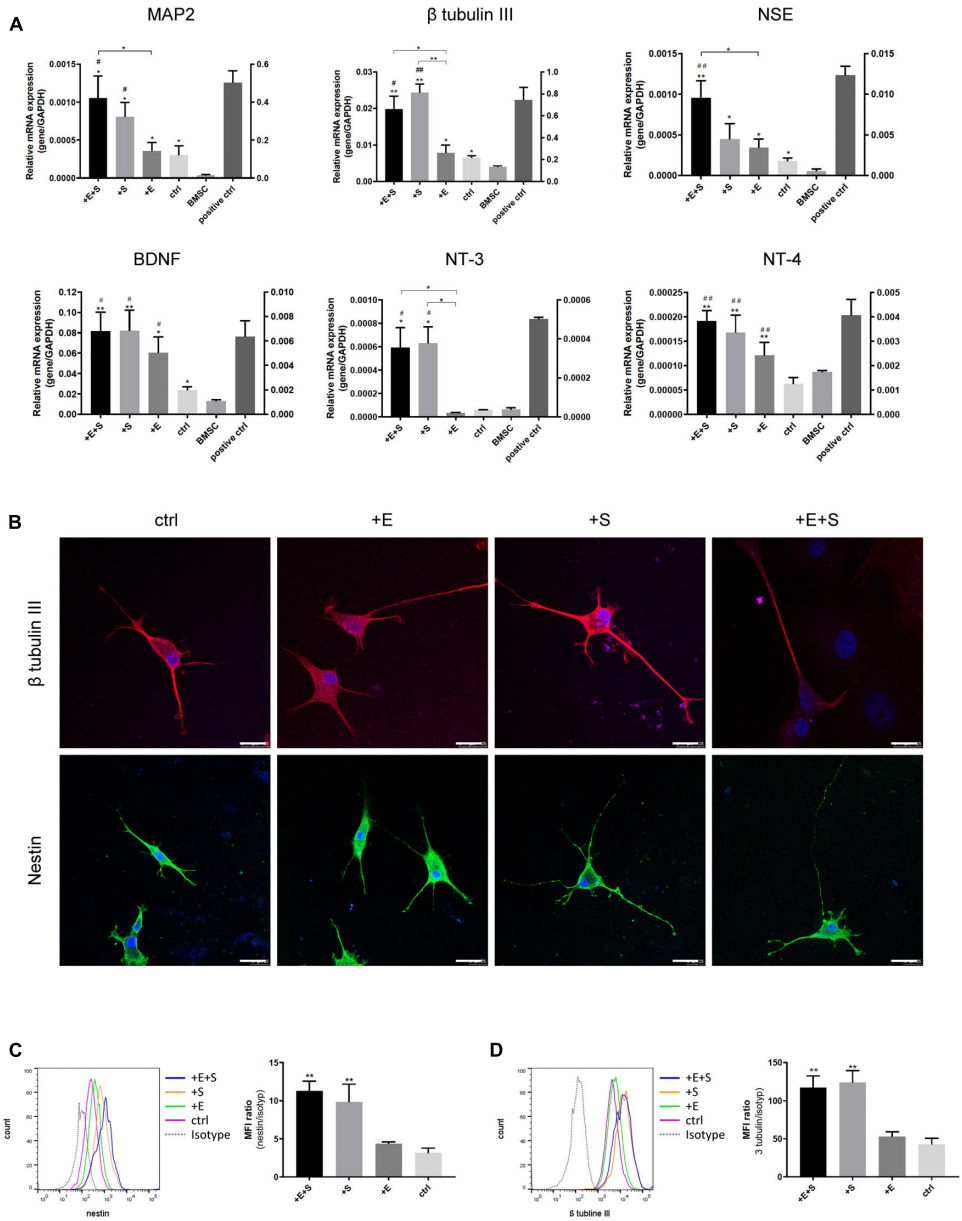

FIGURE 4 | Effects of the strain and electrical stimulation on the neural related gene expressions of BMSCs. **(A)** BMSCs were induced by the neural differentiated medium under static conditions (ctrl) or under cyclic strain (+S, 5% elongation, 0.5 Hz), under electrical stimulation (+E, 1 V/cm, 0.5 Hz), and under co-stimulation (+ E + S) for 24 h. Gene expression of MAP2, β-tubulin III, NSE, BDNF, NT-3, and NT-4 on day 13 was analyzed by real-time RT-PCR and normalized to GAPDH. Normal neonatal rat neurons were used as positive control. Results are shown as mean ± SD ($N = 4$). $^{*}p < 0.05$, $^{**}p < 0.01$ compared to the BMSC, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared to the static control. **(B)** Representative immunostaining images of neural differentiated BMSCs under treatments. Immunocytochemistry detecting β tubulin III (red) and nestin (green) expressions in BMSCs with DAPI (blue) under different treatments (scale bar = 25 μm). Representative flow cytometry histograms showing the protein expression of β tubulin III (**C** left) and nestin (**D** left) and statistical analysis of β tubulin III (**C** right) and nestin (**D** right) expression level under treatments ($n = 3$, $^{**}p < 0.01$).

Cyclic Strain and Electrical Co-stimulation Altered mRNA Expression

We examined the transcriptional changes via RNA sequencing for differentiated cells under strain and/or electrical stimulation and under control conditions. In total, 985, 1,406, and 1,150 DEGs displayed a differential expression between electrical stimulation, strain, and co-stimulation groups compared to no treatment control, respectively (**Figure 6A**). Ninety-four upregulated genes and 18 downregulated genes were screened out in the electrical and strain co-stimulation groups (**Figure 6B**). Hierarchical clustering shows a general overview of the expression pattern among samples (**Figure 6C**).

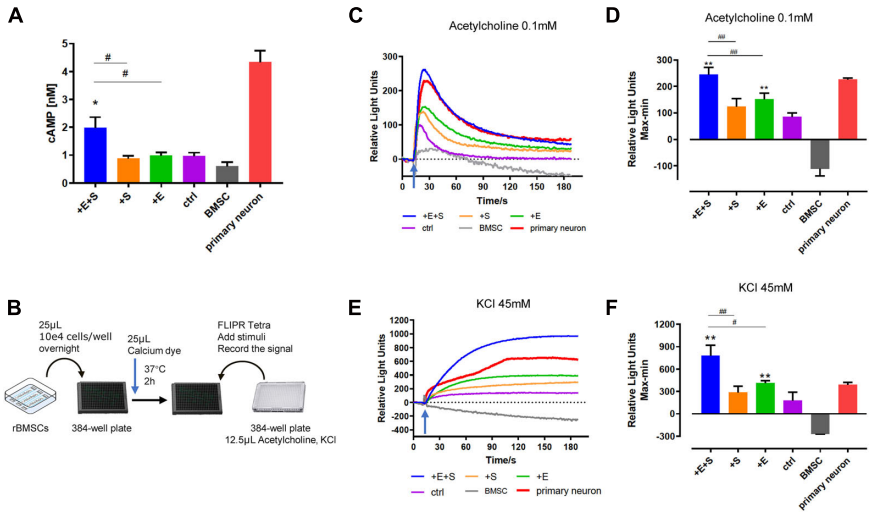

FIGURE 5 | Electrical field and cyclical stretch co-stimulation enhanced the rBMSC-derived neural cell function. **(A)** cAMP level in differentiated cells under static condition (ctrl), strain (+S), electrical stimulation (+E), and co-stimulation (+E +S) (n = 9). **(B)** Schematic of the calcium test process. Calcium signaling triggered (arrows indicate the time point of adding inducer) by acetylcholine (0.1 mM) **(C,D)** and KCl (45 mM) **(E,F)**. The primary neurons cultured *in vitro* for 7 days were used as a positive control, and the undifferentiated BMSCs were the negative control. Representative tracings of calcium signal record by FLIPR after adding acetylcholine **(C)** and KCl **(E)**. Statistical analysis of the peak amplitude **(D,F)**. $^{*}p < 0.05$, $^{**}p < 0.01$ (compared with static control), $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ (ANOVA, n = 5).

The enriched genes for the electrical stimulation or strain vs. co-stimulation comparison are summarized in three main GO categories (molecular function, biological process, cellular component). As shown in **Figures 6D,E**, the genes' differential expression in both electrical stimulation vs. co-stimulation and strain vs. co-stimulation comparison is highly enriched for "binding," "catalytic activity," "cellular process," "metabolic process," and "biological regulation."

Cyclic Strain and Electrical Co-stimulation Activated Pathway Analysis

We next determined the strain and electrical co-stimulation effect on neural differentiation. Comparing EF and strain treatment only, the co-stimulation enriched GO terms are involved in the positive regulation of the ERK1 and ERK2 cascade, negative regulation of cell proliferation, and brain development (**Figure 7A**). In the KEGG pathway analysis, the DEGs are found to be enriched in focal adhesion, ECM–receptor interaction, and axon guidance in both electrical stimulation vs. co-stimulation and strain vs. co-stimulation comparison (**Figure 7B**). Furthermore, the PI3K-AKT signaling pathway is the highest pathway count in electrical stimulation vs. co-stimulation.

To confirm the signaling pathway involved under strain and electrical co-stimulated conditions during neural differentiation, we examined the phosphorylation level of ERK and AKT. Consistent with GO and KEGG pathway analyses, co-stimulation significantly increases the level of phospho-ERK and phospho-AKT than strain and electrical stimulation alone (**Figures 7C,D**). Moreover, the level of phospho-AKT in strained cells is also significantly higher than that in no treatment control cells. These data suggests that strain and electrical co-stimulation could contribute significantly to the activation of ERK and AKT pathways in BMSC neuronal differentiation processes.

Protein and Protein Interaction Analysis Under Strain and Electrical Co-stimulation

To further investigate the differentially expressed genes at the protein level in the differentiation process of BMSCs under co-stimulation, a biological database, search tool/STRING, was used to filter functional genes. The protein–protein interaction was analyzed online to provide an intuitive network for the functional properties of proteins. The STRING analysis shows that in the + E vs. + E + S comparison group, genes for potassium voltage-gated channel subfamily H member 2 and 6 (Kcnh2, Kcnh6) are functionally linked. Besides, nodes Comp, Itga8, and Npnt and nodes Smad6, Smad9, and Nog are linked, respectively (**Figure 8A**). Comp is an extracellular matrix protein, and NPNT binds to integrin alpha-8/beta-1, suggesting a key role in regulating cell adhesion, spreading, and survival. Smad6 and Smad9 encode proteins that are signal transducers and transcriptional modulators which are involved in numerous signaling pathways. Smad6 is highly expressed in mature neurons and can promote cells that differentiate into mature neurons (Hazen et al., 2011; Xie et al., 2011). The Nog gene-encoded protein can regulate neural crest formation. In the + S vs. + E + S comparison group, the most connected protein nodes are Cyp1a1, Gstm3, Gstm5, and Mt1m (**Figure 8B**), which are essential for cell metabolism. Cyp1a1 encodes the cytochrome P450 enzyme. Gstm (Glutathione S-Transferase Mu)3 and 5 are related pathways which are glutathione metabolism and platinum drug resistance. Mt1m encodes a well-known metallothionein.

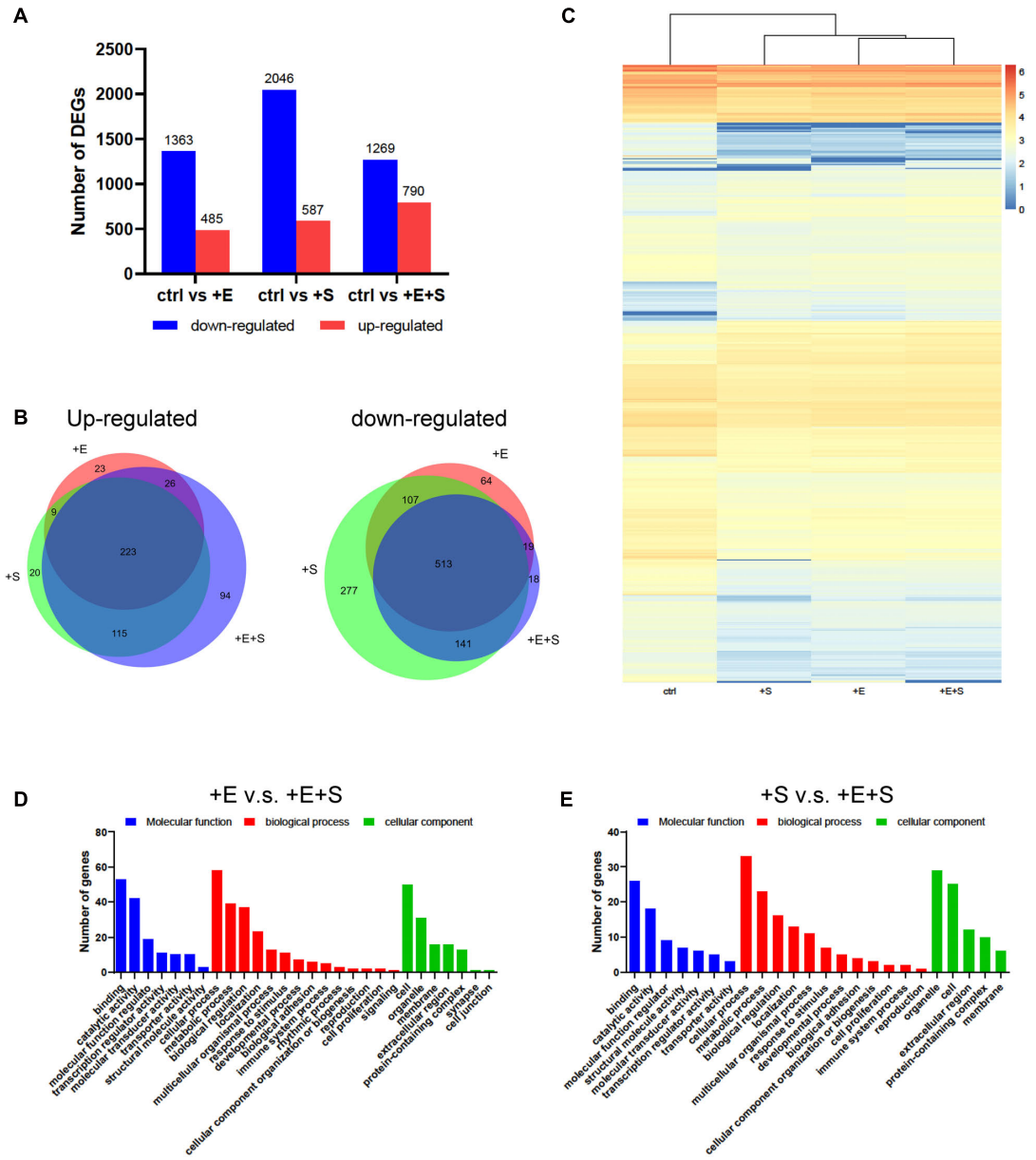

FIGURE 6 | Changes in gene expression profiles of neural differentiated BMSCs under different stimulations. **(A)** Numbers of DEGs compared with only EGF and FGF2 induction with EF and/or stain treatments. **(B)** Venn diagram showed the overlap genes among different treatments. **(C)** Heat map diagrams showed the relative expression levels of total DEGs under different stimulations. **(D)** DEGs between EF and co-stimulation. **(E)** DEGs between strain and co-stimulation.

DISCUSSION

Identify a method that is capable of promoting neural cells different from stem cells is of great interest in treating and repairing nerve damage. A great number of previous investigations have suggested that BMSCs possess the capability of differentiating into neural cells when exposed to defined chemical reagents, trophic factors, or genetic manipulation. Besides, a few reports have investigated that physical cues, such as electrical or mechanical stimulation, could enhance cell neural differentiation (Rajnicek et al., 2006; Thrivikraman et al., 2014; Pires et al., 2015). Our current study demonstrated that electrical and cyclic uniaxial stretching co-stimulation together with EGF and FGF2 could promote BMSC neural differentiation, neurite outgrowth, and active ERK1/2, AKT signaling pathways.

In this study, we used a self-designed device to provide cyclic strain (5%, 0.5 Hz) and electrical field (1 V/cm, 0.5 Hz) simultaneously. Consistent with previous studies suggesting that stretch and EF can regulate cell orientation (Neidlinger-Wilke et al., 2001; Haq et al., 2006; Arocena et al., 2010; Tang-Schomer, 2018), we observed cell reorientation and alignment with the direction of the loading axis and electrical field. In addition,

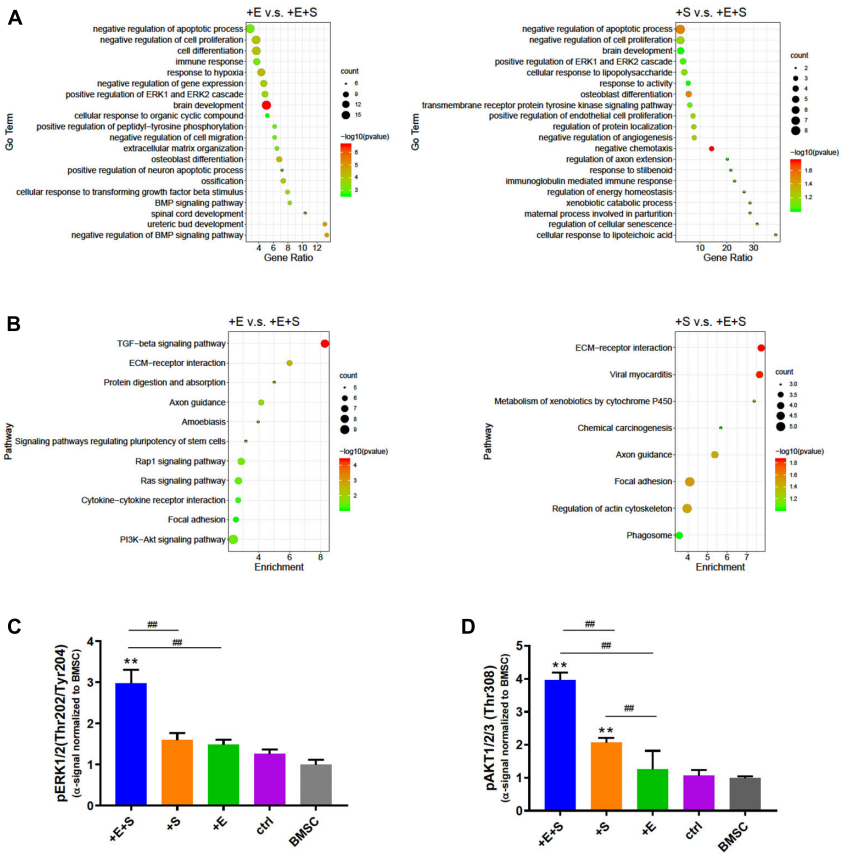

FIGURE 7 | Signaling pathway evaluation under different treatments. **(A)** Go term analysis between EF vs. co-stimulation and strain vs. co-stimulation. **(B)** KEGG pathway enrichment analysis of EF or strain vs. co-stimulation. Phosphorylation of ERK1/2 (Thr202/Tyr204) **(C)** and AKT1/2/3 (Thr308) **(D)** was detected by alpha screening assay. The alpha signal was normalized to that of BMSC ($n = 6$, $^{**}p < 0.01$ compared with static control, $^{\#\#}p < 0.01$, ANOVA).

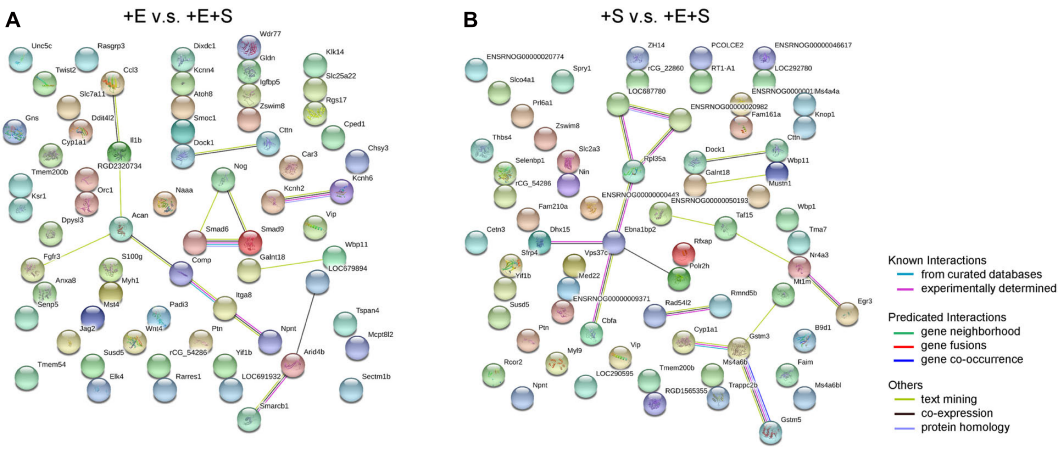

FIGURE 8 | Protein–protein interaction networks by STRING search tool. The up- and downregulated mRNAs (fold change > 1, $p < 0.05$) network between electrical stimulation with co-stimulation **(A)** and strain with co-stimulation **(B)**. Analysis by STRING based on protein–protein interactions. The high confidence score (0.7) was adopted to evaluate the protein interactions for the differentially expressed genes.

cyclic strain and co-stimulation induced longer neurites than did electrical stimulation and static control. Similar findings have been reported that cyclic stretch alone can induce neurite outgrowth of SH-SY5Y (a human neuroblastoma cell line cell, 10%, 0.25 Hz) and PC12 cells (a rat pheochromocytoma cell line, 4%, 1 Hz or 16%, 0.1 Hz) (Haq et al., 2006; Higgins et al., 2013)

and trigger human MSCs to differentiate into neuron-like cells at very low amplitude loading (0.5%, 0.5 Hz) (Leong et al., 2012). Moreover, stretch is also found to stimulate neurite growth of mature neurons. Ten percent cyclic stretch of nerve explants at 0.5 Hz enhanced neurite outgrowth of neurons from rat dorsal root ganglia (Kampanis et al., 2020), and 10 pN of stretch could enhance axon growth and branching (De Vincentiis et al., 2020). However, the conclusions of the amplitude of cyclic strain that can induce neurite outgrowth or neural differentiation are different from these studies. This may be due to the different cell types and the degree of neural cell maturity.

From our study, cyclic strain and electrical co-stimulation showed effects not only on neurite outgrowth but also on neurite branching and filopodia density. There was a significant decrease in the number of roots of neurite under co-stimulation compared with static control, but not with strain or electrical treatment alone. This correlates with a study by Feng et al. (2016) reporting that stretch could reduce the number of neurites because mechanical tension initiated major neurites to grow preferentially near the cell poles closest to the source of tension. In addition, the alternating EF also demonstrated a robust directing effect on axon alignment (Tang-Schomer, 2018). The hypothesis is that stretch and EF have synergetic effects on cell alignment which may last for a longer time than strain or EF treatment alone when physical stimuli are removed. It is also interesting to note that there is a trend that the number of extremities of neurite decreased under strain treatment but only showed a significant decrease when compared with co-stimulation. The possible reason is the increased activation of RhoA GTPase by cyclic strain. Small GTPases, Rho, Rac, and Cdc42 are well-known regulators of the actin cytoskeleton and are critical for neuronal morphogenesis. The activation of RhoA GTPase will induce cell alignment perpendicular to the direction of strain (Kaunas et al., 2005; Goldyn et al., 2009) but inhibit a branch extension of neurons (Lee et al., 2000; Li et al., 2002). Leong et al. reported that Rac1, but not RhoA, activation triggered by low train at 0.5%, 0.5 Hz, was the regulator for hMSC neural differentiation (Leong et al., 2012). The function of Rac1 and RhoA in growth cone of neurons is also verified in electrical field (Rajnicek et al., 2006). Taken together, co-stimulation may cause a different balance of activities of GTPases (Rac, RhoA, Cdc42) from strain alone, under which increased RhoA activation inhibited neurite branching and finally resulted in a different morphological outcome. Moreover, this hypothesis needs to be investigated in future work.

Filopodia play important roles in neuronal branching morphogenesis, sensing the microenvironment, and formation of synaptic connections (Mattila and Lappalainen, 2008; Menna et al., 2011; Heckman and Plummer, 2013; Fischer et al., 2019). There is a marked increase in filopodia density of differentiated BMSCs with electrical stimulation and co-stimulation. This is expected, as electrical stimulation has been reported to promote neurite branching in primary neurons (Stewart et al., 2016), neural stem cells (Stewart et al., 2015), and PC12 cell lines (Manivannan and Terakawa, 1994). The filopodial sprouting strongly related with Ca^{2+} concentration and influx (Manivannan and Terakawa, 1994; Heckman and Plummer, 2013; Hu and Hsueh, 2014), and in return, filopodia increase the neurite sensitivity to stimuli. This was observed in our result (**Figure 5**). Strain-stimulated cells with less filopodia showed lower calcium influx in response to acetylcholine and KCl.

Co-stimulation affects not only the morphological change but also the neural gene expression. Our results show that co-stimulation significantly increased the gene expression of specific neural markers, mature neuronal marker MAP2, neuron marker β-tubulin III, and immature marker nestin. The neurotrophins, BDNF, NT-3, and NT-4 are also upregulated under co-stimulation. Neurotrophins are implicated in multiple roles in the development and function of the nervous system. BDNF plays a vital role in the survival and differentiation of MSC and neural stem cells into neurons (Trzaska et al., 2009; Chen et al., 2017; Li et al., 2017). NT-3 and NT4 were found to improve neurite growth, axonal regeneration, and functional recovery (English et al., 2005; Wu et al., 2008; Hechler et al., 2010). The gene expression level of MAP2 and NSE under co-stimulation seemed a little higher than strain or electrical stimulation alone, but there is no significant difference. Furthermore, the increase of cAMP is observed in cells under co-stimulation. Previous studies have demonstrated the effect of cAMP on neurite outgrowth, axonal growth, and neuron maturation (Cai et al., 2002; Fujioka et al., 2004; Aglah et al., 2008). Moreover, exogenous cAMP is used to induce MSC and neural stem cell differentiation into neuron cells (Deng et al., 2001; Lepski et al., 2013; Shahbazi et al., 2016). As a whole, our results indicate that cyclic strain and electrical co-stimulation can promote neural differentiation of rBMSCs.

ECM and cytoskeletal proteins are reported to be key determinants of neural growth, migration, development, function, and extension of lamellipodia (Olson and Nordheim, 2010; Broadie et al., 2011). KEGG pathway enrichment showed that focal adhesion and ECM–receptor interaction were enriched under strain and electrical co-stimulation conditions. In addition, the protein–protein interaction analysis also shows that the extracellular matrix and membrane integrin are involved in co-stimulation. The rearrangement of the cytoskeleton could activate transducers and transcriptional modulators. Previous research demonstrated that electrical stimulation could increase neurite outgrowth of PC12 cells by activating PKC to increase the NGF-induced phosphorylation of ERK1/2 (Chang et al., 2013). GO and KEGG pathway enrichment analyses and the protein level tested by alpha screen reveal that the phosphorylation of ERK1/2 and AKT is involved in neural differentiation under cyclic strain and electrical co-stimulation. The phosphorylation of ERK1/2 and AKT under co-stimulation was notably increased than under strain and electrical stimulation alone. It is well documented that AKT can improve the survival of neurons (Jo et al., 2012; Wang et al., 2016) and improve axonal growth and branching (Grider et al., 2009), and ERK signaling can promote axonal extension (Huang et al., 2017).

Based on our findings and previous studies, a putative mechanism of cyclic strain and electrical co-stimulated BMSC neural differentiation is proposed (**Figure 9**). Under stretch and electrical stimulation, integrins or other membrane receptors detect the change of ECM and then regulate the remodeling of cytoskeleton and increase the cAMP level and activation

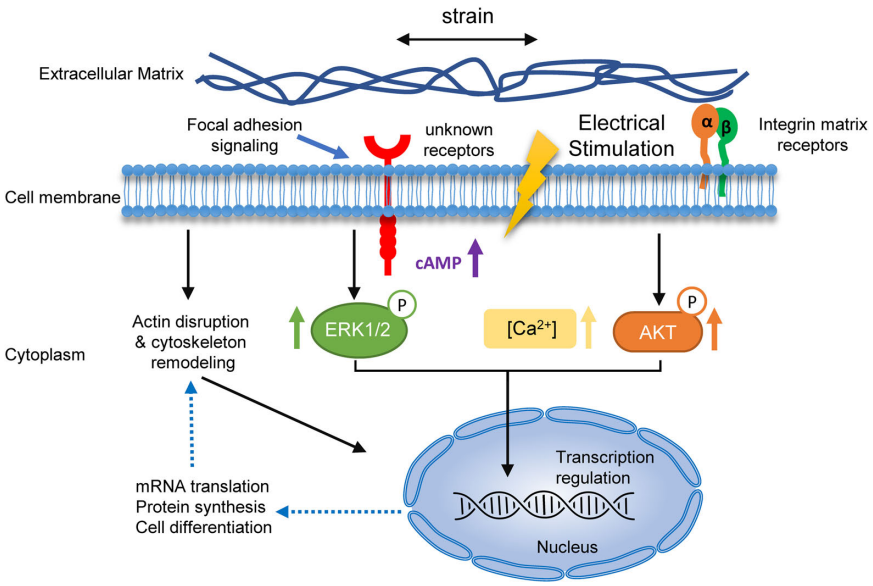

FIGURE 9 | A hypothetical mechanism for the role of cyclic strain and electrical co-stimulation in promoted BMSCs neural differentiation. Schematic summary of the mechanism of strain and EF co-stimulation-induced BMSC neural differentiation. The strain and EF cause changes of ECM, which are sensed by specific receptors and integrins on the cell membrane, resulting in receptor-mediated cell mechanosensing. Activation of these receptors leads to a series of events, including actin disruption and cytoskeleton remodeling, rising of calcium and cAMP, and phosphorylation of ERK and AKT. Signals transduce into the nucleolus and then regulate neural differentiation-related mRNA transcription and protein synthesis, and in return regulate actin formation in neurites.

of certain signaling pathways (such as Ca^{2+} increasing and phosphorylation of ERK and AKT). Then, the signals activate transcription factors to regulate the transcription of neural differentiation genes. Subsequently, neural marker and neurotrophin expressions increase and then regulate actin formation in return, promoting neurite outgrowth and branching. Further molecular experiments are needed to be conducted to discover precise mechanisms of EF and stretch synergetic effects on BMSC neural differentiation.

CONCLUSION

Our findings demonstrate that cyclic strain and electrical co-stimulation have a synergetic effect on EGF and FGF2-induced rat BMSC neural differentiation by upregulating neural markers and neurotropic mediators and increase calcium influx, intracellular cAMP, and phosphorylation of ERK1/2 and AKT. Knowledge of the impact of this strain and electrical co-stimulation on BMSC differentiation provides a better understanding on how cells respond to biomechanical manipulations and suggests new approaches for stem cell neural differentiation.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI BioProject, accession no: PRJNA666744.

ETHICS STATEMENT

The animal study was reviewed and approved by the Beihang University.

AUTHOR CONTRIBUTIONS

HC planned and carried out the experiments, performed the data analyses and interpretation of the results, and wrote the manuscript. YH participated in the planning of the experiments and the revision of the manuscript. WC contributed to the bulk RNA-seq data analysis. JC designed the device and took some of the confocal images. TL, JN, and RW contributed to sample preparation. YH and YF supervised and administered the project. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.624755/full#supplementary-material

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REVIEW
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Postmortem Studies of Fetal Grafts in Parkinson's Disease: What Lessons Have We Learned?

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Neural transplantation is a potential therapeutic method for Parkinson's disease (PD). Fetal dopaminergic (DA) neurons have been important transplantation cell sources in the history of replacement therapy for PD. Several decades of preclinical animal experiments and clinical trials using fetal DA neuron transplantation in PD therapy have shown not only promising results but also problems. In order to reveal possible factors influencing the clinical outcomes, we reviewed fetal DA neuron transplantation therapies from 1970s to present, with a special focus on postmortem studies. Firstly, we gave a general description of the clinical outcomes and neuroanatomy of grafted cases; secondly, we summarized the main available postmortem studies, including the cell survival, reinnervation, and pathology development. In the end, we further discussed the link between function and structure of the grafts, seeking for the possible factors contributing to a functional graft. With our review, we hope to provide references for future transplantation trials from a histological point of view.

Keywords: Parkinson's disease, grafts, Lewy pathology, postmortem studies, transplantation

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INTRODUCTION

Parkinson's disease (PD) is characterized by the loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) of the midbrain. Replacement of the degenerated neurons has been thought to be a promising alternative approach for treating PD. Researchers have studied various cell and tissue types as sources for transplantation; for example, the adrenal medullary tissues from the patients themselves were once used for restoring nigrostriatal function. The method minimized possible immunological problems that might occur postoperatively. In 1982–1985, four patients with severe PD were subjected to an autologous adrenal medulla grafting procedure. Motor improvements were observed in one of the patients for 6 months after grafting (Backlund et al., 1985). Madrazo et al. (1987) grafted two patients with adrenal medullary tissue using a modified microsurgery technique in 1987, bringing the trials using this therapeutic method to a worldwide scale. As more trials were carried out, problems with transplanting adrenal medullary tissue began to appear, with unreproducible results, unsustainable outcomes, and severe side-effects (Goetz et al., 1989; Stoddard et al., 1989). Researchers were again in the urgent need for a promising cell source for PD transplantation therapy.

In the late 1970s, animal experiments demonstrated that the intracerebral grafting of rat fetal DA cells reversed the disease symptoms induced by 6-hydroxydopamine (6-OHDA) lesions (Bjorklund and Stenevi, 1979; Perlow et al., 1979). As one of the main pathological changes in PD is the loss of DA neurons in the SNpc, the method of using healthy cells to directly restore

the decreased dopamine supply targeted the disease on a substantial level (Kalia and Lang, 2015). Repeated preclinical trials have been carried out since then, marking the beginning of using fetal DA neurons as cell source for transplantation to treat PD and giving hopes for the possibility of DA neuron grafting in PD patients (Brundin et al., 1986, 1987). It took nearly a decade before fetal DA neuron transplantation was brought to a clinical trial stage from the lab bench (Lindvall et al., 1988). Lindvall et al. (1988) reported a two patients' 6-month follow-up postsurgery. With no complications shown after surgery, both patients exhibited minor improvements in motor performance accompanied by no striking change in response to drug treatment. However, one of the patients showed significant restored dopamine synthesis and storage in the grafted area, later revealed by 6-L-(18F) fluorodopa (FDOPA) scan (Lindvall et al., 1990). From the late 1980s, clinical follow-ups and postmortem studies on fetal DA transplantation studies in humans have been reported continuously, with diversity in clinical outcomes, sustainability of graft-induced benefits (GIB), survival of the grafts, pathology in grafted neurons, etc., hindering the clinical application of the method. Therefore, a systematic summary and analysis of the lessons we have learned from previous trials is necessary as references to improve the future transplantation therapy in PD. In this review, we have summarized the neuroanatomic studies of previous cases in relation to the clinical follow-ups, focusing on the DA neuron survival, reinnervation and pathology developments, analyzing the possible structure-function interplay of the grafted cells, and innovatively, from a thorough histological point of view, trying to reveal the possible factors contributing to a successful graft.

GENERAL DESCRIPTION OF CASE CLINICAL FOLLOW-UPS AND HISTOLOGY

Clinical trials using fetal DA neurons to treat PD have been continuing since the late 1980s. At the beginning of the 2000s, over 350 patients had been grafted with fetal DA tissues, including both single non-blind cases and double-blind placebo trials (Lindvall and Bjorklund, 2004; Bjorklund and Kordower, 2013). From the follow-up of these studies, promising improvements have been observed in some patients with respect to the following points: (1) improved motor function with speeded up movement, reduced rigidity, and decreased time in "off" periods; (2) maintenance of daily life quality with a reduction in levodopa (L-dopa) treatment; and (3) survival and functionality of grafted tissues observed through FDOPA uptake positron emission tomography (PET) scan. We included in this review (Hagell and Brundin, 2001) studies with detailed description of postmortem results, to reveal the possible relations between neuroanatomy and symptomatic improvements.

In 1990s, autopsy reports with transplanted patients who died shortly after surgery were already reported. Redmond et al. (1990) reported one case grafted at the age of 63, with the patient dying 4 months after transplantation. No major clinical improvements were observed. Postmortem analysis showed no survival of tyrosine hydroxylase-positive (TH+) neurons (Spencer et al., 1992), possibly due to the short survival time postgrafting. The following year, a study with the clinical follow-up of 12 patients showed promising improvements in motor performance with a decreased demand for L-dopa treatment and reduced time spent in the "off" period, in most patients 12 months after operation. Among these patients, five died between 18 and 40 months postsurgery (Henderson et al., 1991). Three of them exhibited TH+ neurons in the graft-host interface, in which some cells showed complete TH staining while others contained neuromelanin (NM). However, there was no clear sign of graft innervation (Henderson et al., 1991). Kordower et al. (1995, 1996) performed a detailed autopsy study on one of these patients, who died 18 months posttransplantation. The patient showed great clinical improvement and striatal graft FDOPA uptake reached 72% of normal levels, 15 months after surgery. Histological analysis presented a massive quantity of cell survival bilaterally with fiber extension into the host putamen (Kordower et al., 1995, 1996). In 1995, a female patient who received grafts at the age of 61 was reported to have benefited greatly from the transplantation with a significant motor functional recovery during the first 12 months postsurgery. She died 19 months postgrafting. Histopathology showed over 138,000 TH+ cells in grafts without any detection of NM. The graft reinnervated 78% of the host putamen. No Lewy pathology was detected in the graft (Freeman et al., 1995). Among the above cases, promising clinical benefits were already present approximately 1-year postoperation, with positive correlation toward the TH+ neuron survival in the grafts.

In the 2000s, fetal neuron transplantation entered a new age with the emergence of double-blind clinical trials. In 2001, a double-blind transplantation study including 40 patients with graft and sham was reported (Freed et al., 2001). In the grafted group, three patients died before the end of study at 30 days, 7 months, and 3 years postoperations, in which the 3-year-old graft induced significant motor improvement for the patient, with a decreased Unified Parkinson's Disease Rating Scale (UPDRS) score and 100% restored FDOPA uptake. All three cases showed numerous amounts of TH+ cells and degenerating NM containing cells in the grafts. TH+ fiber outgrowth was observed from the grafts, particularly seen in the 3-year case, which had extensive fiber reinnervation into the whole putamen (Hagell and Brundin, 2001). In 2005, two autopsy studies were reported by Isacson et al. (Mendez et al., 2000). In both studies, the patients survived longer than 3 years with marked clinical improvements, showing 30–40% decreased UPDRS score and a significant increase in FDOPA uptake during long-term follow-up (Mendez et al., 2002, 2005; Hallett et al., 2014). In the postmortem analysis, the grafts maintained a large number of TH+ neurons with NM present. Extensive reinnervation was observed spreading out from the grafted neurons. Markedly, functional profile of the TH+ neurons was observed with dopamine transporter (DAT) positive staining (Mendez et al., 2002, 2005; Hallett et al., 2014). In these cases, with the rather "young" grafts (7 months to 3 years), no graft containing Lewy pathology had been observed.

In 2008, three groups reported their work on the survival of grafted neurons 12–16 years postsurgery (Lindvall et al., 1990,

1992, 1994; Wenning et al., 1997; Hagell et al., 1999; Hauser et al., 1999; Mendez et al., 2002; Olanow et al., 2003). All three cases showed significant GIBs at the early stages, particularly within the first 10 years, with massively shortened "off" period time and significantly reduced UPDRS scores. Postmortem studies revealed large amounts of survived grafted neurons (Mendez et al., 2005, 2008; Kordower et al., 2008a,b; Li et al., 2008; Chu and Kordower, 2010; Hallett et al., 2014). In 2011, our group reported the postmortem analysis of a patient who received fetal DA neurons 22 years prior to death. The graft did not show clear benefit to the patient, with very mild symptom relief and no change in FDOPA uptake (Lindvall et al., 1988). Only 2,700 neurons were observed in one injection tract, and almost no reinnervation was detected (Kurowska et al., 2011). In 2016, a 24-year case was reported, being the longest surviving case posttransplantation at the time. Unilateral grafts in the right putamen contributed major benefits to the patient in the first 14 years following the surgery. The patient performed well without "off" periods or L-dopa treatment for several years, yet, in the later stage before his death, the condition worsened (Lindvall et al., 1990, 1992, 1994; Wenning et al., 1997; Piccini et al., 1999). Autopsy assessments exhibited a considerable number of survived DA neurons and extensive reinnervation throughout the entire grafted putamen, which correlated to the major improvement in the FDOPA uptake test in the clinical follow-up (Li et al., 2016). In the following year, Kordower et al. (2017) reported a case with 16-year-old grafts, presenting no significant motor improvement; however, large profiles of grafted neurons were shown accompanied by extensive reinnervation into the host putamen. Interestingly, massive Lewy pathology profile was detected in grafted neurons (Olanow et al., 2003; Kordower et al., 2017). Although, survival and reinnervation were assuring in most of these long-term (more than 10 years) cases, GIBs were shown to be absent or unsustainable. Nevertheless, almost all these long-survival grafts showed Lewy pathology development in different extents in the grafted cells, suggesting a possible role of graft Lewy pathology in affecting the graft function and clinical outcomes.

Summary of clinical follow-ups and autopsy reports are shown in **Supplementary Tables 1**, **3**.

SUMMARY OF THE AUTOPSY STUDIES FROM THE GRAFTS

Grafted Neuron Survival

From the cases summarized above, we concluded four aspects to describe the survival of the grafted neurons: (1) time: graft ages ranged from 30 days (Freed et al., 2001; Hagell and Brundin, 2001) to 24 years (Li et al., 2016). TH+-grafted neurons were observed in grafts of 30 days already. Moreover, the 24-year-old case which Li et al. (2016) reported proved that transplanted neurons can survive for long period of time up to a quarter of a century; (2) number: besides the cases where the number of TH+ neurons were not reported (Mendez et al., 2008), the population of survived neurons were mostly from 12,000 (Li et al., 2008) to 330,000 (Kordower et al., 2017), showing large profiles of cell survival; (3) NM: some of the grafted neurons contained NM, with mild or no distinct TH staining, indicating degeneration of DA phenotypes; (4) not all reported autopsy cases showed TH+-grafted neurons, which were seen for example in a study reported by Henderson et al. (1991) where grafted cells in two of the patients failed to show any TH immunoreactivity (Hitchcock et al., 1994; Hagell and Brundin, 2001).

Graft Reinnervation

In order to restore the denervated DA terminals, grafts with large amounts of survived TH+ neurons needed to reinnervate the host putamen or caudate nuclei. Therefore, the autopsy results of TH profile reinnervation were crucial to evaluate the functional aspects of the grafts. Among the cases summarized above, the extent of reinnervation varied. Based on the patterns and percentage of reinnervation, we ranked them into: (1) almost no integration: often shown as very few short fiber outgrowth from the edge of the grafts, such as the postmortem case reported by Kurowska et al. (2011), where the 22-year-old grafts did not integrate into the host striatum sufficiently, with sparsely distributed fibers reaching out from the grafted neurons; (2) partial reinnervation: often presented as fiber outgrowth for several millimeters (Kordower et al., 1997), which extended to parts of the host tissue, forming a patch/matrix pattern (Kordower et al., 2008a) or organotypic shape (Chu and Kordower, 2010); (3) extensive integration: dense TH+ long processes reached out to almost the full width of the host putamen or, in some cases, reinnervating the whole putamen. For example, in the 14-year-old grafts that Mendez et al. (2008) reported, reinnervation was detected throughout the putamen, with TH fiber density comparable with non-PD subjects.

Graft Lewy Pathology

Alpha-synuclein (α-syn) aggregation, which forms the main parts of Lewy bodies (LBs), is one of the main pathological features of PD (Yates, 2019). Therefore, evaluating the Lewy pathology in grafted cells is important to understand the progress of the disease in PD patients' brain. In the cases we summarized, grafts younger than 4 years did not show any LB formation in the grafted cells (Hallett et al., 2014). Although some of the grafted cells were present with increased level of soluble cytoplasmic α-syn, no aggregates were detected (Kordower et al., 2008a). On the contrary, in most of the grafts older than 10 years, grafted neurons contained certain percentages of LBs, ranging from 1.2 to 27%. The morphology of these LBs was undistinguishable from the host nigral aggregates (Kurowska et al., 2011; Kordower et al., 2017). In some cases, the graft LBs were also thioflavin S and ubiquitin positive (Kurowska et al., 2011). Random or abundant Lewy neurites (LNs) were also present occasionally (Li et al., 2016). Nevertheless, there are still ongoing transplantation cases where the patients are still alive to the point of this review. These cases have revealed even longer graft functioning time, such as the two cases reported by Kefalopoulou et al. (2014), where motor improvements sustained for 18 years by 2014, providing a "proof-of-concept" support for the effectiveness of fetal DA neuron transplantation therapy.

Summary of clinical follow-ups and autopsy reports are shown in **Supplementary Tables 1**, **3**.

STRUCTURE AND FUNCTION INTERPLAY OF THE GRAFTED NEURONS

Postmortem studies presented us with the structural status of various DA grafts, including the survival of the grafts, the reinnervation of the grafted tissue, and the Lewy pathology development in grafted cells. How did these histological features reflect the functional outcomes of the grafts? From the summary and analysis of the autopsy cases above, we reasoned that: (1) sufficient survival and reinnervation of the grafted cells was the necessary foundation of functional grafts; (2) Lewy pathology of the grafted cells influenced the sustainability of the graft function.

Grafted Cell Survival and Reinnervation Was Necessary for the Grafts to Function

Survived DA Neuron Number

First, how sufficiently the grafted neurons survived the transplantation was the basic requirement for them to function. In preclinical experiments, the graft survival and integration appeared to be highly relevant to the motor improvements (Nakao et al., 1994). In some clinical trials, FDOPA uptake, which indicated the cell survival and fiber density, was positively correlated with the symptom relief (Hagell et al., 1999; Hauser et al., 1999). The quality of graft survival was firstly reflected by the number of cells present in the transplants. As we discussed above, cell number was mostly above 12,000 in various grafts (Li et al., 2008). It is difficult to determine what the proper number of cells would be the threshold level for a graft to function. However, the presence of very few neurons may contribute to a failed transplantation. For example, a report with graft age ranging from 18 to 40 months showed significant motor improvements in the clinical follow-ups. Postmortem analysis revealed surviving DA neurons in the graft-host interface, partially of which showed intense TH staining (Henderson et al., 1991). On the contrary, in the case Kurowska et al. (2011) reported, few grafted cells were detected in one injection tract. Clinical follow-ups revealed very mild symptom relief correlatedly. Therefore, sufficient amounts of transplanted cells defined the qualifying level for a functional graft.

Functional Profile of the Grafted Neurons

Second, the presence of functional profiles of DA neurons, such as TH, DAT (Olanow et al., 2003; Kordower et al., 2008a; Chu and Kordower, 2010), G-protein-regulated inward-rectifier potassium channel 2 (Girk2) (Mendez et al., 2000, 2002, 2005; Hallett et al., 2014), etc., were also important for the comprehensive functionality of the grafts. From the cases shown, we observed that not all the transplanted cells were TH positive, with some only containing NM. In the trial Spencer et al. (1992) reported, the cells in the 4-month-old graft contained NM; however, no TH+ cells were found (Redmond et al., 1990). Clinical follow-up showed no sign of motor improvements. In the case Kurowska et al. (2011) reported, histological study showed approximately two-thirds of the grafted neurons exhibited TH immunoreactivity (over 27% only NM positive. The long-term clinical follow-up showed transient mild improvements (Lindvall et al., 1988). Similarly, in a case Li et al. (2008) reported in 2008, a large percentage of grafted neurons were shown to be only NM positive. Modest GIBs was observed from the bilateral grafts (Li et al., 2008). Besides TH, DAT immunoreactivity was also observed in some sustainably well-functioned grafts, such as the grafts reported by Mendez et al. (2008), where after 14 years, the graft function was well maintained with UPDRS score up to 50% decrease.

Graft Reinnervation

At last, the reinnervation of the grafts into the host tissue was the key to a beneficial transplantation. As discussed above, there were different degrees of integration from the grafts. Seemingly, there existed a positive correlation between graft survival and reinnervation with the functional recovery. Thorough and extensive integration, especially the ones reinnervated the whole brain region, often resulted in beneficial grafts, such as the cases reported by Kordower et al. (2008a), Li et al. (2016), and Mendez et al. (2008), where patients showed major improvements on motor performance, significantly decreased UPDRS scores and less L-dopa dosage requirements. In all the three cases, postmortem studies revealed dense network of DA profiles extending into the host striatum, forming extensive reinnervation. On the contrary, grafts with sparsely extended TH+ fibers or just reaching out to the surrounding putamen generated mild improvements or unsustainable effects, such as the cases reported by Li et al. (2008), where long processed neurons formed dense networks with the surrounding putamen. Motor improvements appeared to be mild in one of the patients, while in the other, the originally significant GIBs started decaying 3 years postsurgery.

In summary, there existed possible positive correlation between the graft survival and reinnervation with the functionality of the grafted cells and the following clinical outcomes, which the sufficient grafted cell number, functional profile such as DAT immunoreactivity and extensive reinnervation lays out the foundation for a successful graft. However, not all the grafts survived and reinnervated effectively showed GIBs. Among the cases summarized, it is noticeable that the case reported by Kordower et al. (2017) showed no clinical recovery over all, but exhibited large numbers of DA neurons surviving and reinnervating the host tissue. In some cases, grafted cells showed promising benefits in the early stages posttransplantation, however, gradually lost their function along time (Li et al., 2016). Therefore, there may exist factors counteracting to the benefit generated by the originally healthy grafted cells, leading to the decaying of GIBs.

Grafted Cell Neuropathology Development Influenced the Sustainability of Graft Function

Lewy pathology with α-syn aggregation was found to be cellular toxic, by inducing multiple neuronal dysfunctions such as mitochondrial deficiency (Vekrellis et al., 2011; Fares et al., 2021). The development of Lewy pathology in the originally healthy grafted neurons might lead to their early degeneration, therefore causing the gradual loss of GIBs. This was suggested by the

autopsy studies of several long-term cases. For example, the 14-year case Kordower et al. (2008a) reported and the 24-year case reported by our group (Li et al., 2016) both showed significant symptom relief in at least the first 10 years postsurgery, with less or even no L-dopa treatments needed. However, in the later stages, especially after 10 years postoperation, worsening of symptoms began to appear until the GIBs completely disappeared prior to death. Coincidentally, only grafts that were older than 10 years showed LBs in the grafted cells. In both cases, LBs were found in the grafted neurons. In our 24-year case, 12% of the grafted cells contain typical LBs, in which 11% were also ubiquitin immunoreacted (Kordower et al., 2008a; Li et al., 2016). Moreover, study by Kordower et al. (2017), the 16-year-old grafts showed robust survival and extensive reinnervation, however, exhibited 10.7 and 27% LB containing grafted cells bilaterally, contributing possibly to no motor improvements and mild symptom relief in the clinical follow-ups. The decaying and absence of GIBs in these long-term cases suggested that the pathology development in grafted neurons influence the function and sustainability of the grafts. Even a viable graft could lose its function or generate no benefits at all, if heavy loads of Lewy pathology affected the transplants.

The Possible Role of Serotoninergic System

Graft-induced dyskinesia (GID) is defined as unvolunteered movements which appeared after transplantation and could not be eliminated during off-medication period (Winkler et al., 2005). In the transplantation trials, a considerable number of patients experienced GID. In the 34-patient double-blind trial by Olanow et al. (2003), 56% of patients experienced dyskinesia after withdraw of medication. Open-label trial with 14 patients that were followed up to 11 years also showed mild to moderate level of dyskinesia, which did not correlate with the FDOPA uptake (Hagell et al., 2002). Therefore, the appearance of GID was one of the complications affecting clinical outcomes.

There have been discussions about the mechanisms behind GID from a histological point of view; for example, a sustained effects of L-dopa treatments which made the patients more "dyskinetic" (Winkler et al., 2005). Barker and Kuan (2010) discussed the importance of non-dopamine system such as serotoninergic neurons in influencing the nigrostriatal performance. These neurons can convert, store, and release dopamine through nerve terminals. However, due to the lack of DAT (as seen in the postmortem results of several cases), inactivation of dopamine could not be completed (Tanaka et al., 1999). It was shown that in patients with GID, the serotoninergic neurons "hyper-innervated" the grafted striatum (Politis et al., 2010). Researches in humans showed also that serotonergic innervation promoted excessive release of dopamine in the striatum, which contributed to the formation of L-dopa-induced dyskinesia (Espay et al., 2018; Munoz et al., 2020). The accurate mechanism behind GID is still not clear. It may be more of a complicated network problem than a single mechanism induction. However, these still showed that the 5-hydroxy tryptamine (5-HT) cells may play an important role in causing GID, therefore, should be evaluated in the future anatomic studies.

LESSONS WE LEARNED FROM THE AUTOPSY STUDIES—HOW TO IMPROVE TRANSPLANTATION THERAPY

From the discussion above, we confirmed that there existed unneglectable relation between the graft anatomic features and the clinical outcomes. It was undeniable that the effects of cell replacement therapy were not yet optimal. It is necessary for us to analyze and summarize the reported cases and obtain reference on how to improve the fetal DA neuron transplantation.

Improving the Survival and Reinnervation of the Grafted Cells

Brundin et al. (2000) reported a case of five patients grafted with cell suspension pretreated with lazaroid, a lipid peroxidation inhibitor. The trial managed to reach similar level of clinical symptom relief compared with cases without lazaroid addition reported by the same center previously, however, with a decreased amount of graft tissue needed for the transplantation (Brundin et al., 2000). Mendez et al. (2000) reported cases of patients grafted with cell suspension pretreated with glia cell line-derived neurotrophic factor (GDNF). In two of the patients, PET scan showed a mean uptake increase of 107%, suggesting the improvement of cell survival. In the clinical follow-up, both patients had decreased time in "off" phase, with UPDRS score significantly reduced (Mendez et al., 2000). Moreover, in the postmortem analysis of these GDNF-pretreated grafts, the functional profiles of the grafted cells were significantly improved with DAT and Tom20-positive cells present (Mendez et al., 2005; Hallett et al., 2014). It seemed that a positive value of using neurotrophic factors in pretreatment of grafted neurons was shown. Therefore, neurotrophic factor addition may be a potential method for improving the survival and long-term health of the grafted cells. However, the effects of using biomolecules such as GDNF must be investigated more thoroughly before applying to the transplantation therapy. The mechanism of neural protection from neurotrophic factors such as GDNF is the activation of cascades included in specific cell signaling pathway, which then promotes downstream effects such as neurite outgrowth and synaptic plasticity (Airaksinen and Saarma, 2002). Researchers have performed clinical trials using neurotrophic factors for treating PD, both by directly delivering into the brain (Lang et al., 2006) or genetically overexpressing through adeno-associated virus (AAV) vectors (Marks et al., 2010). In placebo-controlled trials, no significant improvements were seen in the primary endpoints (Olanow et al., 2015). Therefore, we should be cautious about drawing conclusions on the effects of pretreating grafted cells with neurotrophic factors.

The age, viability, quantity, and quality of grafted materials defined the start of transplantation trials, therefore possessed great importance in affecting the survival of grafted neurons (Bjorklund and Lindvall, 2017). In mouse experiments, fetal

midbrain tissue obtained around 11–14 days of gestation were shown to yield a high number of TH+ neurons after transplantation, which indicated the narrow window of transplantable fetal tissue, which was the actively developing stages of A9 DA neurons, before the outgrowth of long neurites (Torres et al., 2008; Bye et al., 2012). Therefore, previous trials using fetuses at 6–9 weeks of gestational age had generally large profiles of survived neurons (Hallett et al., 2014; Li et al., 2016). Besides the age of grafted fetuses, the viability of the transplanted cells or tissues also played an important role in determining the efficacy of the grafts. In the trials summarized in **Supplementary Table 2**, both cell suspension and tissue blocks had been used in the preparation of grafted materials. It was suggested that the preparation of cell suspension may compromise the number of viable neurons for transplantation, that only 20% donor cells survived the preparation procedures (Karlsson et al., 2005). Moreover, the transplantation sites can also influence the graft survival (Redmond et al., 2008). From our summarized cases, the putamen grafts in general showed larger amounts of survived neurons and correspondingly better recovery from the patients (Mendez et al., 2005; Li et al., 2008).

Inhibiting the Development of Graft Lewy Pathology

As discussed above, one of the key factors contributing to the loss of grafted neuron function is the development of Lewy pathology in the grafted neurons. Inhibiting the formation and propagation of α-syn aggregates could be a feasible way to sustain the GIBs.

Possible Factors Contributing to Graft Lewy Pathology Development

The development of Lewy pathology in the grafted neurons was possibly influenced by various factors. Firstly, α-syn aggregation in the surrounding or projecting host brain regions may propagate to the originally healthy grafted neurons. Therefore, more severe host brain pathology may also result in a heavier loading of LBs in the grafted cells. It has been reported that α-syn can spread from cell to cell (Hansen et al., 2011; Lee et al., 2012) and from the peripheral tissue to the brain (Braak et al., 2003; Goedert et al., 2010; Holmqvist et al., 2014), inducing multiple aspects of cell dysfunction (Schulz-Schaeffer, 2010; Winslow et al., 2010; Subramaniam and Chesselet, 2013; Wong and Krainc, 2017), such as dopamine release. In the 24-year case, we reported, severe Lewy pathology were detected in multiple regions in the host brain, which resulted in 12% of the grafted cells loaded with LBs (Li et al., 2016). In a recently published work by Hoban et al. (2020), a fraction of the DA neurons transplanted into a humanized rat model, obtained by coinjection of preformed human α-syn fibrils and AAV expressing human wild-type α-syn, developed LBs, suggesting the propagation of α-syn pathology from host to graft. Secondly, the presence of neuroinflammation can contribute to the LB formation in the grafts. In a study reported by Olanow et al. (2019), grafts ranging from 18 months to 16 years were analyzed in the aspects of microglia activation and α-syn aggregation in the grafts. Microglia activation was already present in the 18-month graft, while LB formation only started to appear in 14- and 16-year-old grafts, suggesting the temporally advanced deposition of neuroinflammation compared with Lewy pathology development, which suggested that microglial activation contributed to the development of α-syn pathology in the grafts (Olanow et al., 2019).

Inhibition of α-Syn Aggregation and Propagation

Knowing the factors contributing to α-syn aggregation in grafted neurons, how can we develop pathology-targeted approaches to improve the transplantation outcomes? First, we could seek for methods targeting the accumulation and aggregation process of α-syn from the grafted neuron side. α-Syn propagation follows a "prion"-like manner, where external α-syn seeds template the endogenous overexpressed or mutated α-syn monomers to form secondary aggregation (Goedert et al., 2017; Pineda and Burre, 2017). Therefore, modulating α-syn aggregation in grafted cells may decelerate the Lewy pathology formation. As the toxic species of are mainly the aggregated forms such as oligomers and fibrils (Lashuel et al., 2013; Fares et al., 2021), conformation-specific antibodies which binds to certain species of α-syn, therefore, inhibit the aggregation initiation and progression. Several antibody treatments have been proven effective (De Genst et al., 2010), some of which have already moved on to clinical trial phase, after proven effective in preventing formation of aggregates or deattenuate the misfolded α-syn (Krishnan et al., 2014). Besides strain-specific antibodies, small molecules and chemical compounds have also been proven effective in inhibiting α-syn aggregation. For example, the 3-(1,3-benzodioxol-5-yl)-5-(3-bromophenyl)-1H-pyrazole (anle138b), a compound binding protein aggregates specifically, showed capacity of inhibiting α-syn oligomer formation both *in vitro* and *in vivo* (Wagner et al., 2013). Additionally, protein homeostasis requires the effective clearance of misfolded and aggregated forms of proteins (Vilchez et al., 2014). Therefore, inducing an enhanced protein degradation system can be considered a potential preventing approach for α-syn pathology in the graft. Elevating patient protein degradation system in general may be hard to achieve and can cause side effects, while targeting specifically grafted cells may be feasible. Many approaches can be employed to modulate the protein metabolism, for example, regionally administrating autophagy and lysosome enhancer such as rapamycin, which targets mTOR (Waldner et al., 2016; Moors et al., 2017). Our previous work also demonstrated the effect of bioactive herbal extracts, dihydromerycetin (DHM) and salvianolic acid B (SalB), in inhibiting α-syn aggregation by augmenting chaperone mediated autophagy (CMA) (Wu et al., 2019). However, one should be cautious on excessively reducing α-syn, as the protein plays important physiological roles in neuronal synaptic function. Ninety percent loss of α-syn can induce a significant neuronal toxicity as reported by Gorbatyuk et al. (2010). Native α-syn was also shown to have an immune protective role against RNA viral infections in the central nervous system (CNS) (Beatman et al., 2015). Silencing α-syn expression in the nigrostriatal system resulted in neuronal dysfunction, which sequentially triggered neuroinflammation (Benskey et al., 2018). Therefore, finding a method that can fine tune α-syn level which exhibits a sensitive response to α-syn-level alteration can be the future direction of efforts.

Possible Role of Inhibiting Inflammation

Neuroinflammation plays a crucial role in affecting α-syn aggregation in the grafted cells, as discussed above. Therefore, one could reason that the use of immunosuppression may influence the pathology development in the grafts. As the immunosuppression process may require long-term and frequent medical involvements, which could cause risk of postsurgery complications, there were researchers choosing not to apply immunosuppression. For example, in the double-blind study by Freed et al. (2001) involving 40 patients, none of the patients received immunosuppression. In the cases Lund University researchers reported, patients received long-term triple cocktail immunosuppression similar to that used in organ transplantation (Muntean and Lucan, 2013). In the double-blind trial reported by Olanow et al. (2003), the researchers picked a middle ground using cyclosporine treatments for a short period of time (6 months). The clinical outcomes of these cases failed to indicate a clear advantage of immunosuppression. However, the absence of immunosuppression may preserve a long-term effect of causing neuroinflammatory responses, which may induce malfunction of the grafted tissues. In the postmortem cases, Kordower et al. (2008a) reported from 2008 to 2009, where no immunosuppression was given, CD45-positive microglia were shown to be abundant in grafted regions (Kordower et al., 2008a; Chu and Kordower, 2010). On the contrary, the 2016 case we reported, where triple-cocktail immunosuppression was used, showed no significant activation of microglia comparing with normal subjects (Li et al., 2016). Therefore, the application of immunosuppression may present an antagonym effect toward long-term neuroinflammation and protect the grafted neurons against excessive Lewy pathology. It has been suggested that solid, non-dissociated pieces of grafting tissues, compared with cell suspension, have higher potential to trigger inflammation at the grafted sites, which may in the end result in large amounts of graft Lewy pathology and reduced graft efficacy (Kordower et al., 2008a). The remaining donor blood vessels in non-dissociated tissues, which expressed major histocompatibility complexes class I (MHC I), could trigger the immune reaction in the recipient brains (Geny et al., 1994). It was proven in non-human primate study that solid pieces of tissues induced a stronger inflammation compared with cell suspensions (Redmond et al., 2008).

Other Factors Influencing the Clinical Outcomes of Cell Replacement Therapy

Patient Selection Before Entering Transplantation Trials

Other than improving the survival and reinnervation of the grafted neurons and inhibiting their LB formation, there are certainly many other factors influencing the structure of the grafts and the consequential clinical outcomes, for example, the integrity of the host nigrostriatal pathways. Reinnervation of the grafts into the host striatum was the key to a successfully functional graft; therefore, the host nigrostriatal system should at least be partially present. In animal experiments, it has been shown that the effects of intrastriatal grafts depend on the degeneration severity of the host nigrostriatal pathways (Kirik et al., 2001). A reliable host nigrostriatal system is important for the graft to reach out and re-establish the neuronal network. The disease severity of PD patients can influence the extent of graft innervation, which raised the questions of whether patients should be selected based on the progression of their disease symptoms and even pathology severity in the brain (Piccini et al., 2005; Barker et al., 2013, 2015). Meanwhile, previous open-label cases have obtained promising results in some patients as discussed above. However, the two double-blind placebo-controlled failed to show a statistically significant difference between the grafted group and sham (Freed et al., 2001; Olanow et al., 2003). In one of the double-blind studies, it was observed that the transplantation benefited younger but not older patients (Freed et al., 2001; Ma et al., 2010). Based on these reasoning's, the TRANSEURO consortium was initiated. After summarizing discussion of main investigators involved in previous trials, it was suspected that DA neuron transplantation to earlier stage or less severe PD patients may result in possibly greater recovery post-surgery (Barker et al., 2015). The TRANSEURO program therefore selected younger and early-stage PD patients from different countries and put them into observational cohorts. Following standardized protocols of clinical follow-ups and surgery, the program has now been going on for 10 years. Besides the confirmation that earlier-stage PD patients were more suited for transplantation study, the program has also provided substantial implications and recommendations, such as an established patient-selection criteria and standard operation procedures, for future cell replacement therapy overall, including stem cell-based transplantation (Barker and Consortium, 2019).

Pluripotent Cell-Based Transplantation: New Era of Cell Replacement Therapy in PD

Fetal DA neuron transplantation had its limitation which could not be overcome by methodological improvements, such as the supply of fetal tissue and related ethical problems. Therefore, cell replacement therapy needed a new source of grafting cells to move forward and become clinically scalable. Stem cell-based transplantation therefore came to the forefront. Stem cells such as human embryonic stem cells (hESCs) and human-induced pluripotent cells (hiPSCs) are theoretically unlimited cell sources due to their capabilities to proliferate, therefore eliminating the issues regarding tissue acquiring with fetal cells (Thomson et al., 1998; Takahashi et al., 2007). Their embryonic status gives them the potential of differentiating into different types of adult somatic cell, including DA neurons and neural progenitors. Preclinical experiments have involved both hESCs and hiPSCs (Grealish et al., 2014; Kikuchi et al., 2017; Song et al., 2020; Tao et al., 2021). Compared with hESCs, which are the standards for pluripotent cells, hiPSCs induced concerns previously as their generation process included genetic modification (Li et al., 2015). Even with the optimized reprogramming protocol without any use of viruses, hiPSCs still possess genetic and epigenetic risks in the long run. Therefore, some researchers have chosen hESCs as cell sources for implantation, such as the STEM-PD program (Kirkeby et al., 2017). However, hESCs still bring with them the ethical concerns like fetal DA cells. Moreover, the necessity for immune suppression and the risk of neuroinflammation still remain.

Human-induced pluripotent cells give the possibility of performing autologous transplantation, as they can be derived from the patients' own or HLA-matched individuals' somatic cells, therefore possessing the potential for personalized therapy (Osborn et al., 2020). As we have discussed, neuroinflammation could occur prior to LB formation in grafted neurons, which may therefore facilitate the development of Lewy pathology and affect the sustainability of grafted cell function (Olanow et al., 2019). Minimizing neuroinflammation could benefit the transplantation trial by tempering the accumulation and aggregation of α-syn in grafted cells. Nevertheless, elimination of using immunosuppression could benefit the patients by decreasing the possible risks due to systematic immune system inhibition (Osborn et al., 2020). From a histological point of view, hiPSCs still face the challenges of surviving, reinnervation, and developments of Lewy pathology. As for the enhancement of survival and reinnervation, first, autologous transplantation using hiPSCs has been shown to form extensive reinnervation to the host tissue as proven by several non-human primate trials such as the proof-of-concept work published by Hallett et al. (2015), where grafted hiPSCs derived neuron successfully reinnervate and formed dense synapses with the host putamen. Without the need of immunosuppressing treatment, autologous grafting generated minimal immune responses in the CNS (Hallett et al., 2015). Second, pretreatment using neurotrophic factors such as GDNF was shown to promote DA neuron maturity through activating mitogen-activated protein kinase (MAPK) signaling pathway, therefore leading to better behavioral recovery (Gantner et al., 2020). Although it is still possible that grafted cells derived from hiPSCs will eventually go through the same pathological process as seen in fetal DA transplantation, modification and treatments of iPSCs could be performed prior to the transplantation to increase longevity of functional grafted cells. Besides the strategies to prevent α-syn aggregation as discussed above, genetic editing using CRISPR-Cas9 has been shown to resist Lewy pathology formation, where partial or complete deletion of α-syn allele in hESCs resulted in decreased formation of protein aggregates *in vitro*, upon being challenged by α-syn fibrils (Chen et al., 2019). Therefore, in a summary, autologous transplantation using hiPSCs, when optimized properly and performed under strict regulatory process, could have multiple advantages over allografts.

The gratifying thing is that clinical transplantation trials using hiPSC-derived DA neuron have already begun. In Japan, Takahashi's laboratory has performed their first transplantation trial using clinically graded hiPSC-derived neurons in late 2018 (Takahashi, 2017, 2019, 2020; Doi et al., 2020). Schweitzer et al. (2020) published their clinical follow-up of an idiopathic PD patient receiving autologous graft derived from his own fibroblasts. Without the need for any immunosuppression, the patient showed stabilized and improved clinical symptoms 2 years postsurgery, with PET suggesting the graft survival. Moreover, many groups around the world currently have ongoing or planning trials using iPSC-based transplantation. With over two decades of efforts, several groups have generated "good manufacturing practice (GMP)" level hiPSC-derived DA neurons. Researchers aiming to use iPSC-derived DA neuron for treating PD have decided to join their efforts and share obtained evidences together for maximizing what can be learnt from trials, such as the GForce-PD, formed by groups from the United States, Japan, and Europe (Barker et al., 2017).

SUMMARY

Fetal DA neuron transplantation has been a promising therapeutic method for PD and accelerated the progress of regenerative medicine in PD. In this review, we have summarized postmortem studies of fetal transplantation cases, analyzing cell survival, reinnervation, and graft pathology. We discussed the possible correlation between these histological features and the graft functional outcomes. In the end, we raised several possible factors that could be considered for improving the graft survival and function. With our review, we hope to make a comprehensive summary of the transplantation field and provide references for improving future fetal DA transplantation trials, more importantly cell replacement therapy overall, from a histological point of view.

AUTHOR CONTRIBUTIONS

J-YL and WL written and modified the manuscript. Both authors of this work have made intellectual and substantial contributions to the manuscript and approved it for publication.

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Mouse Embryonic Stem Cells Expressing GDNF Show Enhanced Dopaminergic Differentiation and Promote Behavioral Recovery After Grafting in Parkinsonian Rats

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Parkinson's disease (PD) is characterized by the progressive loss of midbrain dopaminergic neurons (DaNs) of the *substantia nigra pars compacta* and the decrease of dopamine in the brain. Grafting DaN differentiated from embryonic stem cells (ESCs) has been proposed as an alternative therapy for current pharmacological treatments. Intrastriatal grafting of such DaNs differentiated from mouse or human ESCs improves motor performance, restores DA release, and suppresses dopamine receptor supersensitivity. However, a low percentage of grafted neurons survive in the brain. Glial cell line-derived neurotrophic factor (GDNF) is a strong survival factor for DaNs. GDNF has proved to be neurotrophic for DaNs *in vitro* and *in vivo*, and induces axonal sprouting and maturation. Here, we engineered mouse ESCs to constitutively produce human GDNF, to analyze DaN differentiation and the possible neuroprotection by transgenic GDNF after toxic challenges *in vitro*, or after grafting differentiated DaNs into the striatum of Parkinsonian rats. GDNF overexpression throughout *in vitro* differentiation of mouse ESCs increases the proportion of midbrain DaNs. These transgenic cells were less sensitive than control cells to 6-hydroxydopamine *in vitro*. After grafting control or GDNF transgenic DaNs in hemi-Parkinsonian rats, we observed significant recoveries in both pharmacological and non-pharmacological behavioral tests, as well as increased striatal DA release, indicating that DaNs are functional in the brain. The graft volume, the number of surviving neurons, the number of DaNs present in the striatum, and the proportion of DaNs in the grafts were significantly higher in rats transplanted with GDNF-expressing cells, when compared to control cells. Interestingly, no morphological alterations in the brain of rats were found after grafting of GDNF-expressing cells. This approach is novel, because previous works have use co-grafting of DaNs with other cell types that express GDNF, or viral transduction in the host tissue before or after grafting of DaNs. In conclusion, GDNF production by mouse ESCs contributes

to enhanced midbrain differentiation and permits a higher number of surviving DaNs after a 6-hydroxydopamine challenge *in vitro*, as well as post-grafting in the lesioned striatum. These GDNF-expressing ESCs can be useful to improve neuronal survival after transplantation.

Keywords: glial cell line-derived neurotrophic factor, Parkinson's disease, dopaminergic neurons, 6-hydroxydopamine, dopamine release

INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disorder surpassed only by Alzheimer's disease. It is characterized by the progressive loss of dopaminergic neurons (DaNs) in the *substantia nigra pars compacta* (SNpc) and the concomitant denervation of the dorsal striatum (Poewe et al., 2017). Loss of this highly specialized type of neurons results in severe depletion of dopamine (DA) levels in caudate-putamen and is responsible for the pathophysiological features in the disease such as tremor at rest, bradykinesia, and rigidity (Dauer and Przedborski, 2003). Current pharmacological treatments either involves the administration of dopamine receptors agonists or favors DA biosynthesis by supplementation of its precursor L-3,4-dihydroxyphenylalanine (L-DOPA), which is effective in some cases to improve motor symptoms but with associated side effects such as dyskinesias and on-off states. Unfortunately, such treatments lose effectiveness in the long term, since dopaminergic neurodegeneration continues (Lloyd et al., 1975; Hefti et al., 1981; Cenci, 2014). In fact, these treatments do not fully restore the normal release of DA in the brain and affect other systems that require this neurotransmitter, causing side effects (Cenci, 2014).

Survival of DaNs is a key aspect in the installment and progression of PD. A group of neurotrophic factors and neurotrophins such as Glial cell line-derived neurotrophic factor (GDNF), Neuturin (NRTN), and Brain-derived Neurotrophic Factor (BDNF) increase survival of DaNs (Nasrolahi et al., 2018). There are reports that neurotrophic molecules change in the brain of PD patients, specifically, a decrease in BDNF in the *substantia nigra* (Mogi et al., 1999) and a moderate increase of GDNF in the putamen (Mogi et al., 2001). Clinical trials tested the idea of promoting DA neuron survival in the brain by direct infusion of GDNF (Whone et al., 2019a,b) and NRTN (Olanow et al., 2015); however, the overall conclusion is that these neurotrophic molecules were not enough to promote recovery from the classical symptoms and presented important side effects. Experimentally, the use of proteins that increase neuronal viability suggests that neurotrophic factors can prevent neurodegeneration after 6-hydroxydopamine (6-OHDA) (Kearns and Gash, 1995; Åkerud et al., 2001).

Cell therapy with DA neurons from human fetal mesencephalic tissue has been tested in PD patients with variable outcomes (Lindvall et al., 1992; Kordower et al., 1995; Freed et al., 2001; Olanow et al., 2003). Since the availability of fetal tissue is limited, alternative sources to obtain DA neurons have been devised. Embryonic stem cells (ESCs) respond to developmental signaling to differentiate into DA neurons. Such differentiated progeny provides behavioral recovery after grafting in the striatum of experimentally lesioned animals (Chung et al., 2002; Kim et al., 2002; Rodríguez-Gómez et al., 2007; Kriks et al., 2011; Tripathy et al., 2013). However, transplanted DaNs have shown suboptimal survival and poor innervation of the host brain. Additionally, survival, growth, and function of transplanted DaNs are reduced in aged rats due to less trophic support from the host brain (Collier et al., 1999). To circumvent the lack of trophic support, researchers have grafted neural stem cells that express GDNF together with ventral mesencephalic neurons in the brain of lesioned rats and improve recovery and survival (Deng et al., 2013). Recently, a similar strategy expressing GDNF through viral transduction, either before or after transplantation, showed that delayed GDNF increased survival and innervation of grafted DA cells (Gantner et al., 2020).

Cultured cells offer the possibility of effective manipulation *in vitro* prior to transplantation. Here, we engineered mouse ESCs (mESCs) to constitutively produce human GDNF, to analyze DA differentiation and the possible neuroprotection by transgenic GDNF after toxic challenges *in vitro* or after grafting into the striatum of rats lesioned with 6-OHDA.

MATERIALS AND METHODS

Cell Lines

R1 mESCs were transduced with lentivirus containing the empty vector, which are designated control (CTRL-ESC), or with hGDNF (GDNF-ESC), as previously reported (Cortés et al., 2016). Cells were genotyped by end-point PCR for hGDNF detection using a 30-cycle program at 59°C. Primers (in 5′–3′) for transgenic hGDNF: Forward (Fwd), AACAAATGGCAGTGCTTCCT and Reverse (Rev), AGCCGCTGCAGTACCTAAAA; for GAPDH, used as a positive control: Fwd, ATCACCATCTTCCAGGAGCG and Rev, CCTGCTTCACCACCTTCTTG.

In vitro Differentiation of ESCs to DaNs

We used CTRL-ESC and GDNF-ESC, which have been proved to produce spinal motor neurons (Cortés et al., 2016). The dopaminergic differentiation protocol, which consists of five stages, was performed as previously reported (Lee et al., 2000). Briefly, CTRL-ESC and GDNF-ESC were cultured on gelatin-coated tissue cultured plates in the presence of Leukemia Inhibitory Factor in Knockout DMEM medium (Gibco, Carlsbad, CA, United States) supplemented with 15% ESC cell-tested fetal bovine serum (FBS). For the second stage, cells were dissociated with 0.05% Trypsin solution (Gibco) and plated

onto bacterial dishes to induce formation of floating embryoid bodies (EBs) for 4 days. EBs were recovered and seeded onto adherent tissue culture plates in serum-free Insulin-Transferrin-Selenite medium (ITS) supplemented with 5 μg/ml Fibronectin (Invitrogen, Carlsbad, CA, United States) for 6 days, when migration of cells out of the EBs was evident. Expansion of Nestin-positive cells (stage 4) was initiated by seeding the single-cell suspension in glass coverslips precoated with 15 μg/ml poly-L-ornithine (Sigma, St Louis, MO, United States) and 1 μg/ml Fibronectin in N2 medium (Gibco) containing 10 ng/ml of basic fibroblast growth factor (bFGF), 100 ng/ml fibroblast growth factor 8 (FGF-8), and 100 ng/ml of human Sonic Hedgehog (R&D Systems, Minneapolis, MN, United States) during 4–6 days. Terminal differentiation at stage 5 was induced by growth factors removal and feeding with N2 medium with 200 μM ascorbic acid for 6–8 days. This protocol induces preferential differentiation of unmodified mouse ESC to DaNs (20–30% of the differentiated neurons), although lower proportions of serotoninergic (5–10%) are also present (Lee et al., 2000; Kim et al., 2002). Interestingly, DaNs, serotoninergic, and GABAergic neurons are present after grafting in Parkinsonian rats: DaNs represent 20% of surviving neurons, whereas serotonin$^+$ cells are close to 5%, and cells positive to the GABAergic marker GAD67 are less than 2%, after 4–8 weeks (Kim et al., 2002).

Enzyme Linked ImmunoSorbent Assay for GDNF

To measure secreted GDNF, 24 h-conditioned media were obtained from CTRL-ESC and GDNF-ESC during all five stages of DA differentiation. GDNF Emax Immunoassay system kit was purchased from Promega and was used following the manufacturer's booklet.

6-OHDA Toxicity Assay *in vitro*

DA neurons were incubated with 200 μM 6-OHDA (Sigma) for 2 h and incubated at 37°C. Medium was removed and fresh medium was added. Twenty-four hours later, cells were fixed by evaluation by immunocytochemistry. This neurotoxin is taken up by the DA Transporter and therefore does not affect other neurons present in the differentiated cultures.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde, permeabilized, and blocked with 10% normal goat serum and 0.3% Triton X-100 in PBS. Primary antibodies were incubated overnight in 10% normal goat serum in PBS and were applied as follows: mouse anti-OCT3/4, 1:1,000 (BD Biosciences Pharmingen, United States); rabbit anti-SOX2, 1:500 (R&D); mouse anti-β TUBULIN III (TUJ1), 1:1,000 (Covance); rabbit anti-Tyrosine Hydroxylase (TH) antibody, 1:1,000 (Pel-Freez, United States); rabbit anti-FOXA2, 1:500 (Millipore); rabbit anti-LMX1B, 1:200 (Abcam); rabbit anti-Serotonin (Sigma). Appropriate fluorescently labeled secondary antibodies were used alone or in combination, and nuclear detection with Hoechst 33258 1 μg/ml (Sigma, United States) is presented in some cases. For quantification of dopaminergic differentiation efficiency, the number of TH-positive somata were divided by the total number of neurons labeled by the TUJ1 antibodies and multiplied by 100. To further asses DaNs differentiation, we performed an analysis of area with co-localization of TUJ1 and TH signals with the FIJI program, using the JACoP package to obtain the percentage of colocalizing signals, as previously reported (Bolte and Cordelières, 2006; Dunn et al., 2011). Area was set as pixel intensity. The TUJ1 signal was established as the total area and the TH signal was set as the variable. Thus, the % of TH area was normalized using total TUJ1 area. For serotonin neurons, the percentage of neurons labeled with antibodies were normalized by the total number of nuclei. Fluorescent signals were detected using a Nikon A1R HD25 confocal microscope to detect Alexa 488, Alexa 568, Alexa 647, and Hoechst 33258 by exciting with different lasers.

RT-qPCR

RNA was isolated using RNeasy Mini Kit (QIAGEN) and manufacturer's instructions were followed. Complementary DNA (cDNA) was synthesized by SuperScript II Reverse Transcriptase (Thermo Fisher Scientific) from 2 μg of total RNA and used for RT-PCR amplification (Taq DNA Polymerase, Thermo Fisher Scientific). Amplification was performed with QuantiFAst SYBR Green PCR Master Mix (QIAGEN) and a Real-Time PCR Detection System (CFX96; Bio-Rad). Gene expression levels were determined using the following primers: *Oct4*: Fwd, TTG GGC TAG AGA AGG ATG TGG TT; Rev, GGA AAA GGG ACT GAG TAG AGT GTG G. For *Sox2*: Fwd, GCA CAT GAA CGG CTG GAG CAA CG; Rev, TGC TGC GAG TAG GAC ATG CTG TAG G. For *Th*: Fwd, CCA CTG GAG GCT GTG GTA TT; Rev, CCG GGT CTC TAA GTG GTG AA. For *Foxa2*: Fwd, CAG AAA AAG GCC TGA GGT G; Rev, CAG CAT ACT TTA ACT CGC TG. For *Lmx1b*: For *Gapdh*: Fwd, CAT CAC TGC CAC CCA GAA GAC TG; Rev, ATG CCA GTG AGC TTC CCG TTC AG.

Animals

Female Wistar rats weighing 220–250 g were housed at 12 h light-dark cycle with food and water *ad libitum* at 22 ± 2°C. Surgical procedures were approved by the Instituto de Fisiología Celular-UNAM Animal Care and Use Committee (Protocols IV-68-15 and IV-152-19) and compiled local (NOM-062-ZOO-1999) and international guidelines.

6-OHDA Lesion, Apomorphine-Induced Rotational Test, and Non-pharmacological Behavioral Test

6-OHDA (Sigma) lesions were performed as previously described (Ungerstedt, 1968; Díaz-Martínez et al., 2013). This animal model shows behavioral and biochemical improvements after DaNs grafting (Kim et al., 2002; Rodríguez-Gómez et al., 2007) and therefore, we focused our analysis to this type of neurons. Rats were placed in an airtight anesthesia chamber supplied with 3% sevoflurane (Abbot Laboratories) in 95% O_2–5% CO_2 gas mixture. To minimize stress, rats were minimally handled and maintained with inhaled anesthetic (0.5–1.5% sevoflurane). Next, rats were injected with 12 μg of 6-OHDA in the left medial

forebrain bundle with the following stereotactic coordinates in relation to bregma: antero-posterior (AP), −1.0 mm; lateral (L), 1.5 mm; and dorso-ventral (DV), −8.0 mm. The injection was performed over 4 min using a 30G needle. Thereafter, rats were allowed to recover for 30 days before a pharmacological test was performed. Apomorphine (1 mg/kg)-induced rotations were quantified over 60 min, and animals were classified as lesioned when they had more than 360 contralateral turns per hour. To assess motor behavior in the absence of pharmacological stimulation, we conducted the following test:

Adjusting step test: This evaluation was performed as described before (Kim et al., 2002; Díaz-Martínez et al., 2013). The rats were held with one hand, holding and lifting the hindlimbs and securing one of the forelimbs, and then moved in a forward direction over a flat surface for 0.9 m. Each forelimb was independently evaluated by counting the number of steps onto the surface while moving the animal. The value of the lesioned forelimb was normalized with the number of steps registered for the non-lesion value for each group. This evaluation was made blinded to the experimental treatment of animals.

Grafting of DA Neurons Differentiated From CTRL-ESC and GDNF-ESC

All animals were immunosuppressed daily with cyclosporine A (10 mg/kg; GelPharma) starting 24 h before grafting. Hemiparkinsonian rats were grafted in the dorsal striatum with CTRL-ESC or GDNF-ESC derived TH-positive neurons differentiated as described before. DaNs from both cell lines were dissociated at days 2–3 of stage 5 and resuspended at a density of 0.5×10^6 viable cells in 3 μl. Cells were grafted into the lesioned dorsolateral striatum ($n = 7$ for CTRL-ESC and GDNF-ESC) with the following coordinates: AP, 0.0 mm; L, 3.0 mm; DV, four deposits separated by 0.5 mm (from −5.5 mm to −4.0 mm). In sham animals ($n = 8$), 3 μl of N2 medium was injected with the same coordinates. Each animal was evaluated every 2 weeks for apomorphine-induced rotations, for 14 weeks.

DA Quantification

Microdialysis experiments were performed at 14 weeks after grafting to measure DA release in the dorsal striatal region of animals grafted in the striatum with CTRL-ESC or GDNF-ESC and with sham surgery as described (Rodríguez-Gómez et al., 2007; Díaz-Martínez et al., 2013). *In vitro* recovery experiments with the dialysis membranes had values of 15–20% for DA and 3–4, dihydroxyphenylacetic acid (DOPAC). Probes were perfused with artificial cerebrospinal fluid at 2 μl per min for 1 h for tissue stabilization and fractions were collected every 12 min. Chemical depolarization with isosmotic medium (100 mM potassium chloride) was induced through the probe in fraction 4, to stimulate DA release; reversal of DA uptake was performed in fraction 9 by perfusion with 30 μM amphetamine. Monoamines were stabilized by adding 0.1 N perchloric acid, 0.02% EDTA, and 1% ethanol to the collection tubes. Quantification was made by HPLC, using a reversed-phase column (dC18, 3 μm; 2.1 mm × 50 mm; Atlantis, Waters) coupled to a precolumn (Nova-Pack Waters) with a mobile phase solution containing EDTA, 0.054 mM; citric acid, 50 mM; and octasulfonic acid, 0.1 mM dissolved in milli-Q water and mixed with methanol in a proportion of 97:3, respectively (pH = 2.9; flow rate = 0.35 ml/min). DA and DOPAC detection were performed by a single-channel electrochemical amperometric detector (Waters model 2465) at 450 mV with a temperature of 30°C, and quantified by peak height measurements against standard solutions. Resulting concentrations were not corrected by probe recovery.

Stereological Counting

Animals were perfused with 0.9% saline solution and then with 4% paraformaldehyde in PBS. Brains were recovered and cryoprotected subsequently with 10%, 20%, and 30% sucrose. Slices of 30 μm were obtained in a cryostat and immunostained with anti-TH and anti-Tuj1 antibodies for DA neuron counting. Every 5th section was quantified, and the total number of DA neurons was calculated by multiplying the number of TH^+ neurons per slice by the number of slices that contained grafted cells (Coggeshall and Lekan, 1996; Kim et al., 2002). We calculated the mean of the area covered by $TUJ1^+$ cells per mm^3 for at least 12 slices. To calculate the percentage of TH in each slice, the number of DaNs was normalized by the number of $TUJ1^+$ cells.

Statistical Analysis

Results are expressed as mean ± SEM. All experiments were performed at least in duplicate. Statistical differences were identified by one-way ANOVA or *t*-student test. Multiple comparisons were made by two-way ANOVA followed by *post hoc* Dunn's test. GraphPad Prism version 8.0 was used for calculation of probability values.

RESULTS

GDNF Expression in ESCs During Dopaminergic Differentiation

In order to address if transduction with the control vector or with the cDNA of hGDNF affects the pluripotent state of the generated lines of mESCs, cells were cultured for 48 h and we performed immunostaining for the pluripotency-associated markers OCT4 and SOX2. CTRL-ESC and GDNF-ESC presented similar proportions of double-positive cells (**Figure 1A**). NANOG was also analyzed with similar observations (data no shown). In agreement, no differences are detected in the expression of mRNA for *Oct4, Sox2,* and *Nanog* when comparing the parental R1 mESC with CTRL-ESC and GDNF-ESC by RT-qPCR (**Figure 1B**).

After establishing that GDNF-ESC shows normal expression of pluripotency-related markers, we induced its differentiation to DaNs using a method previously described (Lee et al., 2000), which is a stepwise procedure involving five stages (**Figure 2A**). To investigate if GNDF expression and secretion can be detected during this differentiation protocol, conditioned media was collected from CTRL- and GDNF-ESC to quantify GDNF by enzyme linked immunosorbent assay (ELISA).

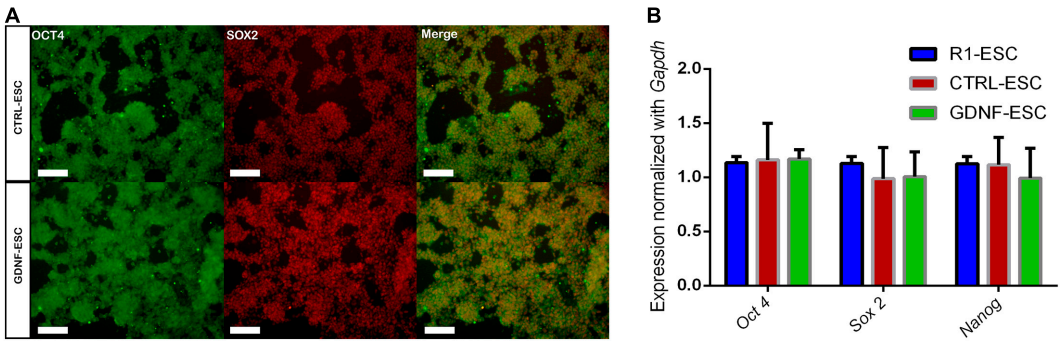

FIGURE 1 | Pluripotency markers are unaffected in CTRL- and GDNF-ESC before differentiation. **(A)** Immunostaining for the pluripotency markers OCT4 and SOX2 in CTRL-ESC and GDNF-ESC, showing a high proportion of double-positive cells in both cell lines. Scale bar = 100 μm. Representative of five independent experiments. **(B)** RT-qPCR for the transcription factors *Oct4, Sox2,* and *Nanog* in both cell lines, normalized by *Gapdh* expression. R1 ESCs were used as a reference. No significant differences were observed; data from four independent experiments.

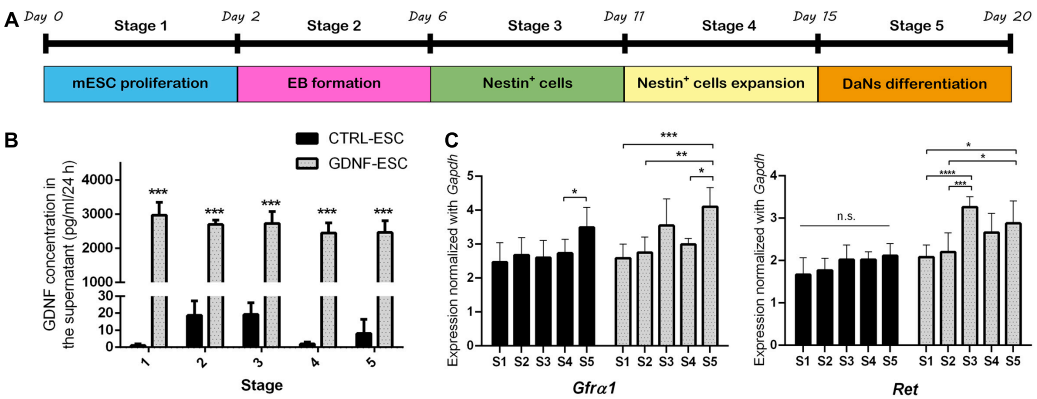

FIGURE 2 | GDNF is released to the medium and its receptors are expressed during different stages of dopaminergic differentiation. **(A)** Scheme of five stages of the dopaminergic neuron differentiation protocol. **(B)** ELISA to quantify GDNF in 24 h-conditioned media from CTRL-ESC or GDNF-ESC during the five stages of differentiation. ***$p < 0.001$ vs. CTRL-ESC. **(C)** RT-qPCR showing mRNA levels of the GDNF receptors *Gfrα1* and *Ret* during differentiation of CTRL-ESC and GDNF-ESC. Values were normalized with *Gapdh* expression. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; and ****$p < 0.0001$. $n = 4$ independent experiments.

GDNF concentration was significantly higher in GDNF-ESC when compared to CTRL-ESC at all stages of differentiation (**Figure 2B**). GDNF must bind to GFRα1 and Ret co-receptors to exert its effects. Therefore, we measured the expression of *Gfrα1* and *Ret* in the five stages of dopaminergic differentiation. No significant differences were observed between GDNF-ESC and CTRL-ESC (**Figure 2C**), but some differences were observed between stages, particularly at stage 5, where the expression of these transcripts was significantly increased compared to all the other stages in both cell lines. Together, these results indicate that GDNF is secreted and their receptors are expressed, suggesting that differentiation of ESCs to DaNs might be modified.

GDNF-ESC Produce More DaNs That Are Resistant to 6-OHDA

DaNs were differentiated from CTRL-ESC and GDNF-ESC. At stage 2, we observed that GDNF-ESC EBs were larger than those generated from CTRL-ESC. Quantification of the diameter showed significant differences (0.98 ± 0.02 μm, CTRL; 1.03 ± 0.03 μm, GDNF; $p < 0.0001$), suggesting that GDNF is increasing proliferation during EB formation, which is consistent with previous findings (Cortés et al., 2016). The final differentiation phase is stage 5, where neurons expressing β-III Tubulin, detected by the TUJ1 antibody, start to express TH, the limiting enzyme in DA production. Postmitotic neurons were evaluated for this marker: a significantly higher number of TH-positive cells were observed in GDNF-ESC compared with CTRL-ESC (**Figures 3A,B**). The increased dopaminergic differentiation in GDNF-ESC was confirmed by measuring the % of TH area, relative to TUJ1 labeling: CTRL-ESC = 21.6 ± 2.9%; GDNF-ESC = 36.8 ± 1.2%; significant difference after *t*-test for $n = 6$ ($p < 0.01$). Furthermore, expression of other markers of DaNs from the ventral mesencephalic area, like FOXA2, LMX1B, GIRK2, and Calbindin, were also significantly increased in GNDF-ESC (**Figures 3C,D**). This was complemented by detection of the dopaminergic cell markers *Th, Foxa2,* and *Lmx1a* through RT-qPCR (**Figure 3E**), showing that GDNF overexpression is increasing efficiency for differentiation into

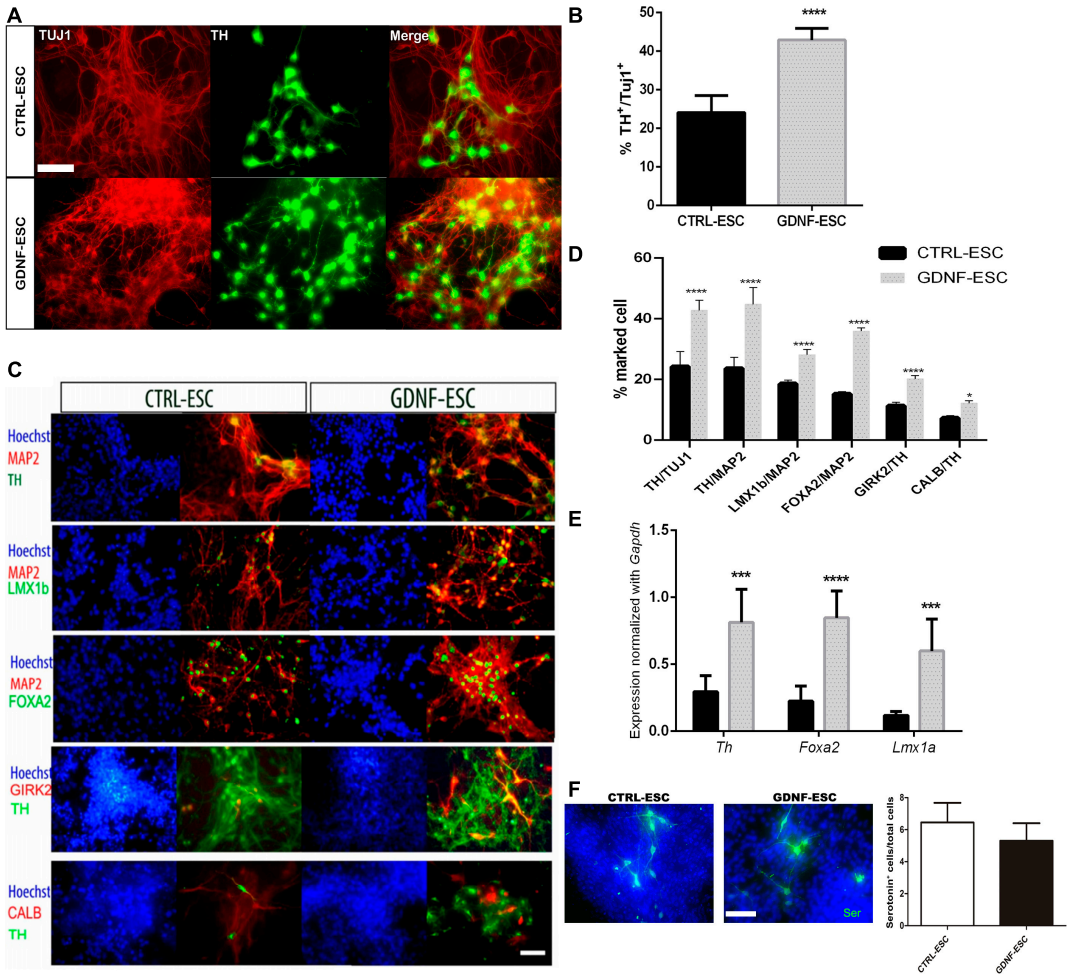

FIGURE 3 | GDNF increases differentiation to DaNs. **(A)** Immunocytochemistry for TH and TUJ1 at 20 days of differentiation of GDNF-ESC or CTRL-ESC. **(B)** Quantification of DaNs, calculated as [total number of TH^+ somas/total number of $TUJ1^+$ somas] x100, to obtain percentage in cultures of 20 days showing a significant increase in TH-positive neurons in GDNF-ESC, compared to control. **(C)** Immunocytochemistry for ventral mesencephalic DaNs markers LMX1B, FOXA2, the A9 marker GIRK2 and Calbindin (CALB), combined with TH or Microtubule Associated Protein 2 (MAP2). **(D)** Quantification of these markers shows an increase that reached statistical significance for GDNF-ESC when quantified in four independent experiments. **(E)** Normalized mRNA expression of genes relevant for dopaminergic differentiation at day 20. Significant increases in expression levels in GDNF-ESC are observed, compared with CTRL-ESC. Data was normalized by *Gapdh* expression. **(F)** Immunocytochemistry and quantification of the percentage of Serotonin$^+$ cells, related to the number of total cells, detected at day 20 of differentiation. Result from four independent experiments. $^*p < 0.05$; $^{***}p < 0.001$; and $^{****}p < 0.0001$. Scale bar = 100 μm.

DaNs. This protocol also produces a low proportion of serotoninergic neurons, so we decide to quantify this neuronal population. The number of serotonin-positive neurons was not modified in GNDF-ESC when compared to CTRL-ESC (**Figure 3F**).

To test if transgenic GDNF has a neurotrophic effect on DaNs after a toxic challenge with the dopaminergic neurotoxin 6-OHDA, differentiated cultures of 20 days were treated with vehicle or 200 μM of 6-OHDA for 2 h, and assessed after 24 h. In CTRL-ESC, 6-OHDA caused a significant decrease: only 27% of TH-positive cells survived, relative to vehicle incubation. In contrast, GDNF-ESC cells were significantly less sensitive to this toxin, since 48% of TH+, relative to vehicle, were present (**Figures 4A,B**). The neuroprotection in DaNs differentiated from GDNF-ESC was also observed by the significant increase in the % of TH^+ area, normalized by TUJ1: CTRL-ESC with 6-OHDA = 9.0 ± 1.5%; GDNF-ESC plus 6-OHDA = 54.2 ± 6.6%; $n = 4$, $p < 0.0001$. Together, these results show that transgenic GDNF increases the proportion of ventral mesencephalic neurons and confer resistance to 6-OHDA cytotoxic challenge.

Transplantation of ESC-Derived DaNs in the Striatum Promotes Behavioral Recovery and DA Release in Hemiparkinsonian Rats

The functionality of *in vitro* differentiated DaNs can be assessed by intrastriatal transplantation in rats lesioned with 6-OHDA (**Figure 5A**). Animals were injected with this

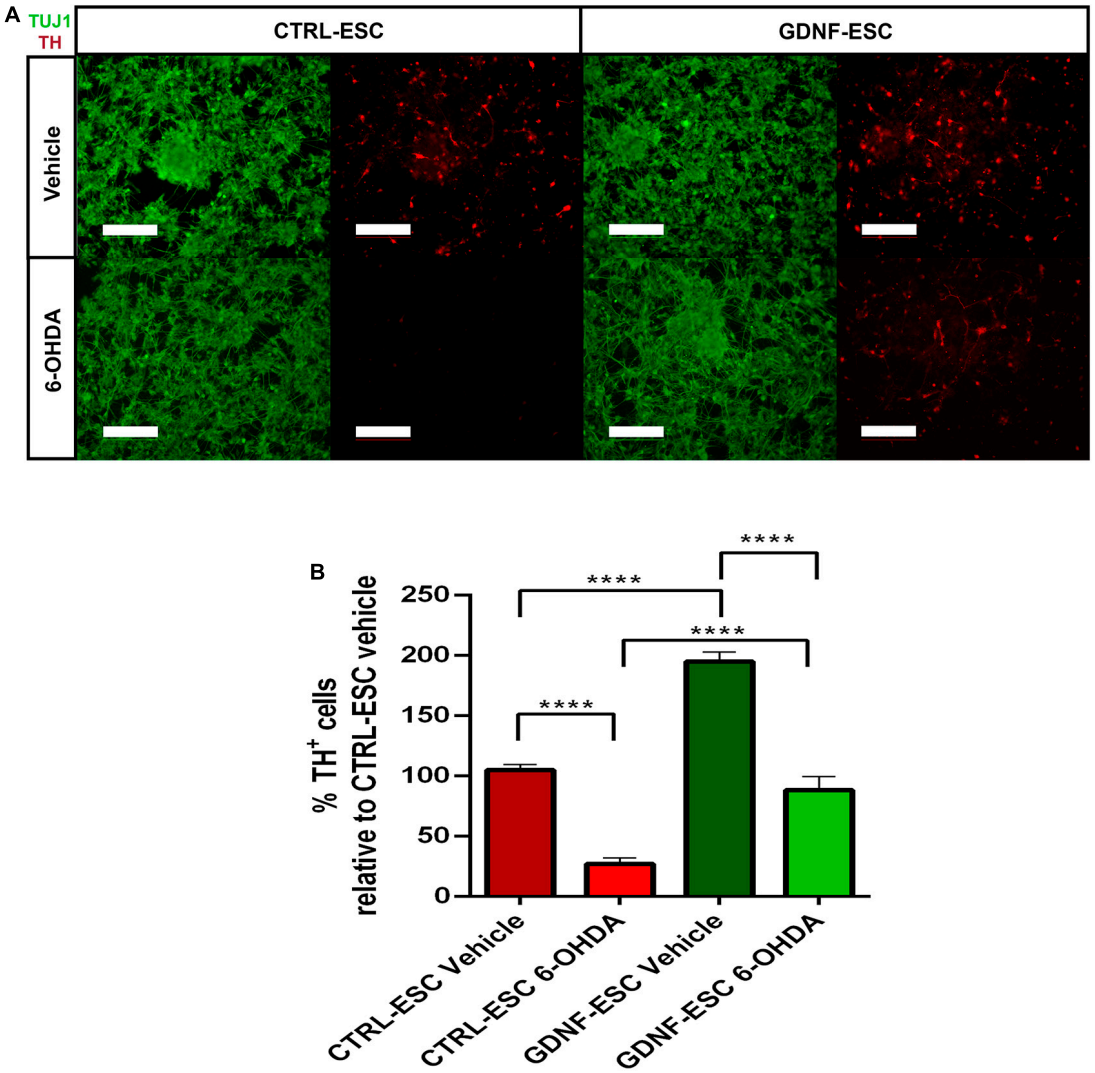

FIGURE 4 | Over-expression of GDNF promotes survival of TH-positive neurons after a challenge with 6-OHDA *in vitro*. **(A)** Immunocytochemistry for TUJ1 and TH after vehicle incubation for 24 h (upper panels) and after 6-OHDA treatment (bottom panels). Note that GDNF-ESC has a higher proportion of DaNs than CTRL-ESC in vehicle and after 6-OHDA. **(B)** Quantification of the % of TH-positive cells in experiments at 20 days of differentiation, related to CTRL-ESC treated with vehicle, which was considered 100%. Results are from four independent experiments. ****$p < 0.0001$. Scale bar = 100 μm.

dopaminergic neurotoxin in the right medial forebrain bundle. A successful lesion was considered when animals presented an apomorphine-induced rotational asymmetry of >360 rotations per hour. This animal model (Ungerstedt and Arbuthnott, 1970) has been widely used to test behavioral alterations and, indirectly, restoration of DA levels. Hemiparkinsonian rats were grafted with 5×10^5 cells from differentiating cultures (day 18) of CTRL-ESC or GDNF-ESC. We measured the behavioral recovery of Parkinsonian rats using the rotational and stepping tests. For rats receiving sham transplantation, the behavioral alterations caused by the lesion were present for 14 weeks post-surgery. Grafts of CTRL-ESC and GDNF-ESC showed a significant recovery in apomorphine-induced rotations (**Figure 5B**) lasting 14 weeks post-transplantation. A similar correction of forelimb asymmetry in the stepping test was found in rats grafted with CTRL-ESC and GDNF-ESC (**Figure 5C**).

To correlate behavioral improvements with DA release in grafted rats, extracellular DA levels were measured in the striatum by microdialysis following two pharmacological challenges applied through the cannula: (a) depolarization induced by K^+ ions (isosmotic medium with 100 mM KCl) and (b) administration of 30 μM amphetamine, which causes the release of DA *via* the DA Transporter. As a control, the non-lesioned striatum of all animals showed a significant potassium-stimulated DA release, compared with basal levels, and a significant DA accumulation after amphetamine application through the dialysis membrane. Intrastriatal grafting of DaNs from CTRL-ESC and GDNF-ESC causes depolarization- and amphetamine-induced DA release in the striatum *in vivo* to a similar extent,

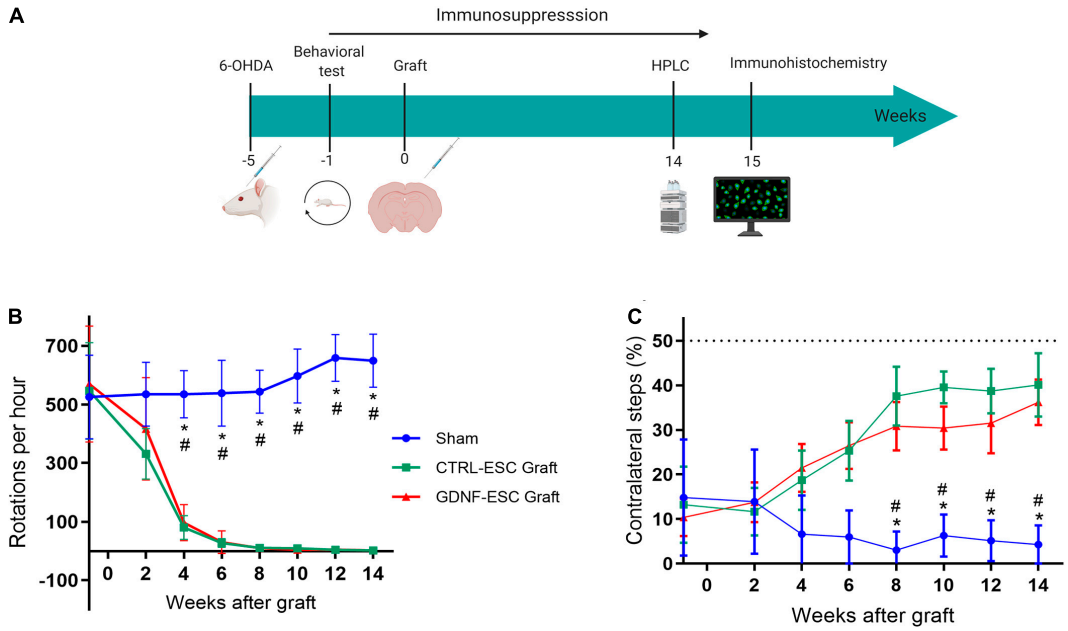

FIGURE 5 | Behavioral evaluation of Hemiparkinsonian rats after grafting. **(A)** Scheme of the protocol for *in vivo* experiments. **(B)** Apomorphine-induced rotations in animals receiving sham, CTRL-ESC, or GDNF-ESC treatments and followed 14 weeks post-grafting. Statistical differences were observed between sham and both types of grafts. The initial values are pre-grafting. **(C)** Grafting of CTRL-ESC and GDNF-ESC caused a significant recovery of forelimb asymmetry in the stepping test, when compared to the sham group. The dotted line represents forelimb use in a non-lesioned animal. $^{*}p < 0.05$ vs. CTRL-ESC; $^{\#}p < 0.05$ vs. GDNF-ESC. The number of animals is eight for the sham group and seven for the grafted groups.

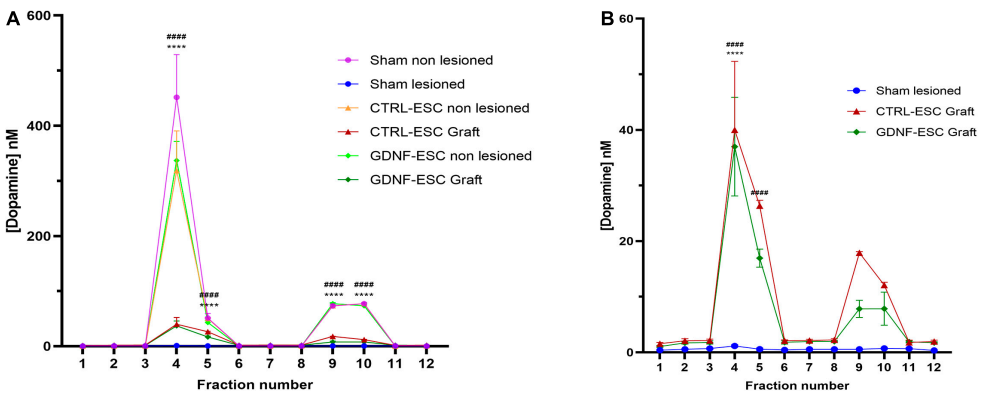

FIGURE 6 | Quantification of DA release *in vivo* in lesioned animals grafted with CTRL-ESC or GDNF-ESC at 14 weeks post-grafting. **(A)** DA concentrations measured by microdialysis and HPLC detection in basal conditions (fractions 1–3), after 100 mM KCl isosmotic medium (fraction 4), and 30 μM amphetamine (fraction 9) in the lesioned and non-lesioned sides of sham and grafted animals. **(B)** Amplification of DA levels shown in panel **(A)** for lesioned sides in grafted CTRL-ESC, GDNF-ESC, and sham groups; $n = 5$; $^{****}p < 0.0001$ vs. fraction 3 or fraction 8 in CTRL-ESC and $^{\#\#\#\#}p < 0.0001$ vs. fraction 3 or 8 in GDNF-ESC.

in agreement with behavioral data. In contrast, the lesioned side from sham rats did not present significant DA increases (**Figures 6A,B**).

Distribution and Engraftment of GDNF-ESC and CTRL-ESC Following Transplantation

To test if GDNF is capable of promoting survival, we analyzed the number of DaNs in the striatum of grafted animals. Both CTRL-ESC and GNDF-ESC showed engraftment and survival of TH^+ cells in the striatum of transplanted animals, 14 weeks after grafting. Both types of grafts presented TH^+ processes into the striatum. In the non-lesioned sides of all groups, TH^+ innervation from the DaNs of the substantia nigra were found, as expected; this innervation was absent in the lesioned side in the sham animals. Interestingly, GDNF-ESC graft size was significantly increased compared to CTRL-ESC (**Figures 7A,B**) and the number of total $TUJ1^+$ was increased, too (**Figures 7C,D**). The number of DaNs was higher in

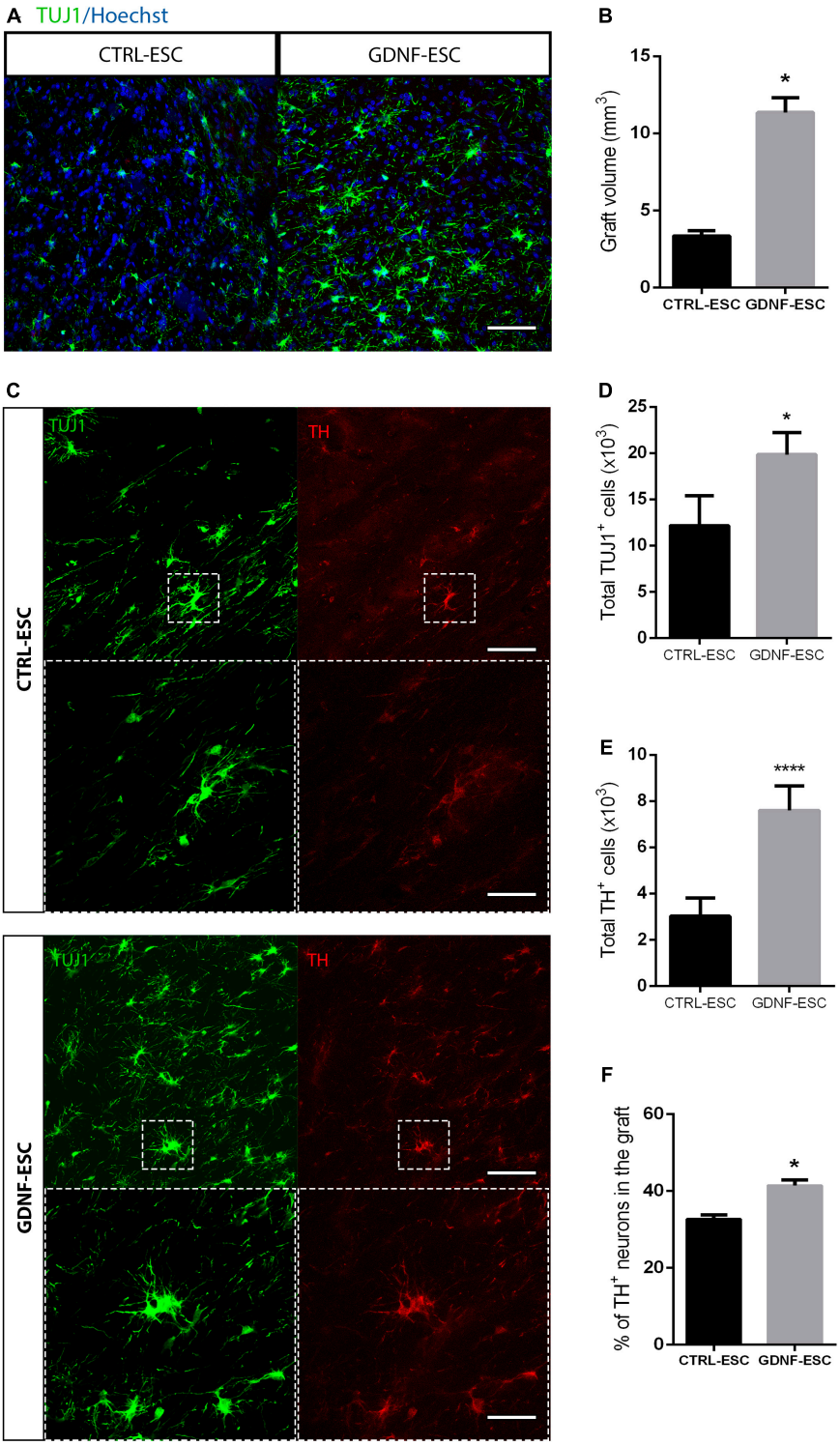

FIGURE 7 | Grafting of GDNF-ESC-derived DaNs into the striatum of lesioned animals results in higher numbers of DaNs, compared to CTRL-ESC. **(A)** Immunostaining for TUJ1 in the striatum of grafted animals at 15 weeks post-graft. Scale bar = 100 mm. **(B)** Quantification of graft volume measured after TUJ1 staining in 12 slices for both grafted groups. **(C)** Immunohistochemistry for TUJ1 and TH in the striatum of grafted animals. GDNF-ESC grafts have increased numbers of TH^+ cells. Scale bar = 100 mm. The bottom panels show the amplification of the doted square. Scale bar = 35 mm. **(D)** Quantification of total $TUJ1^+$ cells in CTRL-ESC and GDNF-ESC grafts. **(E)** Quantification of total TH^+ cells in grafts with CTRL-ESC or GDNF-ESC. **(F)** Quantification of the percentage of TH-positive neurons in the striatum of grafted animals, calculated as follows: [total number of TH^+ somas/total number of $TUJ1^+$ somas] ×100. Results from 7 CTRL-ESC and 7 GDNF-ESC grafted animals. *$p < 0.05$ and ****$p < 0.0001$.

animals grafted with GDNF-ESC (**Figures 7C,E**). Furthermore, the percentage of cells expressing the dopaminergic marker TH in grafts of GDNF-ESC-derived DaNs was significantly higher compared with DaNs from CTRL-ESC (**Figures 7C,F**), indicating that GDNF can promote DaNs survival after grafting. This result was further confirmed by measuring the % of TH area in both groups: CTRL-ESC = 16.2 ± 0.8% vs. GDNF-ESC = 22.7 ± 1.0 ($p < 0.0001$ after comparison with t-test, $n = 7$).

DISCUSSION

We describe a positive effect of continuous GDNF secretion on midbrain dopaminergic differentiation and also an autocrine neurotrophic effect when transgenic DaNs are exposed to 6-OHDA. GDNF-ESC-derived DaNs were grafted in Parkinsonian rats and induced behavioral recovery as well as dopamine release in the striatum, similar to CTRL-ESC-derived DaNs, but with increased numbers of surviving DaNs, indicating that the continuous release of GDNF does not cause undesired effects and promotes DaNs survival after grafting.

In the current study, we have characterized mESCs that constitutively express and release GDNF, a strong neurotrophic factor that promotes survival and maturation of DaNs (Lin et al., 1993; Sauer et al., 1995; Åkerud et al., 2001). This GDNF-secreting cell line differentiated more efficiently to DaNs compared to CTRL-ESC. Although these ESCs express receptors for this neurotrophic factor, in our experiments, pluripotency-associated factors such as Oct4, Sox2, and Nanog were not altered, suggesting that cells remain pluripotent. It is well known that GDNF is a strong inducer of proliferation and differentiation of enteric nervous system and parasympathetic ganglia precursors *in vivo* (Enomoto et al., 2000; Rossi et al., 2000; Airaksinen and Saarma, 2002; Gianino, 2003; Cortés et al., 2017); however, its role in early differentiation of ESCs to DaNs *in vitro* is still unclear. Interestingly, during EB formation the size of these aggregates increased significantly in GDNF-ESC; furthermore, the number of Nestin$^+$ cells migrating away from the EBs, during the first 2 days of stage 3, was significantly increased in ESC-GDNF (data not shown), suggesting that GDNF influence the proliferation and migration of neural precursors. This is consistent with previous work reporting that human recombinant GNDF can increase the proliferation rate, the size of EBs, and spinal motor neuron differentiation form pluripotent stem cells (Lamas et al., 2014; Cortés et al., 2016). The cell population that is responsive to GDNF in EBs, and the mechanisms triggered by this trophic factor, remain as an open question, requiring further investigations. Interestingly, the effect of recombinant GDNF on control ESC differentiated to motor neurons was abolished by the addition of anti-GDNF antibodies. The conditions of exogenous addition of recombinant GDNF would be mimicked by the addition of conditioned medium from GDNF-ESC to differentiating CTRL-ESC to test if an increase of DaNs is observed, which would be an interesting follow-up experiment.

Most of the protocols for the differentiation of pluripotent stem cells to dopaminergic lineages include GNDF as part of the supplementation of the medium, especially at late stages to increase the survival of TH$^+$ cells (Lee et al., 2000; Kriks et al., 2011; Kirkeby et al., 2017). In this work cells expressed GDNF during all the differentiation protocols and we observed an increase in the number of TH$^+$ cells vs. CTRL-ESC. Moreover, other mesencephalic markers were increased, such as Lmx1b, Foxa2. In the midbrain there are two dopaminergic populations: neurons from ventral tegmental area (VTA) express Calbindin and those from the SNpc are positive for Girk2, which is a G-protein-regulated inward-rectifier potassium channel (Inanobe et al., 1999; Neuhoff et al., 2002; Roeper, 2013). We assessed the proportion of Calbindin- and GIRK2-positive cells and found significant increases in both dopaminergic cell types. As previously reported, GDNF can provide survival signals to different niches of neurons such as sympathetic, enteric, motor, and other catecholaminergic neurons such as noradrenergic (Lin et al., 1993; Coulpier and Ibáñez, 2004; Pascual et al., 2008; Cortés et al., 2016; Ito and Enomoto, 2016; Enterría-Morales et al., 2020b,a). Interestingly, the proportion of differentiated serotonin neurons was unchanged in GDNF-ESC. This suggests that GDNF might increase the number of DaNs derived from ESC by promoting cell survival but also by having an effect as an inductor of dopaminergic differentiation.

DaNs differentiated from GDNF-ESC were more resistant to the cytotoxic challenge with 6-OHDA *in vitro*, as expected given the well-described neuroprotective effect of GDNF (Lin et al., 1993; Sauer et al., 1995; Kramer and Liss, 2015; Meka et al., 2015). However, this effect has only been observed when exogenous GDNF is added to the medium (Meyer et al., 2001) or when DaNs are co-cultured with cells that secrete GDNF (Åkerud et al., 2001), but not when the same cell population is producing GDNF. This type of system could generate an autocrine effect in differentiated DaNs, leading to increased cell survival *in vitro* or *in vivo*, especially after grafting in animal models of PD (Zurn et al., 2001). In this manner, additional allogenic transplants or viral methods for delivery of GDNF, such as AAV2 systems, can be avoided (Kells et al., 2012; Tenenbaum and Humbert-Claude, 2017). Recent works have shown that co-grafting of DaNs with systems that in a constitutively (Gantner et al., 2020) or regulated way (Perez-Bouza et al., 2017; Widmer, 2018) secretes GDNF, can promote behavioral recovery in animal models of PD. However, as mentioned before, none of these systems express the GDNF by the grafted cells and require another cell type to express this neurotrophic factor, such as mesenchymal stem cells, myoblasts, or by direct infusion of GDNF (Yurek, 1998; Wyse et al., 2014).

The loss of striatal dopamine results in motor dysfunction, including resting tremor, muscular rigidity, bradykinesia, and postural instability in PD patients (Dauer and Przedborski, 2003). Currently, pharmacologic DA supplementation is the most used strategy to reduce the symptoms of PD. Transplantation of DaNs has widely been used in laboratory animal models and in several clinical trials (Kordower et al., 1995; Piccini et al., 1999; Kordower et al., 2017). The most popular procedures have involved ectopic transplantation of healthy DaNs into the striatum, but this technique has proven unsatisfactory in clinical trials for multiple reasons, one of them the number of survival neurons after the graft (Björklund and Lindvall, 2000; Brundin et al., 2010). For

this reason, new strategies have emerged focusing on preventing the progressive loss of neurons at early stages of the disease using neurotrophic factors (Axelsen and Woldbye, 2018; Nasrolahi et al., 2018; Gouel et al., 2019; Wang et al., 2020). However, clinical trials of GDNF have shown that this therapy has serious limitations such as intraputaminal delivery and the poor capacity of diffusion, added to the fact that there was no motor improvement in the patients (Whone et al., 2019a,b). Thus, in this work, we used both cell replacement and GDNF delivery to test enhanced survival of grafted DaNs in Hemiparkinsonian rats. It is well known that injection of 6-OHDA results in a severe and acute loss of SNpc DaNs and causes behavioral deficits measured by apomorphine-induced rotations (Ungerstedt, 1968; Ungerstedt and Arbuthnott, 1970). Our study shows that grafts of ESC-derived DaNs that express constitutively GNDF can promote behavioral recovery in animals that were previously lesioned with 6-OHDA in both pharmacological and non-pharmacological tests. Interestingly, no difference in recovery time was observed between control cell and GDNF-expressing cells. As previously reported, consistent behavioral recovery has been reported after grafting of ESC-derived DaNs in the dorsal striatum (Kim et al., 2002; Kriks et al., 2011; Díaz-Martínez et al., 2013; Kirkeby et al., 2017; Gantner et al., 2020) 4 weeks after transplantation of mouse ESC-derived DaNs, and 12 weeks using human ESC-derived DaNs. Interestingly, continuous GDNF presence in the graft did not result in uncontrolled growth since the aspect of grafts for both cell types studied here is similar, although the volume was increased in GDNF-ESC.

GDNF is a strong regulator of excitability in DaNs (Yang et al., 2001) and it has been demonstrated that human DaNs differentiated from ESCs are capable of regulating DA synthesis when GDNF is delivered and released at multiple times (Gantner et al., 2020). Grafts of DaNs differentiated from mESCs or human ESCs can increase the levels of DA in the striatum of transplanted animals without GDNF over-expression (Piccini et al., 1999; Rodríguez-Gómez et al., 2007; Díaz-Martínez et al., 2013). Dopamine levels in the lesioned striatum of grafted animals were higher in comparison with sham animals, but no difference between CTRL-ESC and GDNF-ESC was observed. This is in contrast with previous reports that demonstrate that GDNF can increase dopamine levels *via* the regulation of *TH* gene expression in a *Ret*-dependent response in human neuroblastoma cell lines (Xiao et al., 2002) and in grafts of human ESC-derived DaNs into an environment of GDNF overexpression (Gantner et al., 2020). However, in other studies, sustained GDNF expression induces down-regulation of DA levels, as well as of DAT activity, without altering *TH* mRNA levels as a compensatory mechanism and, thus, reduces dopaminergic function (Georgievska et al., 2002; Yang et al., 2009; Barroso-Chinea et al., 2016; Chtarto et al., 2016; Tenenbaum and Humbert-Claude, 2017). For these reasons, GDNF concentration and expression should ideally be regulated in future studies to avoid unwanted compensatory mechanisms and prevent the downregulation of DA release in grafts of ESC-derived DaNs.

We demonstrate that the sustained expression of GDNF increases the number of TH-positive neurons after grafting of ESC-derived DaNs. Unfortunately, this increased number did not improve the time of recovery nor increased DA release in the brain, showing that sustained expression of GDNF does not alter the beneficial effects of grafted DaNs. It has been proposed that integration and survival in the host tissue of DaNs are key features to improve functional recovery (Brundin et al., 2000; Karlsson et al., 2000); however, our results show that both grafted groups had similar recovery in pharmacological and no pharmacological tests, suggesting that the number of DaNs in the grafts of CTRL-ESC were sufficient to induce behavioral and neurochemical improvements. Recent works have proposed that cell survival induced by GDNF is not necessarily the main key to improve motor symptoms, but increased fiber growth or axonal sprouting (Clavreul et al., 2006; Grealish et al., 2014; Perez-Bouza et al., 2017). Furthermore, it seems that the time in which GDNF is present relative to grafting determines how efficient the axonal sprouting and the improvement in non-pharmacological tests will be (Gantner et al., 2020). This could be associated to the "candy-store" effect (Santosa et al., 2013; Marquardt et al., 2015) where the axons only grow to the major concentration of GDNF.

In summary, we provide evidence for the following: (1) Transgenic GDNF does not affect pluripotency of mESCs; (2) The positive effects of sustained GDNF release on dopaminergic differentiation *in vitro*; (3) GDNF-ESC are less sensitive to the toxic action of 6-OHDA; (4) After grafting, both CTRL- and GDNF-ESC induced behavioral recovery and DA release in the brain of lesioned animals; (5) GDNF in genetically modified mESC-derived DaNs increased the number of surviving DaNs in the brains of a rodent model of PD. Although CTRL-ESC can promote behavioral recovery in lesioned animals after grafting, the number of TH-positive neurons is lower than in GDNF-ESC grafts. This difference in the number of TH^+ cells has no effect in pharmacological and non-pharmacological tests or neither elevating DA release measured by the HPLC. Our findings suggest that a better strategy to deliver GDNF in grafts should be considered, such as a regulable expression system activated by tetracycline, which proved to be effective in stem cells (Marquardt et al., 2015; Das et al., 2016; Guo et al., 2017; Bara et al., 2018; Ge et al., 2018).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Instituto de Fisiología Celular-UNAM Animal Care and Use Committee (Protocols IV-68-15 and IV-152-19) and compiled local (NOM-062-ZOO-1999) and international guidelines.

AUTHOR CONTRIBUTIONS

RL-R: conception and design, collection and assembly of data, analysis and interpretation, manuscript writing, and

final approval of manuscript. DC, AL-O, KS, FC, LR, and EE: collection and assembly of data and final approval of manuscript. IV: financial support, data analysis and interpretation, manuscript writing, and final approval of manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Stem Cell Therapies for Progressive Multiple Sclerosis

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Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system characterized by demyelination and axonal degeneration. MS patients typically present with a relapsing-remitting (RR) disease course, manifesting as sporadic attacks of neurological symptoms including ataxia, fatigue, and sensory impairment. While there are several effective disease-modifying therapies able to address the inflammatory relapses associated with RRMS, most patients will inevitably advance to a progressive disease course marked by a gradual and irreversible accrual of disabilities. Therapeutic intervention in progressive MS (PMS) suffers from a lack of well-characterized biological targets and, hence, a dearth of successful drugs. The few medications approved for the treatment of PMS are typically limited in their efficacy to *active* forms of the disease, have little impact on slowing degeneration, and fail to promote repair. In looking to address these unmet needs, the multifactorial therapeutic benefits of stem cell therapies are particularly compelling. Ostensibly providing neurotrophic support, immunomodulation and cell replacement, stem cell transplantation holds substantial promise in combatting the complex pathology of chronic neuroinflammation. Herein, we explore the current state of preclinical and clinical evidence supporting the use of stem cells in treating PMS and we discuss prospective hurdles impeding their translation into revolutionary regenerative medicines.

Keywords: progressive multiple sclerosis, neural stem cell, regenerative neuroimmunology, mesenchymal stem cell, stem cell therapy, clinical trial

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INTRODUCTION

Multiple sclerosis (MS) is a chronic neuroinflammatory condition that affects over 2 million people worldwide (Stenager, 2019). The disease typically manifests in a relapsing-remitting (RR) form marked by sporadic attacks of neurological dysfunction (i.e., clinical relapses) followed by a (full or partial) functional recovery. While advances in the development of immunomodulatory disease-modifying therapies (DMTs) have had a substantial impact on the severity and frequency of relapses (Derfuss et al., 2020), within 30 years of diagnosis, two-thirds of RRMS patients will ultimately transition into the debilitating secondary progressive (SP) phase of the disease (Scalfari et al., 2014). During this phase, patients experience a gradual and ongoing accumulation of disability despite a lack of clinically evident relapses (Confavreux and Vukusic, 2014). In addition, 10–15% of MS

patients present with a progressive form of the disease from the outset, a condition known as primary progressive (PP) MS.

Both forms of progressive MS (PMS) represent unmet clinical needs, as no available therapy is capable of arresting and repairing central nervous system (CNS) damage once progression ensues. Therefore, PMS therapeutic options (beyond conventional DMTs) should be devised to address the core drivers of this process to reduce chronic CNS compartmentalized neuroinflammation, enhance remyelination, and promote neural plasticity/regeneration.

In this sense, an extensive body of preclinical data supports the capacity of stem cell therapies to modulate the deleterious host immune responses and to facilitate neuroprotection in the CNS, which may be key to treat PMS.

UNDERSTANDING THE PATHOPHYSIOLOGY OF PMS

The current success of DMTs mostly stems from their action on the adaptive immune system, a key driver of disease pathogenesis in the RR phase. Here, DMTs work to prevent acute inflammatory insults by limiting infiltration of activated T cells, B cells, and macrophages into the CNS where they contribute to the initial demyelinating insult that eventually leads to axonal loss and neurological disability (Mallucci et al., 2015). However, with increasing age and the subsequent transition of patients into the progressive phase of the disease, conventional DMTs no longer have a clear rationale of use nor provide obvious clinical benefits (Cunniffe et al., 2021). In fact, the progressive form of MS differs from RRMS in that it is a distinct neurodegenerative process shaped by persistent inflammation behind a *closed* blood-brain barrier (BBB) involving mostly activated microglia/macrophages, and only partially T and B cells.

Understanding the pathological correlates of disease that contribute to the transition from RRMS to PMS and identifying dysfunctional mechanisms in PMS that lead to a persistent inflammatory disease state in the CNS is pivotal to identify new therapeutic strategies. Key mechanisms are discussed in the following sections and summarized graphically in **Figure 1**.

The Role of Lymphocytes

The activation of lymphocytes and their infiltration into the CNS, which is typical of the RR forms of MS, is significantly decreased in PMS (Frischer et al., 2009). In early progressive disease, infiltration of lymphocytes (T and B cells) is compartmentalized at the leptomeninges and blood vessels of the CNS, beyond an intact BBB (Hochmeister et al., 2006). The extent of T cell infiltrate found within the meninges is directly correlated with the degree of axonal loss in the normal appearing white matter (NAWM) (Androdias et al., 2010; Lassmann, 2018). This implies that T cells, through the secretion of inflammatory factors, may contribute to CNS inflammation and damage. B cells have also been found to play a significant role in PMS by accumulating in the meninges and creating *de novo* structures called *ectopic follicles* (Serafini et al., 2004; Magliozzi et al., 2013). Here, B cells produce antibodies, secrete cytokines, and present antigens which further contribute to the persistence of inflammation (Aloisi and Pujol-Borrell, 2006). In fact, PMS patients presenting with follicle-like structures have a higher rate of disability and show a faster disease progression, associated with an increased lesion burden (Howell et al., 2011). This is thought to be at least partially the result of the extent and severity of meningeal inflammation in the formation of subpial cortical lesions (Howell et al., 2011; Choi et al., 2012). However, not all PMS patients present with inflammatory follicles, which suggests the presence of factors other than CNS B cells contributing to the persistent inflammation observed behind the intact BBB (Magliozzi et al., 2007). A number of studies have identified the presence of meningeal follicles only in SPMS and absent in PPMS (Magliozzi et al., 2007; Choi et al., 2012), yet a few others have identified follicular structures in PPMS cases associated with a rapid disease progression (Kutzelnigg et al., 2005; Haider et al., 2016; Cencioni et al., 2021).

The Role of Mononuclear Phagocytes

In PMS, persistent tissue injury is associated with the activation of mononuclear phagocytes (MPs), consisting of both the CNS tissue resident microglia and the infiltrating monocytes that differentiate into macrophages (Lassmann et al., 2012). Interestingly, activation of MPs is found in many other neurodegenerative diseases without the associated pathological changes observed in MS (such as demyelination), suggesting that a specific mechanism may be required in MS to induce disease progression.

MPs in demyelinating lesions produce reactive oxygen species (ROS), reactive nitrogen species (RNS), and secrete pro-inflammatory cytokines and chemokines, including interleukin (IL)-1ß, IL-6, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ, leading to oligodendrocyte and neuronal cell death. MPs are found in *smoldering* lesions, which are lesions unique to PMS patients that slowly expand due to clusters of activated MPs at the edge of these lesions (Reich et al., 2018). The continued activation and spreading of MPs in *smoldering* lesions contributes to further disease progression by increasing the size of the lesion and associated axonal damage (Kutzelnigg et al., 2005). Activated MPs, identified through their expression of CD68, are also found in the NAWM and normal appearing gray matter of patients with PMS. The number of activated MPs within the NAWM correlates with the extent of axonal injury, as measured by axonal swelling and degeneration, suggesting the neurodegenerative impact of diffuse MP inflammation (De Groot et al., 2001; Kutzelnigg et al., 2005; Absinta et al., 2019).

The Role of Oligodendrocytes, Astrocytes and Neurons

Failure of the damaged CNS to regenerate and remyelinate is currently under intense study as the primary reason behind the transition from RRMS to PMS, since the presence of demyelinated axons in post-mortem neural tissue is highly prevalent during this stage of the disease (Franklin and Ffrench-Constant, 2008).

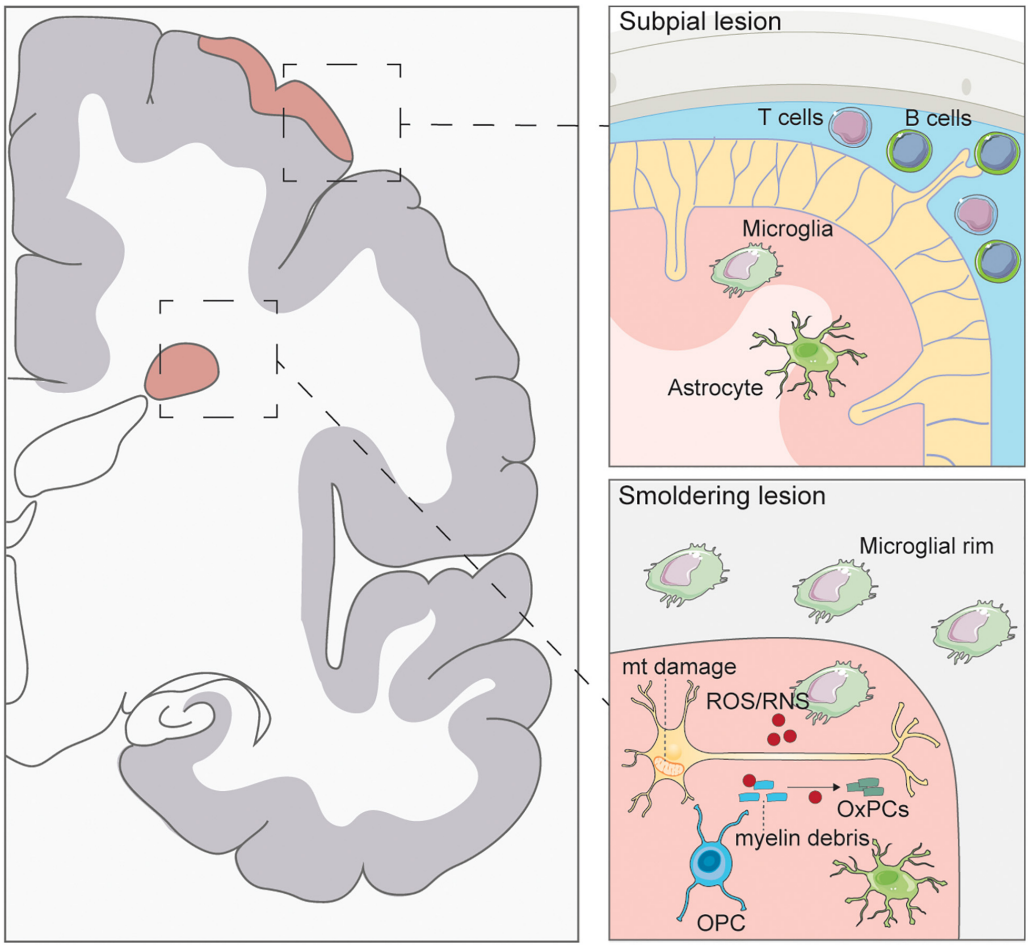

FIGURE 1 | Pathology of progressive multiple sclerosis. Subpial lesions are typically found in PMS which are characterized by lymphocyte accumulation in the meninges. Activated T and B cells can secrete inflammatory cytokines causing microglia and astrocyte activation and ensuing demyelination of the cortex. Smoldering lesions are characterized by the degeneration of demyelinated neurons and surrounding microglial rim. Demyelinated axons have been found to have mitochondrial (mt) damage caused by ROS/RNS secretion from activated microglia. ROS/RNS can also oxidize myelin debris to generate oxidized phosphatidylcholines (OxPCs), which are toxic to neurons. Few oligodendrocyte progenitor cells (OPCs) are seen in these lesions, with no remyelination.

Findings from pre-clinical rodent models of MS have suggested that oligodendrocyte progenitor cells (OPCs) are functionally capable of remyelinating demyelinated axons (Franklin and Ffrench-Constant, 2017). However, recent findings from human studies have determined that the presence of myelinating oligodendrocytes in shadow plaques match the biological age of the patient. This suggests that myelinating oligodendrocytes within the plaque arise from the rearrangement of pre-existing oligodendrocytes rather than from newly generated OPCs (Yeung et al., 2019). Further work in humans using single cell RNA sequencing has revealed a depletion of OPCs in the NAWM of the PMS brain, implicating mature oligodendrocytes in early remyelination. However, significant differences in the transcriptome of mature oligodendrocyte populations were identified, including a decrease in intermediate oligodendrocytes and a skewed distribution of mature oligodendrocytes, suggesting a decreased regenerative potential (Jäkel et al., 2019). There remains significant debate as to whether OPCs are capable of remyelination in the human disease, if they are impeded by external and intrinsic factors, or if remyelination is primarily initiated by mature oligodendrocytes.

Astrocytes have been implicated in perpetuating CNS damage through secretion of inflammatory molecules, such as TNF-α and ROS that lead to both oligodendrocyte and neuronal cell death (Yi et al., 2019). In fact, the inhibition of astrocyte reactivity during experimental autoimmune encephalomyelitis (EAE), a mouse model of MS, ameliorates disease activity, suggesting a key role in neuroinflammation (Mayo et al., 2014). In chronic MS lesions, astrocytes maintain a hypertrophic response and form a glial scar in order to prevent spread of tissue destruction (Holley et al., 2003). However, the astrocytic scar can inhibit both remyelination and axonal regeneration, for example via the secretion of fibroblast growth factor-2 which promotes OPC proliferation but prevents differentiation (Goddard et al., 1999; Thümmler et al., 2019). Astrocytes have also been implicated in the production of hyaluronan, a glycosaminoglycan that

accumulates in MS lesions, which can interact with CD44, a receptor found on neural stem cells (NSCs) (Pluchino et al., 2005), OPCs, astrocytes, and T cells (Sherman et al., 2002). The activation of CD44 on T cells induces proliferation and a cytokine response (Baaten et al., 2010), while treatment of OPCs with hyaluronan prevents their maturation into oligodendrocytes (Back et al., 2005).

Astrocytes play a key role in perpetuating inflammation in the MS CNS through the recruitment and activation of immune cells. The passage of leukocytes through the BBB is facilitated through increased expression of adhesion molecules such as vascular adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1 on astrocytes. The expression of VCAM-1 on astrocytes is necessary for the entry and retention of T cells in the CNS parenchyma of EAE animals, as well as ensuing neurological disease (Gimenez et al., 2004). Further, the release of pro-inflammatory factors by astrocytes at the BBB such as IL-1ß, TNF-α, and chemokines C-C motif ligand 3 (CCL3) and C-X-C motif ligand 12 (CXCL12), attracts peripheral immune cells and increases permeability, thus allowing for their passage into the CNS (Minagar and Alexander, 2003; Calderon et al., 2006). Lastly, astrocytes affect the phenotype of T cells and microglial/macrophage activity in the CNS. Cytokines secreted by astrocytes promote the polarization of T cells and microglia/macrophages into pro-inflammatory states (Th1/Th17 and M1-like, respectively) (Saikali et al., 2010; Toft-Hansen et al., 2011; Zhou et al., 2011).

Neuronal damage is the key driver of brain atrophy, the prominent pathological feature of PMS (Bermel and Bakshi, 2006). Secondary, immune-mediated damage to neurons by peripheral lymphocytes is more common in the relapsing stage of the disease (Dutta and Trapp, 2011). On the other hand, primary neuronal damage is the key mechanism of damage in the progressive phase of the disease. Persistent demyelination of the axon – especially that driven by inflammatory factors such as ROS and cytokines – also renders the neuron more vulnerable to damage.

In PMS, neurons show impaired mitochondrial activity as evidenced by the decreased density of mitochondrial complexes I and III, resulting in a deficiency in the ability to generate energy to sustain normal cellular function (Friese et al., 2014). Mitochondrial DNA (mtDNA) deletions in genes that code for catalytic complexes' subunits necessary for oxidative phosphorylation have been identified in PMS (Campbell et al., 2011), and analysis of common mtDNA sequence variations in MS populations identified a specific haplotype associated with an elevated risk of incurring mtDNA deletions in PMS (Tranah et al., 2015). The cumulative effect of mitochondrial abnormalities in neurons leads to the increased vulnerability of axons to external damaging stimuli contributing to their eventual degeneration. Interestingly, both brain atrophy and the number of transected axons correlate with the degree of inflammation in PMS lesions, suggesting a link between immune cell activation and neuronal damage (Frischer et al., 2009).

In this sense, ROS and RNS generated from both subsets of MPs may be the key drivers affecting mitochondrial functionality in neurons, thus leading to a highly destructive environment permissive to continued neuronal death.

The Role of Cell Metabolism and Oxidative Stress

Metabolic signatures, such as differences in glycerophospholipids, have emerged as important readouts of PMS pathology and may aid in the diagnosis and the understanding of disease progression (Stoessel et al., 2018).

Several metabolites, or breakdown and intermediate products of cellular metabolism, are known to play important roles in regulating the inflammatory activity of immune and nervous system cells (Rothhammer et al., 2016). Succinate, an intermediate metabolite of the tricarboxylic acid cycle (TCA), increases and accumulates in the cerebrospinal fluid (CSF) of animals with EAE. Here, extracellular succinate exacerbates the pro-inflammatory activity of MPs, which further increased tissue damage (Peruzzotti-Jametti et al., 2018).

Clinically, analysis of CSF samples from MS patients has identified increased levels of lactate and altered levels of glucose in patients with PMS (Lynch et al., 1993; Simone et al., 1996). Follow-up untargeted mass spectrometry-based metabolomic studies have also identified alterations in lipid and energy metabolism in the CSF that are associated with a more severe disease progression in PMS patients. This may reflect the overall decrease in lipid content associated with increased demyelination (Villoslada et al., 2017).

Oxidized phosphatidylcholines (OxPCs) are another possible driver of neurodegeneration in PMS lesions. OxPCs are generated when myelin debris encounters free radicals leading to oxidized myelin. Prominent depositions of OxPCs are found in white matter lesions in PMS brains and associated with activated microglia. An *in vitro* study found OxPCs to be toxic to cultured neurons and oligodendroctyes (Dong et al., 2021). The combination of mitochondrial dysfunction, inflammatory metabolites (such as succinate) and inflammatory ROS/RNS may further generate OxPCs, thus contributing to the progression of MS pathology.

The Role of Aging

Age is a prominent factor in the transition to PMS (Sanai et al., 2016; Scalfari et al., 2016). Several of the hallmarks that are associated with the aging process, including telomere attrition, mitochondrial dysfunction, cellular senescence, and stem cell exhaustion, have been linked to PMS (López-Otín et al., 2013; Oost et al., 2018). Cellular senescence is a biological process that can be induced via stress or replicative fatigue, triggering a variety of intrinsic cell processes including cell cycle arrest and secretion of a pro-inflammatory senescence associated secretory phenotype, which can have deleterious effects on the tissue microenvironment (Coppe et al., 2010).

Senescent progenitor cells are identified in lesions of PMS patients and associated with increased secretion of the pro-inflammatory alarmin high mobility group box 1 protein, which impairs OPC differentiation *in vitro* (Nicaise et al., 2019). Recent evidence further demonstrates that, with age, rodent OPCs are

incapable of differentiating into mature oligodendrocytes which impairs their potential to regenerate lesioned areas (Neumann et al., 2019). Changes in OPCs due to aging and inflammation, such as DNA damage and reduced mitochondrial function, may account for the loss of remyelination in PMS.

Immunosenescence of macrophages and T and B cells could be another potential mechanism behind the decreased regenerative potential of the diseased CNS in PMS. Indeed, senescence of the immune compartment is understood to play a role in driving systemic aging in solid organs, including in immune privileged organs such as the brain (Yousefzadeh et al., 2021). Here, extracellular cholesterol generated from myelin breakdown overwhelms the phagocytic capability of aged macrophages and drastically impairs their ability to clear areas of inhibitory and damaging cellular debris (Cantuti-Castelvetri et al., 2018). T cell senescence has been observed in with RRMS and PMS patients, correlating perturbation of the immune system with age (Thewissen et al., 2005). Additionally, shorter telomeres, associated with senescent cells, have been identified in leukocytes of patients with PMS, with shorter telomere length correlated with increasing disability (Krysko et al., 2019).

Overall, a significant effect of aging is the increase in secreted inflammatory factors from senescent cells which can promote MS progression.

CURRENT AND EMERGING THERAPIES FOR PMS

DMTs and Their Role in PMS

In RRMS DMTs are largely used to reduce and prevent relapses. IFN-ß and glatiramer acetate (GA) are immunomodulatory agents commonly recommended as first-line DMTs for RRMS. Other first-line DMTs for RRMS include teriflunomide and dimethyl fumarate (DMF), which are typically recommended as an option only if patients do not have a highly active or rapidly evolving severe disease. When these first-line DMTs are ineffective, the alternative is (1) switching to another first line DMT or (2) starting a second-line DMT (e.g., natalizumab, fingolimod, cladribine, alemtuzumab, or rituximab), which has greater efficacy but also more severe side-effects. This approach is defined as *escalation* therapy and is used for most RRMS patients.

In patients presenting with aggressive inflammatory disease at onset, consensus is that a more beneficial approach is to employ an *induction immune* therapy using second-line DMTs from the beginning of treatment (Roos et al., 2020), conferring a significantly lower risk of SPMS conversion (versus first-line *escalation* therapy) (Brown et al., 2019). Such an approach is more effective in reducing the risk of reaching a disability milestone, albeit with a worse safety profile (Prosperini et al., 2020).

Despite DMT treatment, the majority of RRMS patients will eventually experience a change in their MS, with fewer or no relapses over time but increasing disability and a decline in neurological function, reflecting an SPMS pattern (Inojosa et al., 2019). The transition from predominantly relapsing forms to more progressive forms of MS is gradual, and the two phenotypes inherently overlap for a period. In these *transitional* forms of MS, clinicians tend to continue the use of DMTs because of uncertainty in making a firm SPMS diagnosis, reluctance to stop treatment, and patients' fear of disease activity returning upon withdrawal. However, the overall benefits of this approach are dubious, as many DMTs approved for RRMS showed negative or inconsistent results in clinical trials centered on SPMS patients (Fox et al., 2012). IFN-ß (Leary et al., 2003), GA (Wolinsky et al., 2007), fingolimod (clinicaltrials.gov identifier NCT00731692), and natalizumab (NCT01416181) (Kapoor et al., 2018) have all shown no clear efficacy in PMS patients.

Until recently, IFN-ß was the only DMT approved by the United Kingdom National Institute for Clinical Excellence (NICE) for people with SPMS, but only in the case of patients experiencing continuing relapses (i.e., *active* SPMS) (La Mantia et al., 2013). This recommendation came from evidence suggesting that IFN-ß reduced relapse risk in SPMS patients but was unable to significantly slow disability progression versus placebo (Panitch et al., 2004). The antineoplastic mitoxantrone was also approved as a potential therapy for SPMS by the US Food and Drug Administration (FDA), despite serious adverse effects related involving cardiotoxicity and therapy-related acute leukemia (Martinelli Boneschi et al., 2013).

Ocrelizumab (Ocrevus), a humanized anti-CD20 monoclonal antibody, was approved for PPMS patients by the FDA in 2017 and by NICE in 2019. The use of anti-CD20 antibodies stems from the initial observation that a single intravenous course of the anti-CD20 antibody rituximab reduces the inflammatory brain lesions in RRMS patients (Hauser et al., 2008). These data provided evidence of B-cell involvement in the pathophysiology of MS and prompted the use of anti-CD20 antibodies in PMS patients. Despite initial setbacks (Hawker et al., 2009), ocrelizumab was approved for patients with *active* PPMS thanks to the results of the ORATORIO study (Montalban et al., 2017); it is recommended for PPMS patients fulfilling specific clinical and radiological criteria consistent with *early active* disease.

In 2019, siponimod (Mayzent), a modulator of the sphingosine-1-phosphate (S1P) receptor (−1 and −5) (Gergely et al., 2012), was approved by the FDA as the first ever oral treatment for people with *active* SPMS, with NICE approval following the next year. Siponimod is a close structural analog of S1P, a naturally occurring bioactive sphingolipid that plays a key role in inflammation and repair processes. The S1P receptor is expressed by several CNS cells, including astrocytes, oligodendrocytes, neurons, microglia, and dendritic cells (Groves et al., 2013). By acting as a functional antagonist on S1P1 receptors on lymphocytes, siponimod prevents egress from lymph nodes, reducing the recirculation of T cells into the CNS to limit central inflammation. Moreover, siponimod can penetrate into the CNS and distribute into the white matter. Siponimod approval came after the results of the phase 3 EXPAND study (Kappos et al., 2018); it is recommended for treating *active* SPMS in adults.

Despite advances in PMS treatment, major hurdles still exist as these DMTs are limited to use in patients with an Expanded Disability Status Scale (EDSS) $\leq$ 6.5 due to lack of evidence in

those with more severe disability. Moreover, no treatments are available for progressive patients who do not experience an *active* form of disease, making the identification of new therapies a key priority of MS research.

Emerging Therapies for PMS

In recent decades several experimental or repurposed drugs have been tested in PMS but failed to advance past early phases of clinical testing due to a lack of efficacy (Ontaneda et al., 2017). These negative outcomes were disheartening, but also fostered the formation of several consortia aimed at identifying novel candidates for PMS treatment.

A recent combined systematic approach has reviewed existing evidence of human safety, BBB penetrance, demonstrable efficacy, and mechanistic targeting of licensed drugs for repurposing in PMS (Cunniffe et al., 2021). By focusing on processes and mechanisms of action that are specifically relevant to the pathogenesis of progression, four treatments were recommended for immediate testing in PMS: (R)-α-lipoic acid, metformin, the combination treatment of both (R)-α-lipoic acid and metformin, and niacin.

(R)-α-lipoic acid is a cofactor for at least five enzyme systems including pyruvate and α-ketoglutarate dehydrogenases, key enzymes of the TCA cycle. Results of a phase 2/3 trial in MS showed that treatment with lipoic acid induced a 68% reduction in annualized Percent Change Brain Volume while maintaining favorable safety, tolerability, and compliance over 2 years (Spain et al., 2017). Metformin, a synthetic derivative of guanidine commonly used as an oral antidiabetic, can reverse aging-associated remyelination failure, suggesting a possible application in PMS (Neumann et al., 2019). Niacin, a nicotinamide adenine dinucleotide precursor used for the treatment of hypercholesterolemia, has been shown to be protective against activated microglial-induced neurotoxicity and to promote oligodendrocyte proliferation *in vitro* (Kaneko et al., 2006). These mechanisms of action could be exploited in promoting regeneration and repair in PMS.

Besides these repurposed drugs, other therapies currently being tested in PMS include simvastatin, biotin, cladribrine, masitinib, ibudilast, and epigallocatechin-3-gallate (Faissner et al., 2019). Simvastatin has been studied in MS for its neuroprotective effects, which in part depend on the improvement of cerebrovascular hemodynamic (Neuhaus et al., 2004). A randomized, double-blind, placebo-controlled phase 2 clinical trial (MS-STAT) has shown that high dose simvastatin significantly reduces brain atrophy and radiological lesions in SPMS patients (Chataway et al., 2014). A larger phase 3 follow-up (MS-STAT2; NCT03387670) is now ongoing and will hopefully confirm these benefits. Biotin (vitamin B7) has been shown to (i) activate myelin formation in oligodendrocytes through its role as a cofactor for acetyl-CoA carboxylase, and to (ii) increase ATP production in axonal mitochondria, being a co-enzyme for three carboxylases (including the pyruvate carboxylase) of the TCA cycle (Sedel et al., 2015). The preliminary results of a phase 3 trial (NCT02220933) have shown that high dose daily administration has an impact on SPMS in reducing disease progression (Tourbah et al., 2016). A bigger cohort in a phase 3 clinical trial is currently being recruited. Cladribrine is approved for use in RRMS patients but a previous study found no significant treatment effects in terms of changes in EDSS scores in PMS (Rice et al., 2000). Nevertheless, cladribine produced significant sustained reductions in radiological lesion loads. These positive (but limited) outcomes sparked a new phase 3 trial, ChariotMS, looking to assess the beneficial role on cladribine on upper limb function in advanced PMS patients (EDSS between 6.5 and 8.5) (NCT04695080).

To summarize, therapies for PMS patients are beginning to emerge and hopefully we will experience a new era of therapeutics acting on the core drivers of disease progression. Most likely, successful therapeutic agents will have to interact with multiple processes, modifying chronic inflammation while enhancing the intrinsic repair of the damaged CNS. While more rigorous clinical trial design with appropriate endpoints and longer follow-up times may aid in the successful identification of safe and efficacious PMS DMTs (Huntemann et al., 2021), the innate multifunctionality of stem cell therapies offer a promising alternative route toward addressing the unmet needs of neuroprotection and neuroregeneration.

THE BASIS OF STEM CELL THERAPIES FOR THE TREATMENT OF PMS

Despite considerable success in treating RRMS, and a growing armamentarium of DMTs for combating *active* forms of PMS, there are substantial unmet needs for interventions capable of halting and reversing the chronic degeneration associated with PMS. In this light, there has been considerable interest in the presumed regenerative capabilities of stem cell therapies.

Stem cell therapy is a broad concept comprising the transplantation of different stem cell types sourced from various tissues into prospective patients for therapeutic effect. The choice of one cell type over another is based on multiple factors, but optimal outcomes will necessitate marrying appropriate mechanisms of action to the pathobiology being addressed.

Hematopoietic Stem Cell Sources

A first important delineation in terms of therapeutic functionality exists between hematopoietic and non-hematopoietic therapies.

Hematopoietic stem cell (HSC) transplantation was the earliest cell therapy to emerge for the treatment of MS and it is currently the only clinically validated approach, having been imported from the field of hematology where it is routinely used in treating malignancies (Muraro et al., 2017a). HSC transplantation works by resetting the immune system by means of conventional immunoablation followed by reconstitution of the immune system by the stem cell graft. This results in the development of a novel immune system deprived of pathogenic auto-immune cells. Notably, whether the transplanted HSCs only provide means to overcome the cytopenia and toxicity caused by the immunosuppressive conditioning regimen, or whether there is a distinct transplant-associated anti-inflammatory effect, remains a matter of debate (Miller et al., 2021). Nonetheless, HSCs have little *regenerative*

impact on the CNS as HSCs lack the ability to differentiate into neurons, astrocytes or oligodendrocytes (Gavriilaki et al., 2019). Thus, HSC transplantation is primarily efficacious for the treatment of clinical forms of MS with high inflammatory activity (i.e., RRMS or *active* PMS with clinical and/or radiological evidence of inflammation), but has limited efficiency in the case of inactive PMS, failing to address the degenerative component of the disease (Muraro et al., 2017a; Gavriilaki et al., 2019).

Consequently, *non-hematopoietic* stem cell (nHSC) transplantation has been embraced as a potential means to regenerate the damaged CNS in an attempt to offer a therapeutic solution for PMS.

Non-hematopoietic Stem Cell Sources

While early studies of therapeutic nHSC transplantation typically sourced cells from embryonic or fetal tissue, the safety, practical, and ethical issues surrounding their prospective clinical use have caused current research efforts to shift toward the study of nHSCs derived from adult tissue (Hentze et al., 2009; Volarevic et al., 2018).

The most common nHSC types employed in preclinical and clinical studies of CNS disorders include: (1) mesenchymal stromal cells (MSCs), a heterogeneous class of multipotent cells derived from various tissues (Sharma et al., 2014; Hsuan et al., 2016; Martin et al., 2019); (2) bona fide neural stem cells (NSCs) derived from neurogenic brain niches (Boese et al., 2018); (3) pluripotent stem cell-derived NSCs, produced *ex vivo* through neural lineage differentiation of isolated embryonic stem cells (ESCs) (Cao et al., 2011; Tang et al., 2017; Oh et al., 2018; Zhao and Moore, 2018) or from differentiation of induced pluripotent stem cells (iPSCs), themselves generated by reprogramming of somatic cells such as fibroblasts acquired through a minimally invasive procedure (Takahashi and Yamanaka, 2006; Yu et al., 2007; Tang et al., 2017); and, most recently, (4) induced NSCs (iNSCs) obtained by *direct* reprogramming of a patient's somatic cells, bypassing a pluripotent state (Kim et al., 2011; Han et al., 2012; Lujan et al., 2012; Thier et al., 2012; Daekee et al., 2019).

Mesenchymal stromal cells are a convenient source of nHSCs, being derived from various autologous or allogeneic tissues including bone marrow (BM-MSCs), adipose tissue (AD-MSCs), and the umbilical cord (UC-MSCs). While MSCs have also been reported to be able to differentiate toward non-mesodermal cells including neurons, astrocytes, and oligodendrocytes both *in vitro* and *in vivo*, their propensity for neural differentiation *in vivo* is limited (Wei et al., 2013; Squillaro et al., 2016). MSCs from different donors, various sources from the same donor, and even fractions of the same cell population are highly heterogenous, making it difficult to accurately establish their therapeutic efficacy (Bortolotti et al., 2015). Additionally, MSCs have been reported to exert immunosuppressive properties, raising concerns regarding patients potentially being at a greater risk of developing cancer due to the impaired surveillance activity of the immune system (Hasan et al., 2017).

Somatic NSCs possess several advantages for CNS applications over other stem cell sources, including their inherent commitment to the neural lineage, patient-specificity, and a low tumorigenic risk thanks to their lack of pluripotency and limited proliferation rate. However, the latter property comes at the expense of their low expandability *in vitro*, limiting the practicality of using somatic NSCs in large quantities. Additionally, extraction of NSCs from the neurogenic regions of the brain is difficult, invasive and carries significant risks (Nam et al., 2015). Indeed, human adult neurogenesis and the existence of NSC niches within the adult human brain has been a source of controversy in the scientific community (Lucassen et al., 2020). Instead, NSCs are almost exclusively sourced from fetal tissue, limiting their accessibility and raising ethical concerns.

These caveats led to the search for and identification of alternative sources of NSCs such as those obtained from ESCs or iPSCs, as well as iNSCs. These derived NSCs can recapitulate the properties, potency, and therapeutic potential of bona fide NSCs, making them ideal candidates to pursue regeneration of the CNS. Additionally, ESCs, iPSCs and iNSCs are readily expandable *in vitro*, and, in the case of iPSCs/iNSCs, autologous origins can minimize issues relating to immunogenicity, although not necessarily negating them completely (Wood et al., 2016). iNSCs offer a number of potential therapeutic advantages over pluripotent sources in that they are readily sourced, ostensibly easier, faster, and more cost-efficient to generate than iPSCs, and bypass a problematic pluripotency stage associated with tumorigenic risks (Erharter et al., 2019). In several cases, these multipotent sources have been further differentiated to specific neural lineages for study in a preclinical transplantation studies, with iPSC-derived oligodendrocyte progenitor cells (iPSC-OPCs) being a key example in the context of MS (Chanoumidou et al., 2020).

Nonetheless, it remains unclear to what extent autologous patient-derived NSCs may retain disease-specific epigenetic marks could hamper their therapeutic potential or have deleterious effects on other CNS cells (Nicaise et al., 2019).

Mechanisms of Action for Non-hematopoietic Stem Cells

Non-hematopoietic stem cells exert their therapeutic function in a multifaceted fashion, targeting a broad range of deleterious disease processes, often in a tissue-specific manner, making them ideal candidates for treating diseases such as PMS with multiple overlapping pathological mechanisms.

Originally, nHSC stem cell therapy was explored as a means to revert CNS damage by replacement of damaged cells by virtue of the self-renewal and potency properties of the graft. This view was supported by observations of successful engraftment and differentiation of stem cells into the CNS following transplantation in preclinical and clinical studies of CNS disorders. Indeed, multiple studies assessing stem cell engraftment and differentiation efficiency in several CNS conditions such as amyotrophic lateral sclerosis, spinal cord injury, and stroke have reported the successful synaptic integration of the graft and subsequent regeneration (Zhang et al., 2019). As touched upon below, transplanted nHSCs can also integrate *in vivo* without differentiation, instead exerting some of their therapeutic effects through mechanisms implying cell-to-cell interactions with the host. However, it

is becoming increasingly clear that the therapeutic properties of nHSC transplantation extend well beyond differentiation, cell replacement and integration, factors shown to play only a secondary role in preclinical studies (Pluchino et al., 2005, 2009; Scolding et al., 2017). The currently accepted scientific view is that nHSC transplantation primarily exerts its beneficial effects by regulating the local environment through paracrine effects including trophic support, immunomodulation and metabolic signaling (**Figure 2**).

The neuroprotective and neurotrophic actions of nHSCs are directly exerted through the secretion of various factors (Willis et al., 2020). For example, one of the most clinically advanced NSC products, the CTX fetal NSC line, has been reported to secrete a complex cocktail of cytokines and growth factors that promote neurogenesis, axonal sprouting, and angiogenesis (Sinden et al., 2017). Likewise, it has been shown that MSCs secrete factors that induce axonal outgrowth and increase the survival of cells *in vitro* (Cho et al., 2009; Kim et al., 2010). Interestingly, MSCs have exhibited neuroprotective effects at a distance, without the need of their direct transplantation into the CNS, as a result of their capacity to secrete paracrine neurotrophic factors via extracellular vesicles (EVs) (Li et al., 2019; Mansoor et al., 2019). As membranous vectors of intercellular communication secreted by cells into the extracellular space, EVs are capable of influencing physiological and pathophysiological functions by trafficking bioactive cargoes such as proteins, lipids, and nucleic acids to recipient cells (van Niel et al., 2018; Xiao et al., 2021). EVs originating from various cell types are understood to influence the pathogenesis of MS and EAE (Dolcetti et al., 2020), highlighting their utility as biomarkers of disease. Conversely, exogenous stem cell-derived EVs act as delivery vehicles for prospective therapeutic factors (Wiklander et al., 2019), even demonstrating the ability to cross the BBB (Jan et al., 2017; Banks et al., 2020). On this basis, EV-based therapies are now being explored as a promising acellular alternative to stem cell transplantation, taking advantage of the stem cell secretome while minimizing disadvantages such as immunogenicity (Drago et al., 2013; Riazifar et al., 2017; Vogel et al., 2018; Branscome et al., 2020).

Mesenchymal stromal cells exhibit immunomodulatory functions via both direct and paracrine interaction with immune cells. Specifically, MSCs suppress T cell proliferation, inhibit the production of pro-inflammatory cytokines, and regulate the ratio of Th2/Th1 activated cells (Glennie et al., 2005; Puissant et al., 2005; Yanez et al., 2006). MSCs can also arrest B cell cycling and inhibit their division and antibody production (Corcione et al., 2006). Moreover, MSCs affect natural killer cells and dendritic cells by inhibiting their activation and maturation (Ramasamy et al., 2007; Burchell et al., 2010), and exert immunosuppressive activity by modulation of regulatory T cell function (Selmani et al., 2008). Several MSCs-derived soluble factors including transforming growth factor (TGF)-β1, prostaglandin E2 (PGE2), hepatocyte growth factor (HGF), indoleamine-pyrrole 2,3-dioxygenase, NO, and IL-10 have been proposed to mediate the immunosuppressive effects of MSCs (Gao et al., 2016). Importantly, previous studies showed that secretory factors vary greatly in relation to both the origin of MSCs and their environmental conditions (Mansoor et al., 2019).

Immunomodulatory functions of NSC transplantation have been primarily described to occur through juxtracrine and paracrine T cell signaling (Pluchino and Cossetti, 2013), as a result of their release of factors such as the cytokines IL-10 (Yang et al., 2009), leukemia inhibitory factor (LIF) (Cao et al., 2011), and TGF-β2 (De Feo et al., 2017), as well as NO and PGE2 (Wang et al., 2009). Consequently, NSC transplantation inhibits the peripheral and perivascular activation of proinflammatory T cells and increases the numbers of anti-inflammatory T regulatory cells (Tregs) in animal models of MS (Pluchino et al., 2005; Einstein et al., 2007). In addition to their paracrine functions, transplanted NSCs exert important anti-inflammatory effects on the adaptive immune system through cell-to-cell interactions, leading to reduced proliferation, decreased activation, and increased apoptosis of proinflammatory T lymphocytes (Pluchino et al., 2005; Fainstein et al., 2008). Transplanted NSCs have also demonstrated immunomodulatory properties through their direct interaction with local cells of the innate immune system, including macrophages (Cossetti et al., 2012). These observations are of particular importance for the treatment of PMS, when macrophages play a pivotal role in pathogenesis. Specifically, analysis of cell-to-cell interactions at the perivascular niches revealed the presence of tight contacts between NSCs and macrophages, established via connexin 43 (Cusimano et al., 2012).

Metabolic signaling has been described as a distinct mechanism by which stem cells mediate part of their immunomodulatory function. In local inflammatory microenvironments NSCs have been shown to engage in a homeostatic cell competition with MPs by changing their own metabolism and depleting the extracellular milieu of inflammatory immunometabolites such as succinate (Peruzzotti-Jametti et al., 2018). Additionally, cytokine-primed NSCs increase the secretion of extracellular arginase-1, inhibiting the proliferation of lymph node cells (Drago et al., 2016), and EVs derived from NSCs exposed to a pro-inflammatory stimulus were found to shuttle receptor-bound IFN-γ to recipient cells wherein it activates Stat1 signaling (Cossetti et al., 2014). NSCs-derived also harbor functional metabolic enzymes and thus act as independent metabolic units (Iraci et al., 2017). Conversely, addressing the chronic activation of mononuclear phagocytes by means of modulating their mitochondrial metabolism is expected to be a key target of future molecular and cellular therapies for PMS (Peruzzotti-Jametti and Pluchino, 2018).

A new phenomenon of mitochondrial transfer has been found to take place between MSCs and immune cells, modulating the function of the latter. Macrophages reportedly enhance their phagocytic capacity and bioenergetics profiles, and shift to an anti-inflammatory phenotype, following EV-mediated transfer of mitochondria from MSCs (Han et al., 2020). A similar phenomenon has been reported in NSCs, which release EV-trafficked mitochondria that are taken up by lipopolysaccharide-activated MPs and integrated into the host mitochondrial network, metabolically reprogramming

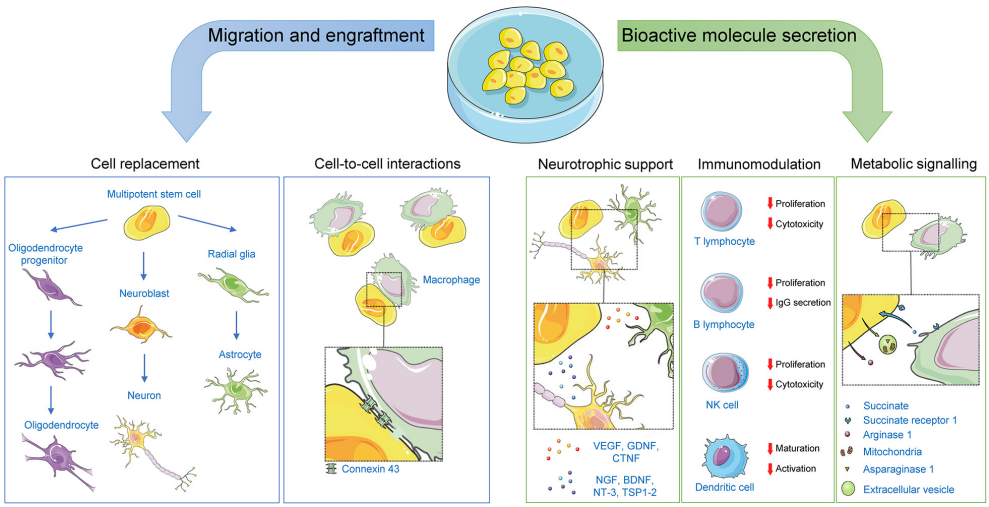

FIGURE 2 | Mechanisms of action for non-hematopoietic stem cells. Following transplantation, non-hematopoietic stem cells can exert their therapeutic effects by: (1) replacing damaged CNS cells; (2) offering neurotrophic support to CNS cells via paracrine and juxtracrine signaling; (3) affecting immunomodulatory functions on both the innate and adaptative immune systems via paracrine and juxtracrine signaling, or via direct cell-to-cell contacts; and (4) engaging in metabolic signaling with cells within their niche. Representative examples of key players in each mechanism are illustrated. BDNF, brain-derived neurotrophic factor; CNTF, ciliary neurotrophic factor; GDNF, glial cell–derived neurotrophic factor; NGF, nerve growth factor; NT-3, neurotrophin-3; TSP1-2, thrombospondins 1 and 2; VEGF, vascular endothelial growth factor.

the MPs and reducing their pro-inflammatory activation (Peruzzotti-Jametti et al., 2021).

Despite findings suggesting nHSCs as promising therapeutic tools for diseases such as PMS in which tissue repair is needed and/or inflammation is extensive, clinical translation of nHSC transplantation has met some barriers. These include limited engraftment duration, little *in vivo* differentiation, and restricted accessibility into damaged sites (Yousefi et al., 2019). Additionally, the complex functionality of stem cells makes it difficult to clearly correlate their distinct therapeutic mechanisms of action and resultant outcomes in clinical studies. In an attempt to increase the translational success of stem cell-based therapies, the use of cell-free stem cell products (i.e., stem cell-derived secretomes or extracellular vesicles) is currently being proposed as an alternative therapeutic strategy (Bai et al., 2012). The use of stem cell derivatives may circumvent many of hurdles of cell therapy including tumorigenicity, immune rejection, high costs, and time-consuming manufacturing. Moreover, there is increasing interest in engineering of stem cell lines to increase their production of paracrine regulators. For instance, NSC lines have been engineered to overexpress specific neurotrophic factors (Marsh and Blurton-Jones, 2017) while the neuroregulatory platform of the MSC-derived secretome has been modified by dynamic culturing of MSCs in computer-controlled bioreactors (Yousefi et al., 2019).

Practical Considerations of Stem Cell Transplantation Therapy

The specific parameters regarding the optimal administration route, dose and timing for successful stem cell interventions are dictated by the cell type being administered and their mechanisms of action. Multiple prospective administration routes are available for CNS applications, each of which comes with its own pros and cons. The least invasive route is the intravenous (IV) injection (Wang et al., 2017). Of note, this systemic route is characterized by poor engraftment efficiency due to the entrapment of stem cells in organs including the lungs, spleen, bone marrow, and kidneys, preventing most cells from reaching the CNS parenchyma (Fischer et al., 2009; DePaul et al., 2015). Nevertheless, while IV injection of stem cells may not be the optimal method for direct cell replacement, it has instead the potential to be highly effective for cells which primarily exert their therapeutic effect through long distance paracrine modulation, such as MSCs. Alternatively, intraarterial injection of stem cells has been proposed to prevent the collection of cells in systemic organs and increase the incidence of cells reaching their CNS targets (Walczak et al., 2008; Argibay et al., 2017; Na Kim et al., 2017). However, caution should be taken when using this administration route as it runs the risk of causing microembolisms if the volume and concentration of injected cells are not carefully controlled (Argibay et al., 2017; Na Kim et al., 2017).

More invasive administration routes ranging from intrathecal (IT), intracerebroventricular (ICV), and even direct intraparenchymal (IP) injection have been proposed as efficient means of facilitating intracranial migration to sites of damage (Muir et al., 2011; Hurst et al., 2013; Donega et al., 2014). IP injection may not be a feasible option for PMS stem-cell transplantation because of the multicentric and/or diffuse nature of PMS lesions. Thus, administration of NSCs into the CSF by IT or ICV injection establishes a useful compromise between the invasiveness of the procedure and circumvention of biological barriers and may represent the best options with

regards to the treatment of PMS (Scolding et al., 2017). Lastly, the transplantation of stem cells through the nasal cavity has been described as a novel non-invasive approach that ostensibly results in an effective migration into the CNS. However, the safety, migratory mechanisms, and overall efficacy of this procedure are still being studied, and additional reports are needed to properly assess the safety and reproducibly of this administration route (Li et al., 2015).

The optimal window for stem-cell administration is another key consideration to ensure the best clinical outcome. Specifically, while the early administration of nHSCs may prove beneficial as the graft counteracts neuroinflammation and provides neurotrophic support, the pro-inflammatory CNS environment which characterizes early RRMS and *active* forms of PMS can adversely affect cell engraftment, differentiation, and survival (Smith et al., 2020).

It is thus essential that the suitability of the recipient with respect to the clinical form of MS, the stage of progression, and the nature of the therapy being delivered are thoroughly assessed before stem cells are administered.

PRECLINICAL EVIDENCE FOR STEM CELL THERAPIES IN TREATING PMS

While there is no perfect animal analog of human MS, various aspects of the disease (acute and chronic inflammation and demyelination) can be recapitulated across a variety of *in vivo* models (Procaccini et al., 2015; Burrows et al., 2019).

Preclinical evidence for the efficacy of stem cell therapy in PMS has relied heavily on EAE mouse models to recapitulate the immunologically driven tissue damage observed in PMS. EAE is typically induced through administration of myelin-derived antigens (proteolipid protein or myelin oligodendrocyte glycoprotein). Other methods commonly used to invoke MS-like pathology include cuprizone- or lysophosphatidylcholine-induced demyelination, JHM-strain murine hepatitis virus (JHMV)-caused encephalomyelitis and demyelination in susceptible rodents, and the shiverer model of congenital hypomyelination. Promising preclinical evidence of the safety and efficacy evidence of stem cell transplantation has been acquired with both HSC and nHSC sources, with a common theme of transplants resulting in immunomodulation, neuroprotection, and neurotrophic support in the chronically inflamed CNS. Below we briefly summarize key findings in this area, with a particular focus on nHSC experiments (as summarized in **Table 1**).

Preclinical Evidence for HSCs

Immunoablation and subsequent reconstitution by means of autologous HSC transplantation (HSCT) is now seen as a viable clinical treatment option for *early active* MS (see section "Clinical Studies of Stem Cell Therapies in Progressive Multiple Sclerosis"), with a strong body of supporting preclinical data from EAE studies extending back several decades (van Bekkum, 2004; Van Wijmeersch et al., 2008).

In key supporting *in vivo* rodent experiments, the effectiveness of post-conditioning syngeneic HSCT (in the form of a bone marrow transplant) in combatting EAE was found to be highly dependent upon the timing of the intervention, with early treatment having substantial preventative outcomes (Karussis et al., 1992; Karussis et al., 1993) but little effect during the chronic stages of EAE (Karussis et al., 1993; van Gelder et al., 1993; Burt et al., 1998; Herrmann et al., 2005). Bone marrow transplant during the peak of disease greatly reduced spontaneous relapse rates (van Gelder et al., 1993), while a study of non-myeloablative conditioning in mice suggests the long-term EAE remission is dependent on a transplant-associated induction of regulatory T cells (Meng et al., 2011). Notably, pseudo-autologous transplants, obtained from syngeneic bone marrow donor mice in the same stage of EAE as the recipient, were found to be associated with higher levels of induced relapses than typical syngeneic treatments, despite similar efficacy with regards to disease prevention (van Gelder and van Bekkum, 1996).

Allogeneic transplants have typically been found more effective than syngeneic or pseudo-autologous sources in preventing relapses in EAE rodents (van Gelder and van Bekkum, 1995, 1996). There may be implications here with regards to the optimal choice of allogeneic versus autologous HSC sources for therapeutic use, however, clinical HSCT routinely employs autologous sources, backed by assuring efficacy and safety evidence (Alexander et al., 2021; Miller et al., 2021).

Preclinical Evidence for MSCs

Mesenchymal stromal cells have seen extensive study across multiple disease models, including CNS disorders, as they are an easily accessible source of autologous or allogeneic somatic stem cells with the capacity to differentiate into multiple lineages including mesodermal, ectodermic, and endodermic cells (Gugliandolo et al., 2020). However, the limited ability of MSCs to differentiate *in vivo* (Nombela-Arrieta et al., 2011) means that the therapeutic effects of MSCs stem largely from their paracrine effects, secreting cytokines, growth factors, small RNAs, and EVs (Gugliandolo et al., 2020). The IV injection of BM-MSCs in mouse models of EAE at the onset (10 days post-immunization [dpi]) and peak (15 dpi) of disease, but not during the chronic phase (24 dpi), improves functional outcomes, decreases inflammatory cell infiltration, induces T cell anergy, and suppresses pathogenic B cells (Zappia et al., 2005; Gerdoni et al., 2007). Further evidence for the immunomodulatory role of BM-MSCs arises from a study where MSCs were transplanted intraperitoneally at the peak (14 dpi) and again at the chronic (20 dpi) stages of EAE in mice (Xin et al., 2020). Amelioration of clinical scores was observed, correlating with reduced $CD4^+$ and $CD8^+$ T cell activation and proliferation, increased numbers of Tregs in the spleen, and a shift in the polarization of macrophages from M1 to M2, ultimately reducing the splenic production of pro-inflammatory cytokines, IFN-γ and IL-17. Despite these positive outcomes, transplanted cells were rarely observed in the CNS parenchyma, in line with notion that the therapeutic effects of MSCs transplants arise primarily through paracrine mechanisms taking place peripherally.

TABLE 1 | Preclinical evidence of non-hematopoietic stem cells in treating MS.

Study	Cell Type/Source	Injection Route/Cell Dose	Timing	Animal Model of MS	Key Findings
MSCs					
Zappia et al., 2005	BM-MSCs of adult male WT mice	IV, 1×10^6 cells	10, 15, or 24 dpi	MOG-induced chronic-EAE in WT female mice	• Improved EAE outcomes when transplanted before the chronic phase (before 24 dpi) • Grafted cells migrated and survived in the spinal cord and lymphoid organs • Decreased inflammatory infiltrates • Induction of T cell anergy
Gerdoni et al., 2007	BM-MSCs from adult WT mice	IV, 10^6 cells	Onset of disease (ca. 12 dpi)	PLP-induced relapsing-remitting EAE in WT female mice	• Improved EAE outcomes with fewer relapses • Decreased inflammatory infiltrates, demyelination, and axonal loss • No evidence of graft integration in brain parenchyma • PLP-specific immune response reduced in MSC-treated mice
Bai et al., 2009	Human BM-MSCs	IV, 3×10^6 cells	16 or 27 dpi	MOG-induced chronic-EAE and PLP-induced relapsing-remitting EAE in WT female mice	• Improved chronic and relapsing-remitting EAE outcomes • Survival and migration of grafted cells into demyelinating areas • Increased oligodendrocyte lineage cells surrounding lesions • Reduced inflammatory cell infiltration and demyelination in chronic EAE
Constantin et al., 2009	AD-MSCs from adult WT mice	IV, 1×10^6 cells	3 and 8 dpi or 3 and 28 dpi	MOG-induced chronic EAE in WT female mice	• Improved EAE outcomes when transplanted before onset or chronically • Reduced demyelination and axonal loss • Shift toward Th2 T cell population • Survival of grafted cells and differentiation into OPCs
Guo et al., 2013	Human BM-MSCs	IV, 1×10^6 cells	3 or 12 dpi	MOG-induced chronic EAE in WT female mice	• Improved EAE outcomes • Reduced inflammatory cell infiltration and demyelination • Increase in anti-inflammatory Th2 cells and anti-inflammatory cytokines • Lower serum levels of IL-6 and TNF-α
Anderson et al., 2017	AD-MSCs from adult WT male mice	IP, 1×10^6 cells	At onset or the acute phase of disease (on the basis of clinical score)	MOG-induced chronic-EAE in WT female mice	• Improved EAE outcomes • Reduced inflammatory cell infiltration and demyelination • Reduced dendritic cell activation in the draining lymph nodes • Reduced autoantigen-specific T-cell function
Xin et al., 2020	BM-MSCs from adult WT mice	IP, 1×10^7 cells	14 and 20 dpi	MOG-induced chronic-EAE in WT female mice	• Improved EAE outcomes • Shifted the polarization of macrophages from M1 to M2 • Inhibited the activation and proliferation of T cells
Laso-García et al., 2018	EVs from human AD-MSCs	IV, 25 μg EVs	60 days post-infection	TMEV-induced demyelination in WT female mice	• Improved motor function • Reduced brain atrophy • Decreased inflammatory infiltrates • Increased myelin expression • Reduced plasma levels of Th1/Th17 cytokines
Jafarinia et al., 2020	EVs from human AD-MSCs or human AD-MSCs	IV, 60 μg EVs or 1×10^6 cells	10 dpi	MOG-induced chronic-EAE in WT female mice	• Improved EAE outcomes with EVs or cells • Decreased inflammatory infiltrates • Reduced demyelination
Clark et al., 2019	EVs from human placental MSCs or human placental MSCs	IV, 1×10^7 or 1×10^{10} EVs, or 1×10^6 cells	19 dpi	MOG-induced chronic-EAE in WT male and female mice	• Improved motor function outcomes with high-dose EVs or cells • Reduced oligodendroglial DNA damage • Increased myelination in the spinal cord
Riazifar et al., 2019	EVs from human BM-MSCs (native or IFN-γ) stimulated	IV, 150 μg EVs	18 dpi	MOG-induced chronic-EAE in WT female mice	• Improved EAE outcomes • Reduced demyelination • Decreased neuroinflammation • Upregulated Treg numbers

(Continued)

TABLE 1 | Continued

Study	Cell Type/Source	Injection Route/Cell Dose	Timing	Animal Model of MS	Key Findings
Li et al., 2019	EVs from rat BM-MSCs	IV, 150 or 400 μg EVs, or 1×10^6 cells	On day of induction	Spinal cord homogenate-induced EAE in WT female mice	• Dose-responsive improved EAE outcomes with EVs or BM-MSCs • Decreased inflammatory infiltrates • Decreased demyelination • Increased expression of M2-like cytokines (IL-10, TGF-β) • Decreased expression of M1-like cytokines (TNF-α, IL-12)
Hosseini Shamili et al., 2019	EVs from mouse BM-MSCs	IV, 200 μg EVs or aptamer-modified EVs	1, 3, 6 dpi (prophylactic), or 12, 15, 18 dpi (therapeutic)	MOG-induced chronic-EAE in WT female mice	• Improved EAE outcomes by aptamer-modified EVs in prophylactic treatment regime • Decreased demyelination • Decreased inflammatory infiltrates
NSCs					
Pluchino et al., 2003	NSCs from SVZ of adult WT mice	ICV or IV, 1×10^6 cells	10, 15, or 22 dpi	MOG-induced chronic-EAE in WT mice	• Improved EAE outcomes • Survival, integration, and neural differentiation of NSCs • Increased the quantity of OPCs • Reduced astrogliosis
Pluchino et al., 2005	NSCs from SVZ of adult WT mice	IV, 1×10^6 cells	13 or 31 dpi	PLP-induced relapsing-remitting EAE in WT mice	• Improved EAE outcomes • Survival and integration of NSCs, but retention of immature stem-cell phenotype • Localization of grated NSCs around blood vessels • VLA-4 expression by NSCs • BMPs, noggin, notch-1, VEGF, and jagged-1 all upregulated
Pluchino et al., 2009	Human fetal NSCs	IV, 6×10^6 cells, or IT, 2×10^6 cells	Disease onset	Human MOG-induced EAE in common marmosets	• Improved EAE outcomes and increased survival • Effect more significant in IV-administered group • Graft survived undifferentiated for up to 3 months post-administration • Localization to perivascular inflammatory CNS regions and draining lymph nodes • Attenuated T cell proliferation and dendritic cell maturation
Yang et al., 2009	NSCs from SVZ of adult WT mice engineered to express IL-10	ICV or IV, 1.5×10^6 cells	10, 22, or 30 dpi	MOG-induced chronic-EAE in WT mice	• Improved EAE outcomes when applied at onset, 10, 22, or 30 dpi • Differentiation into neurons and oligodendrocytes • Reduced demyelination, increased remyelination
Peruzzotti-Jametti et al., 2018	NSCs from SVZ of adult WT mice	ICV, 1×10^6 cells	3 days post-onset (14–21 dpi)	MOG-induced chronic-EAE in WT female mice	• Improved EAE outcomes • Survival and integration of grafted cells • Accumulation within perivascular infiltrates • Reduction of extracellular succinate • Reprogramming of immune cells toward anti-inflammatory phenotype • Reduced demyelination and axonal loss
ESCs					
Aharonowiz et al., 2008	Human ESC-NSCs	ICV, 5×10^5 cells	10 dpi	MOG-induced chronic-EAE in WT female mice	• Improved EAE outcomes • Reduced axonal damage and demyelination • Reduction of encephalitogenic T cells
Cao et al., 2011	Mouse ESC-NSC	IV, 2×10^6 cells	0 or 10 dpi	MOG-induced chronic-EAE in WT mice; sex not reported	• When administered 0 dpi, disease onset was delayed, symptoms were reduced, inflammation and demyelination were decreased • When administered 10 dpi, ameliorated EAE symptoms, inhibited proliferation and cytokine production of T cells via LIF
Chen et al., 2014	Human ESC-NSCs	Intraspinal, 2.5×10^5 cells	14 dpi	JHMV-induced encephalomyelitis; sex not reported	• Improved functional outcomes • Reduced neuroinflammation • Reduced accumulation of $CD5^+$ T cells • Increase in regulatory T cell populations • Decreased demyelination and enhanced remyelination

(Continued)

TABLE 1 | Continued

Study	Cell Type/Source	Injection Route/Cell Dose	Timing	Animal Model of MS	Key Findings
iPSCs					
Laterza et al., 2013	iPSC-NSCs from MEFs	IT, 1×10^6 cells	4 days post-onset (dpi not reported)	MOG-induced chronic-EAE in WT female mice	• Improved EAE outcomes through neuroprotection, not cell replacement • Survival of grafted cells and accumulation within perivascular infiltrates • Reduced demyelination, enhanced remyelination via activation of LIF pathway
Thiruvalluvan et al., 2016	Human iPSCs-OPCs from human dermal fibroblasts	Injection in the corpus callosum, 5×10^4 cells	79 dpi in marmosets, at onset in mice (dpi not reported)	MOG-induced EAE in female common marmosets; cuprizone in female mice; MOG-induced chronic-EAE in WT female mice	• Survival and migration of grafted cells to CNS lesions in EAE marmosets; differentiation into mature and myelin-forming oligodendrocytes in EAE marmosets • Improved EAE outcomes in mice through reduced inflammatory cell infiltration and demyelination in EAE mice, but no cell engraftment • Grafted cell survival in cuprizone mice and partial differentiation into mature oligodendrocytes
Zhang et al., 2016	iPSC-NSC from MEFs	ICV, 2×10^5 cells	18 dpi	MOG-induced chronic-EAE in WT male mice	• Improved EAE outcomes • Reduction of T cell infiltration • Ameliorated white matter damage
iNSCs					
Lujan et al., 2012	iNSCs from MEFs	Intracerebellar, 1×10^5 cells	1 day postnatal	Shiverer mouse model of congenital hypomyelination	• Remyelination in the white matter tracts of the cerebellum
Peruzzotti-Jametti et al., 2018	iNSCs from MEFs	ICV, 1×10^6 cells	3 days post-onset (14–21 dpi)	MOG-induced chronic-EAE in WT female mice	• Improved EAE outcomes • Survival and integration of grafted cells • Accumulation within perivascular infiltrates • Reduction of extracellular succinate • Reprogramming of immune cells toward anti-inflammatory phenotype • Reduced demyelination and axonal loss
Sullivan et al., 2020	iNSCs from MEFs	Corpus callosum injection, 1×10^4 cells	12-week cuprizone diet	Cuprizone-induced chronic demyelination in WT male mice	• Astroglial and oligodendroglial differentiation; many undifferentiated • Demyelination unaffected but increased endogenous oligodendrocytes and proliferating OPCs • Reduced astrogliosis • Amelioration of motor deficits

BMP, bone morphogenic protein; CNS, central nervous system; dpi, days post-immunization; EAE, experimental autoimmune encephalomyelitis; ESC, embryonic stem cell; ICV, intracerebroventricular; IL, interleukin; iNSC, induced neural stem cell; IP, intraperitoneal; iPSC, induced pluripotent stem cell; IV, intravenous; JHMV, JHM-strain murine hepatitis virus; LIF, leukemia inhibitory factor; MEF, mouse embryonic fibroblast; MOG, myelin oligodendrocyte glycoprotein; MSC, mesenchymal stromal cell; NSC, neural stem cell; OPC, oligodendrocyte progenitor cell; PLP, proteolipid protein; SUCNR1, succinate receptor 1; SVZ, subventricular zone; TMEV, Theiler's murine encephalomyelitis virus; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor; WT, wild-type.

Nonetheless, cases of neural differentiation and integration have been reported. IV injected AD-MSCs successfully integrated into the spinal cord of mice with EAE at 3 months; most cells had differentiated into OPCs, but a limited differentiation into astrocytes or mature oligodendrocytes was also observed (Constantin et al., 2009). When administered before EAE onset (3 and 8 dpi) AD-MSCs improved functional outcomes and reduced inflammation. When administered in the chronic phase (23 and 28 dpi) functional deficits were again significantly ameliorated, endogenous OPCs were increased around the demyelinating lesions, and the number of anti-inflammatory Th2 cells was increased (Constantin et al., 2009). In a similar study, AD-MSCs sourced from either mice or humans were transplanted intraperitoneally at onset or during the acute phase of EAE and, in both cases, clinical scores were improved (Anderson et al., 2017). Further *in vitro* and *in vivo* investigation revealed that in mice with EAE, the transplantation of syngeneic AD-MSCs improved symptoms by reducing both autoantigen-specific T cell function and the activation of dendritic cells in draining lymph nodes.

Xenografts of human MSCs in mouse models of EAE have had similar success when compared to their mouse counterparts. The IV transplantation of human BM-MSCs at the peak (16 dpi) or chronic stage (27 dpi) of EAE improved functional outcomes and reduced inflammatory cell infiltration and demyelination (Bai et al., 2009). In contrast to previous findings from mouse MSC grafts (Zappia et al., 2005; Gerdoni et al., 2007), human BM-MSCs migrated to the CNS within 24 h, where they persisted for up to 45 days (albeit with decreasing numbers). Similarly, the IV transplantation of human BM-MSCs at 3 dpi or at onset (12 dpi) improved functional outcomes and reduced inflammatory cell infiltration, including pro-inflammatory Th1 and Th17 cells, while increasing the number of anti-inflammatory Th2 cells

and levels of anti-inflammatory cytokines (Guo et al., 2013). Furthermore, greater quantities of oligodendrocyte lineage cells were observed surrounding lesions.

Beyond the cells themselves, MSC-derived EVs have received considerable attention as a prospective MS therapy. Human AD-MSC EVs, when delivered IV to Theiler's murine encephalomyelitis virus-induced demyelinating disease mice (Laso-García et al., 2018) or EAE mice (Jafarinia et al., 2020), were found to improve functional outcomes and decrease inflammatory infiltrates, with evidence of reduced demyelination in the latter model. Likewise, upon IV administration at peak mouse EAE, human placental MSC-derived EVs yielded improved motor function and myelination (Clark et al., 2019) while EVs from IFN-γ stimulated human BM-MSCs increased Treg cell numbers with a concomitant decrease in neuroinflammation and demyelination (Riazifar et al., 2019). Early/prophylactic IV administration of rat BM-MSC EVs, when utilized in a spinal cord homogenate-induced rat EAE model, were found to induce a substantial polarization of CNS microglia toward an anti-inflammatory M2-like state, with improved behavioral outcomes and decreased inflammation and demyelination (Li et al., 2019). *Ex vivo* functionalization of EVs is also a promising aspect of this acellular approach, with, for example, mouse BM-MSC derived EVs adorned with a myelin-binding aptamer found to suppress inflammation and demyelination in EAE mice (Hosseini Shamili et al., 2019). The aptamer-modified EVs yielded improved clinical scores compared to the non-modified EVs when employed prophylactically. While EVs of various sources are under investigation as a distinct therapeutic intervention in several clinical trials, none are yet being conducted in the context of MS.

Extensive preclinical evidence supporting the paracrine and immunomodulatory effects of MSCs (or their acellular products) in preclinical models of MS, as well as safety and feasibility data from clinical study in other disease contexts, has seen their adoption as the main nHSC source for clinical trials to date.

Preclinical Evidence for NSCs

As self-renewing, multipotent cells capable of differentiating into functional neurons and glial cells, NSCs are apposite to therapeutic applications in the CNS. Small populations of NSCs are found in the subcortical white matter (Nunes et al., 2003) and the regions of adult neurogenesis (subventricular zone (SVZ) and sub-granular zone of the hippocampus), and the discovery that proliferating NSCs can be maintained in culture (Reynolds and Weiss, 1992) inspired the study of NSC transplantation as a therapeutic approach for CNS pathologies, including PMS.

In the context of EAE, the IV and ICV transplantation of SVZ NSCs before disease onset (10 dpi), at onset (15 dpi), and the peak of disease (22 dpi) have ameliorated functional deficiencies in mice. Transplanted NSCs localized to areas of brain with demyelination and axonal loss within one-month post-transplantation (Pluchino et al., 2003), with migration to the lesion via chemotaxis reliant on the expression of very late antigen-4 (VLA-4) adhesion molecules and the activation of G-coupled protein receptors (Pluchino et al., 2005). Histopathological analysis demonstrated that a significant fraction of transplanted NSCs differentiated into OPCs, and ultimately reduced glial scarring at the lesion sites. Additionally, the IV transplantation of NSCs during chronic EAE also improved functional outcomes by promoting apoptosis of pro-inflammatory T-cells and reducing inflammatory immune cell infiltrate (Pluchino et al., 2005). To further enhance the anti-inflammatory effects of NSC transplants in EAE, NSCs from the SVZ were genetically engineered to secrete the anti-inflammatory cytokine IL-10 (Yang et al., 2009). When transplanted IV or ICV before disease onset (10 dpi), at the peak of disease (22 dpi), or during the chronic phase (30 dpi), grafted IL-10-secreting NSCs were found to amplify the therapeutic effects observed with control NSC transplants (Yang et al., 2009). The therapeutic properties of human NSCs have also been demonstrated in non-human primate models of EAE, where decreased disease severity and improved functional outcomes were observed in both IT- and IV-injected animals, with the IV-treated cohort demonstrating the greater improvement along with a substantial survival benefit (Pluchino et al., 2009). Human NSC xenografts survived undifferentiated for up to 3 months after administration, distributing to perivascular inflammatory CNS regions and attenuating T cell proliferation and dendritic cell maturation.

The initial expectation of NSC therapeutics was differentiation into neural cells and incorporation into the damaged CNS. However, it has become clear from more recent preclinical studies that these outcomes are secondary to the effects of immunomodulation and the promotion of neuroprotection and homeostasis (Smith et al., 2020), the so-called '*bystander effect,*' with NSCs possessed of a therapeutic plasticity allowing them to respond to endogenous (patho)physiological stimuli (Martino and Pluchino, 2006). In the context of PMS, the immunomodulation of MPs by transplanted NSCs is very promising. Preclinical evidence from mouse models of EAE suggests that NSCs can alter the proinflammatory phenotypes of MPs by sequestering the extracellular immunometabolite succinate and secreting anti-inflammatory PGE2 in response (Peruzzotti-Jametti et al., 2018). Transplanted NSCs home to meningeal perivascular areas where they localize in close contact with MPs, which in turn undergo metabolic reprogramming toward anti-inflammatory oxidative phosphorylation, with a resultant amelioration of chronic neuroinflammation and functional recovery in EAE mice. Notably, similar results were found using both somatic NSCs and iNSCs (Peruzzotti-Jametti et al., 2018).

Given these promising findings but otherwise limited accessibility of NSCs, there is a demand for more practical sources of NSCs to support clinical study, thus inspiring preclinical investigations of ESCs, iPSCs, and iNSCs.

Preclinical Evidence for ESC-Derived Cells

Embryonic stem cells are pluripotent cells derived from the inner cell mass of the blastocyst and – of interest to MS therapeutic applications – they can be differentiated into NSCs or OPCs (Piao et al., 2015; Zhao and Moore, 2018; Chanoumidou et al., 2020). An IV injection of ESC-NSCs on the day of EAE immunization

delayed the onset of disease in mice, reduced clinical scores, and decreased both inflammation and demyelination (Cao et al., 2011). When administered at 10 dpi, the transplants still ameliorated EAE symptoms, inhibiting the proliferation and cytokine production of T cells via the secretion of LIF.

As observed with MSCs, xenografts of human ESC-NSCs yielded similar results. Human ESC-NSCs transplanted ICV before disease onset (10 dpi) in mouse models of EAE reduced axonal damage and demyelination, decreased the quantity of encephalitogenic T cells, and ultimately improved clinical scores (Aharonowiz et al., 2008). While transplanted human ESC-NSCs were found to migrate to the brain parenchyma, differentiation into mature oligodendrocytes was not observed and the extent of remyelination was negligible. Similarly, in JHMV mouse models of MS, intraspinal injection of human ESC-NSCs were found to survive in the spinal cord parenchyma for only 1 week post-transplantation, but through immunomodulatory and paracrine effects the cells improved functional outcomes, reduced demyelination/increased remyelination, and decreased neuroinflammation with increased $CD4^+CD25^+FOXP3^+$ Tregs and depleted $CD5^+$ T cells in the spinal cord (Chen et al., 2014). *In vitro* studies found that human ESC-NSCs reduced T cell proliferation and, as observed *in vivo*, increased the number of Tregs.

Although ESC-derived NSCs have shown promising preclinical results, their use is burdened with ethical concerns over the source of the cells, as the collection of ESCs destroys the donor blastocyte. Furthermore, residual pluripotency from contaminating undifferentiated ESCs remains a safety issue. Thus, alternative non-ESC sources are favored for future clinical applications.

Preclinical Evidence for iPSC-Derived Cells

Preclinical evidence for the feasibility of mouse iPSC-derived cells for autologous transplantation in MS was first demonstrated in EAE (Laterza et al., 2013). At the peak of disease, iPSC-derived NSCs were transplanted ICV into the cisterna magna, resulting in reduced demyelination and axonal damage, lower quantities of infiltrating inflammatory cells, improved functional outcomes, and the activation of the LIF pathway, as described for ESC-NSCs (Cao et al., 2011). Neither toxicity nor tumorigenicity was observed (Laterza et al., 2013). Furthermore, iPSC-NSCs displayed homing effects like those of somatic NSCs (Pluchino et al., 2003), and were found to localize to either demyelinating lesions or sites of increased inflammatory cell infiltration. In line with the *bystander* hypothesis, most of the transplanted cells did not differentiate and replace damaged neural cells, but rather their regenerative effects stemmed from the iPSC-NSC secretion of LIF, a neuroprotective, trophic cytokine that promotes endogenous OPC and oligodendrocyte growth (Laterza et al., 2013). Similarly, a later study performed ICV transplants of iPSC-NSCs at peak of disease (18 dpi) and observed amelioration of EAE symptoms and decreased T cell infiltration (Zhang et al., 2016).

Induced pluripotent stem cells can be differentiated into specific neural cell types *in vitro*, including oligodendrocytes or OPCs. In PMS there is a loss of endogenous OPC functionality and thus attempts have been made to replace these cells with pre-differentiated iPSC-OPC transplants. When transplanted ICV into marmoset and mouse models of EAE, iPSC-OPCs were shown to decrease inflammatory cell infiltration, reduce demyelination, and improve functional outcomes through bystander effects, with minimal transplanted cells surviving within the CNS parenchyma. However, if transplanted directly into the parenchyma, histopathological analysis revealed that the majority of iPSC-OPCs differentiated into mature oligodendrocytes capable of remyelinating axons, as observed via electron microscopy, while the remainder retained their OPC characteristics or differentiated into astrocytes (Thiruvalluvan et al., 2016).

Nevertheless, iPSC technology faces a number of hurdles to be addressed before successful clinical translation. There is growing evidence that the epigenetic signature of the donor cell can be maintained after iPSC induction. This can lead to issues of immune rejection of the transplants (Zhao et al., 2011), or unexpected iPSC functions. In one example, iPSC-NSCs generated from blood samples of PPMS patients lacked the neuroprotective phenotype observed in control iPSC-NSCs when transplanted into cuprizone-induced mouse models of demyelination (Nicaise et al., 2017). Furthermore, the two-step induction process required to generate iPSC derived neural cells and the expansion needed to produce sufficient cell counts for transplants is very lengthy, increasing the chance of genetic instability leading to tumorigenicity upon transplantation (Lee et al., 2013).

Thus, despite potentially exciting *in vivo* results, iPSCs and iPSC-derived neural cells still face substantial barriers to clinical translation.

Preclinical Evidence for iNSCs

In recent years iNSC technology has emerged as a promising stem cell-based approach to CNS regeneration. iNSCs can be directly transdifferentiated from somatic cells such as fibroblasts, bypassing a potentially hazardous pluripotency stage and, in the case of autologous transplants, largely circumventing immunogenicity concerns.

Several studies have shown that iNSCs can be stably expanded *in vitro* and, like NSCs, secrete pro-regenerative molecules such as glia cell-derived neurotrophic factor (GDNF) and brain derived neurotrophic factor (BDNF) (Gao et al., 2017). Moreover, transplanted iNSCs have demonstrated long-term functional integration into the CNS *in vivo*, highlighting their potential for regenerative applications (Hemmer et al., 2014).

In shiverer mouse models of dysmyelination, iNSCs transplanted into the cerebellum of post-natal day 1 pups were able to differentiate, albeit slowly over 10 weeks, into functional oligodendrocytes capable of myelination (Lujan et al., 2012). Similarly, iNSCs grafted into the chronically demyelinated corpus callosum of cuprizone-treated mice were found to differentiate along oligodendrocyte and astrocyte lineages, although many remained undifferentiated

(Sullivan et al., 2020). While iNSC transplantation was not found to mitigate demyelination in this model, endogenous oligodendrocytes and proliferating oligodendrocyte progenitors were increased in iNSC recipients. Notably, astrogliosis was significantly reduced in mice receiving transplanted iNSCs, with a concomitant amelioration of motor deficits (Sullivan et al., 2020). In the more PMS-relevant model of EAE, iNSCs transplanted ICV were found to be therapeutically equivalent to their somatic NSC counterparts, migrating to meningeal perivascular areas of EAE-affected mice where they reprogrammed pro-inflammatory MPs with resulting amelioration of chronic neuroinflammation and associated behavioral deficits (Peruzzotti-Jametti et al., 2018).

Overall, several putative stem cell therapies have demonstrated compelling preclinical safety and efficacy data in the context of MS models, with a consensus on the beneficial bystander/paracrine effects of the transplants, including during more chronic timepoints. Going forward, further characterization of the mechanisms of action, in support of optimized administration variables such as the timing, route, and dose of cells delivered, will be invaluable in translating preclinical evidence into clinical study.

CLINICAL STUDIES OF STEM CELL THERAPIES IN PROGRESSIVE MULTIPLE SCLEROSIS

Despite a wealth of preclinical data supporting the potential of cell therapies in treating neuroinflammatory conditions such as MS, definitive clinical evidence is lacking (Smith et al., 2020). While the last two decades have seen numerous early phase clinical trials of putative stem cell therapies in treating MS, with compelling evidence of the safety and feasibility of the approach, there is a strong need for more robust efficacy data to assert the translational potential of the approach. Most published clinical trial data relates to small safety and tolerability studies not statistically powered or appropriately controlled to infer therapeutic benefit, while the heterogeneity of larger studies has made it difficult to measure the efficaciousness of specific interventions. Reported trials exhibit variability in a diversity of parameters, including the makeup of the patient cohort, trial design, and nature of the therapy (Smith et al., 2020). While suggestions of beneficial outcomes often emerge from these clinical studies, larger and/or more rigorous studies are needed to discern signal from noise before we can gauge the potential impact of emerging stem cell therapies on the lives of PMS patients (Pluchino et al., 2020). The following is an overview of clinical studies in support of the therapeutic use of stem cell therapies, with a particular focus on nHSC interventions (summarized in **Supplementary Table 1**).

Clinical Evidence for HSCs in PMS

Some of the earliest clinical evidence of the utility of cell therapy in treating MS has arisen serendipitously, with reports of MS patients experiencing clinical improvements after undergoing immunoablation and allogeneic bone marrow transplantation for the treatment of concomitant leukemic or lymphoid malignancies (van Bekkum et al., 1996; McAllister et al., 1997).

Chemotherapeutic depletion of autoreactive immune cells followed by reconstitution through HSC transplantation (HSCT, employing bone marrow, peripheral blood, or umbilical cord blood sources) has been undergoing clinical study for several decades (Bakhuraysah et al., 2016; Muraro et al., 2017a; Oliveira et al., 2021), including in patients with PMS (Fassas et al., 1997), with beneficial functional outcomes commonly associated with the treatment. While intervention-associated morbidity and mortality has decreased substantially since the earliest reports (Snowden et al., 2017; Das et al., 2019), there remain ongoing efforts to delineate the optimal compromise between efficacy and safety with regards to the immunoablative conditioning regime (Das et al., 2019; Miller et al., 2021). Autologous HSCT is preferred over allogeneic transplants due to a significantly higher risk of adverse events such as graft-versus-host disease in the latter (Lu et al., 2010), as well as a lack of supporting clinical data for allogeneic grafts (Sharrack et al., 2020).

Evidence from long-term clinical studies points to beneficial effects of autologous HSCT in impeding disability worsening over the 5–10 years post-transplant (Muraro et al., 2017b; Boffa et al., 2021) with an impact exceeding that of DMTs (Muraro et al., 2017a), however, the efficacy of the treatment appears to favor younger patients, those with RRMS, and those with lower disability scores. On the recommendations of groups such as the (United States) National Multiple Sclerosis Society (Miller et al., 2021) and European Society for Blood and Marrow Transplantation (Sharrack et al., 2020), autologous HSCT may be beneficial for the treatment of *active* RRMS that is refractive to current DMTs (or in cases wherein these are contraindicated). Indeed, some appraisals suggests that HSCT may represent a cost-effective alternative to DMTs for the treatment of RRMS (Burt et al., 2020), however, larger studies are ongoing to ascertain whether HSCT is truly advantageous to current best standards of care [e.g., (Burt et al., 2019; Zhukovsky et al., 2021), and the BEAT-MS trial, NCT04047628]. While promising for the treatment of RRMS, the weight of clinical evidence suggests that autologous HSCT has limited efficacy in treating chronic PMS without superimposed disease activity (Bowen et al., 2012; Miller et al., 2021).

This observation supports the concept of a switch from an adaptive immune response mediated by infiltrating lymphoid cells during *active/relapsing* MS toward a compartmentalized CNS inflammation with a substantial (non-hematopoietic) MP-driven innate immunity component during PMS (Pluchino et al., 2020).

Clinical Evidence for MSCs in PMS

In terms of a putative "*regenerative*" therapies, non-hematopoietic cell sources are typically employed. MSCs have received the most attention, both with regards to applications in MS (Oliveira et al., 2019) and more generally (Tavakoli et al., 2020), although their therapeutic activity in the neural context appears to derive primarily from immunomodulatory and trophic effects on the pathobiology rather than integration

and cell replacement (Freedman et al., 2010; Dulamea, 2015; Wu et al., 2020).

Most of these studies have employed autologous BM-MSCs or AD-MSCs (occasionally in the form of a stromal vascular fraction, AD-SVF, a heterogeneous cell fraction including MSCs, HSCs, and various myeloid and lymphoid cells, amongst others), but allogeneic MSCs from umbilical cord or placental sources have also been trialed. A number of phase 1 clinical studies have demonstrated the feasibility and safety of the IV (Cohen et al., 2018; Feng et al., 2019; Iacobaeus et al., 2019) or IT (Mohyeddin Bonab et al., 2007; Sahraian et al., 2019) administration of BM-MSCs, although in one study a large combined IT and intracisternal dose of MSCs (100×10^6 cells) was associated with transient encephalopathy, with seizures in one recipient (Yamout et al., 2010). In several of these pilot studies hints of therapeutic benefit were observed, but small enrolment sizes and a lack of controls preclude an assessment of efficacy.

An ongoing phase 1 study (NCT03069170) is comparing IV and IT BM-MSC administration routes with respect to primary outcomes of magnetic resonance imaging (MRI) metrics, safety, and functional changes, while a recent case report describes reduced radiological inflammatory activity and clinical stabilization in a RRMS patient receiving multiple IT and IV treatments over a period of 4 years (Hou et al., 2013). Notably, the patient received treatments of both autologous BM-MSCs and allogeneic UC-MSCs with adverse events reported only with the allogeneic treatment (Hou et al., 2013), but these were minor and may relate to the larger dosages employed in the IV UC-MSC transplants.

In other phase 1 clinical study of allogeneic sources serious but transient adverse events (including an anaphylactoid reaction and superficial thrombophlebitis) were reported in the highest-dose (600×10^6 cells) IV placental MSC treatment group of a placebo-controlled study (Lublin et al., 2014), whereas only minor transient reactions (dizziness, headache, irritation) were reported in a very small study of multiple UC-MSC treatments (seven IV administrations per patient, each $1–6 \times 10^6$ cells/kg body weight) (Meng et al., 2018). Both studies included participants with SPMS and generated some early evidence of disease stabilization and mitigation of clinical symptoms.

In phase 1 safety studies of autologous AD-SVFs, three IT administrations of up to 14.2×10^6 cells did not lead to serious adverse events (Siennicka et al., 2016), whereas a study of participants receiving between 1 and 15 ICV cell administrations revealed several instances of treatment-related transient meningismus and additional complications related to the use of implanted conduits in delivering the cells (Duma et al., 2019). The safety of autologous endometrial MSCs administered IV to women with SPMS is also currently being explored in a placebo-controlled phase 1 study in Iran (Iranian Registry of Clinical Trials: IRCT20190711044175N1).

Studies of the efficacy of MSCs on MS have likewise involved both autologous and allogeneic sources, with a mix of IV and IT administration. Uncontrolled phase 1/2 studies have further affirmed the safety of the intervention, but with clinical outcomes ranging from no significant effect (Dahbour et al., 2017) through to short-to-intermediate-term stabilization of disease activity and/or progression (Stepien et al., 2016), modest improvements in function or quality of life (Riordan et al., 2018), and long-term reductions in relapse occurrence (Lu et al., 2020).

In a small, placebo-controlled phase 2 crossover clinical study of IV-delivered autologous BM-MSCs, evidence of non-significant decreases in MRI lesion activity and circulating Th1 cell counts is reported (Llufriu et al., 2014).

A further phase 2 study of multiple IV administrations of UC-MSCs found treatment-associated reductions in clinical scores and symptoms, and number of relapses, with serum analyses suggesting a shift from Th1-like (pro-inflammatory) to Th2-like (anti-inflammatory) immune responses in treated patients (Li et al., 2014).

The ambitious MEsenchymal StEm cells for Multiple Sclerosis (MESEMS) study, a placebo-controlled, crossover phase 1/2 study of autologous BM-MSCs, incorporated multiple partially independent studies from different centers under harmonized protocols to overcome funding constraints and improve statistical power (Uccelli et al., 2019). Preliminary results support the safety of the approach (single IV injection of $1–2 \times 10^6$ MSCs per kg of body weight), but with no impact on the number of contrast-enhancing lesions by MRI at 24 weeks, the primary efficacy endpoint (Uccelli et al., 2020).

Any benefit of MSC transplantation in treating PMS specifically is often obfuscated by trial cohorts including both RRMS and PMS patients. Even those trials focused specifically on PMS often enroll participants with *active* PMS, a population apparently more responsive to the immunomodulatory effects of MSCs.

In a placebo-controlled phase 2 crossover study comparing the IT and IV administration of autologous BM-MSCs to participants with PPMS and SPMS, those having had a relapse or MRI activity in the year prior to treatment were more responsive to treatment (Petrou et al., 2020). Overall, the inhibition of disease progression and beneficial functional outcomes were observed, with IT administration eliciting a more significant effect than IV, and two MSC injections (at a 6-month interval) proving more efficacious than a single dose. This study was a follow-up to an earlier single-arm phase 1/2 trial of IT administration of autologous BM-MSCs to MS and amyotrophic lateral sclerosis patients in which acute immunomodulatory effects and a statistically significant improvement in EDSS scores were observed in the MS cohort during 6 months of follow-up (Karussis et al., 2010).

Other PMS-specific trials include a phase 2 study of IT autologous BM-MSC administration in which no significant improvements where observed in PMS patients (Bonab et al., 2012), and a placebo-controlled, dose-ranging phase 1/2 study of IV autologous AD-MSCs in which inconclusive signs of efficacy in SPMS were inferred from changes in the number of MRI lesions and evoked potential parameters (Fernandez et al., 2018).

On the other hand, a phase 1/2 study of combined IT and IV UC-MSCs in SPMS patients showed decreased relapse frequency and/or lesion severity, improvement in clinical scores, and evidence of peripheral immunomodulation (Lu et al., 2013). Similarly, a single-arm phase 1/2a study of IV autologous BM-MSCs in SPMS patients revealed improvements in visual acuity, reduction of visual evoked response latencies,

and protection of the optic nerve area by optical coherence tomography (OCT) in the 6 months following transplantation (Connick et al., 2011, 2012).

Several additional randomized phase 1/2 trials have been completed but the results not yet reported, including placebo-controlled studies of IV-administered autologous BM-MSCs in RRMS patients and a crossover study in RRMS and SPMS participants, and a study comparing the beneficial effects of combined IT and IV administration of UC-MSCs (and a follow-up booster of MSC-conditioned media) with supervised physical therapy (Alghwiri et al., 2020).

Ongoing clinical studies include a unique phase 1 study (NCT02795052) comparing IV administration of autologous BM-MSCs with a combination of IV and intranasal delivery, currently recruiting participants across various neurological conditions, including MS (Weiss and Levy, 2016), as well as a larger, controlled phase 2/3 study of autologous BM-MSCs on RRMS relapse rates and clinical/radiological outcomes employing an initial IV dose followed up with an IT booster (IRCT20191004044975N1).

Progressive MS-specific trials currently underway include a phase 1/2 placebo-controlled crossover study of IT autologous BM-MSCs exploring neurophysiological, functional, and quality-of-life outcomes in both PPMS and SPMS patients (NCT04749667), a single-arm phase 1/2 studies of the effects autologous AD-MSCs clinical, radiological, and immunological measures in SPMS patients (NCT03696485 and IRCT20091127002778N1), and a single-arm phase 2 study of BrainStorm Cell Therapeutics' NurOwn product (neurotrophic factor-expressing autologous BM-MSCs) upon multiple IT administration to PMS patients (NCT03799718).

In order to increase the regenerative potential of MSCs, a number of clinical studies by the Tisch MS Research Center of New York have employed MSC neural progenitors (MSC-NPs), a subpopulation of mesenchymal cells that exhibit neuroectodermal lineage characteristics and are likely to be more CNS compatible while retaining the immunomodulatory and trophic capabilities of common MSCs (Harris et al., 2012). The tolerability of the treatment was established by a phase 1 dose-escalating IT administration study in people with PMS, which supported feasibility and long-term safety with post-treatment improvement of clinical scores in 4 (out of 6) participants (Harris et al., 2016). A follow-up single-arm phase 1 study, comprising 3 IT treatments of MSC-NPs administered at 3-month intervals, found an improved mean EDSS score over 12 months of follow-up sustained by 7 (out of 20) participants at 2 years (Harris et al., 2018, 2021). Effects were found to be more pronounced in ambulatory (low/medium-disability) SPMS participants, rather than (non-ambulatory), PPMS participants. Clinical outcomes also included signs of improved muscle strength (70% of participants) and bladder function (50% of participants) (Harris et al., 2018), while cerebrospinal fluid biomarker changes, including a decrease in CCL2 and increases in IL-8, HGF, and CXCL12, were found to reflect treatment-related immunoregulatory and trophic effects (Harris et al., 2021). These outcomes have inspired an ongoing placebo-controlled phase 2 crossover trial (NCT03355365), which is recruiting 50 PMS participants to receive a total of 6 IT treatments at 2-month intervals, with functional outcomes (including clinical scores and bladder function) assessed over 36 months of follow-up. Participants in this trial are to be ambulatory (i.e., EDSS $\leq$ 6.5) and will be stratified according to baseline EDSS score and disease subtype (PPMS or SPMS); an expanded-access study for those that do not meet eligibility criteria is also planned (NCT03822858).

Clinical Evidence for NSCs and Pluripotent Stem Cells in PMS

Two dose-response phase 1 safety and tolerability clinical studies of bona fide allogeneic fetal-derived NSC transplantation in PMS patients have been conducted.

Clinical trial NCT03269071 (IRCCS Ospedale San Raffaele) has enrolled 4 cohorts of 3 PMS participants each to receive an IT dose of 0.7–5.7 $\times$ 10^6 cells/kg, with quality-of-life outcomes to be assessed, while trial NCT03282760 (Casa Sollievo della Sofferenza IRCCS) has treated 24 SPMS participants with an ICV dose of 5–24 $\times$ 10^6 cells each, examining functional, cognitive, and neurophysiological changes post-treatment. Both trials have been completed but outcomes are yet to be reported at the time of writing.

While iPSC-derived cells have begun to see study in other clinical contexts [e.g., iPSC-derived NSCs in spinal cord injury (Nagoshi et al., 2020) and iPSC-derived retinal pigment epithelial cells for treating age-related macular degeneration (Mandai et al., 2017)], the technology has yet to undergo trials in MS despite its potential (Fossati and Douvaras, 2014). Concerns regarding the safety of pluripotent cells sources is likely to be a key factor impeding their clinical development (Ortuño-Costela et al., 2019), although case reports of *embryonic* stem cell transplants in MS patients have been published (Shroff, 2016). The use of iNSCs, generated by direct reprogramming of somatic cells and thus bypassing the pluripotent state, may represent an attractive alternative approach (Xie et al., 2016).

Thus, evidence to date supports the safety and feasibility of HSC and MSC therapies in MS, with immunomodulatory and/or trophic mechanisms of action providing modest, transient clinical benefits in RRMS or *active* PMS. More robust clinical studies and systematic reviews (see e.g., Rahim et al., 2019) will be required to establish the extent of these benefits and whether cell therapies provide advantages over current best standards of care. There is little current clinical evidence supporting the efficacy of HSC or MSC transplants in treating PMS, which will likely require novel mechanisms through which to not only halt disease progression but also foster CNS repair. NSCs, somatic or induced, may be key to providing the regenerative potential necessary to combat the gradual accumulation of disability arising from PMS, but clinical studies in this area are in their infancy.

CONCLUSION

While the treatment of MS continues to advance, therapeutic options remain restricted to ameliorating or preventing relapses and acute inflammation events in RRMS or *active* PMS. There are

no proven interventions able to halt the gradual accumulation of disability associated with PMS, let alone effectively promote repair of the damaged CNS. The prospect of stem cell therapy brings with it great promises of regenerative potential, yet the weight of preclinical and clinical evidence to date points to immunomodulatory and trophic effects that are most advantageous to addressing *relapsing/active* forms of the disease. The best hopes for an impact on PMS perhaps lay with NSCs, either somatic or transdifferentiated, which have given rise to compelling preclinical evidence for the amelioration of chronic neuroinflammation through novel mechanisms of action, but have yet to see substantial clinical study in the context of MS. Ultimately, while the safety and feasibility of stem cell transplantation has been demonstrated across various cell types and administration routes, there remains a need for larger and/or more rigorous studies to quantify the benefits of stem cell therapy and demonstrate an advantage over current best standards of care. Whether stem cell therapies have the potential to repair the PMS CNS in a clinical setting remains to be seen.

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SUPPLEMENTARY MATERIAL

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Exosomes Derived From Adipose-Derived Mesenchymal Stem Cells Ameliorate Radiation-Induced Brain Injury by Activating the SIRT1 Pathway

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Objective: Studies have shown that the therapeutic effects of mesenchymal stem cells (MSCs) are mediated in a paracrine manner, mainly through extracellular vesicles such as exosomes. Here, we designed a study to investigate whether exosomes derived from adipose-derived mesenchymal stem cells (ADMSC-Exos) had protective effects in a rat model of radiation-induced brain injury and in microglia.

Methods: Male adult Sprague-Dawley (SD) rats were randomly divided into three groups: the control group, the radiation group (30 Gy), and the radiation + exosomes group (30 Gy + 100 ug exosomes). Meanwhile, microglia were divided into four groups: the control group, the radiation group (10 Gy), the radiation + exosomes group (10 Gy + 4 ug exosomes), and radiation + exosomes + EX527 group (10 Gy + 4 ug exosomes + 100 nM EX527). Tissue samples and the levels of oxidative stress and inflammatory factors in each group were compared.

Results: Statistical analysis showed that after irradiation, ADMSC-Exos intervention *in vivo* significantly reduced the levels of caspase-3, malondialdehyde (MDA), 8-hydroxydeoxyguanosine (8-OHdG), tumor necrosis factor-α (TNF-α), interleukin-4 (IL-4), and promoted the recovery of superoxide dismutase (SOD), catalase (CAT), IL-4, and IL-10. Moreover, ADMSC-Exos intervention inhibited microglial infiltration and promoted the expression of SIRT1. Furthermore, the results *in vitro* showed that the above effects of ADMSC-Exos could be reversed by SIRT-1 inhibitor EX527.

Conclusion: This study demonstrated that ADMSC-Exos exerted protective effects against radiation-induced brain injury by reducing oxidative stress, inflammation and microglial infiltration via activating the SIRT1 pathway. ADMSC-Exos may serve as a promising therapeutic tool for radiation-induced brain injury.

Keywords: radiation, brain injury, exosomes, mesenchymal stem cells, oxidative stress

INTRODUCTION

Radiotherapy plays an important role in the treatment of brain tumors, head and neck cancers, and arteriovenous malformations (Na et al., 2014). However, radiotherapy not only causes tumor cell necrosis but also affects the surrounding healthy tissue, and patients who receive radiotherapy are at risk of developing radiation-induced brain injury (RIBI) (Rahmathulla et al., 2013). The process of radiation-induced brain injury is a complicated, cascaded and dynamic process, and several theories have been proposed to explain the development of radiation-induced brain injury, including direct injury from radiation (Oh et al., 2013), damage to the cerebrovascular system (Kamiryo et al., 2001), immunoinflammatory responses (Lumniczky et al., 2017; Bostrom et al., 2018), and oxidative stress (Liao et al., 2017). In recent years, several preclinical studies have demonstrated that interventions such as modulating inflammation and reducing oxidative stress injury can prevent or ameliorate radiation-induced brain injury. One of those interventions is mesenchymal stem cell (MSC) therapy (Balentova and Adamkov, 2015).

Mesenchymal stem cells have emerged as promising therapeutic measures for different clinical applications, including tissue engineering, autoimmune diseases, and graft-versus-host diseases (Kiang, 2016; Drommelschmidt et al., 2017). Previous studies have demonstrated that MSCs could protect against radiation-induced liver injury (Francois et al., 2013), promote wound healing in radiation-induced skin injury (Xie et al., 2013; Sun et al., 2019), improve survival and mitigate gastrointestinal syndrome in irradiated mice (Hu et al., 2010; Kiang, 2016). However, in recent years, an increasing number of studies have shown that the therapeutic effects of MSCs are mediated in a paracrine manner, mainly through extracellular vesicles such as exosomes (Drommelschmidt et al., 2017; Giebel et al., 2017). Exosomes are small biological lipid membrane vesicles with diameters of 40–100 nm (Trams et al., 1981). They serve as vital extracellular communicators by transporting their contents, such as microRNAs (miRNAs), messenger RNAs (mRNAs), cell adhesion molecules, cytokines and proteins, to target recipient cells (Ren, 2018). Whether exosomes from MSCs can alleviate radiation-induced brain injury is still unclear.

Thus, we designed this study to investigate whether exosomes derived from adipose-derived MSCs (ADMSC-Exos) had neuroprotective effects on the injured brain tissues of irradiated animals.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley (SD) rats weighing between 250 and 300 g were provided by the Experimental Animal Center of the Air Force Military Medical University. All rats were housed five per cage under a constant 12-h light/dark cycle at room temperature. Food and water were available *ad libitum*. All experimental protocols and animal handling procedures were performed in accordance with the National Institutes of Health (NIH) guidelines for the use of experimental animals and were approved by the Institutional Animal Care and Use Committee of the Air Force Military Medical University.

Irradiation

Irradiation was performed using a ^{60}Co gamma-ray facility (Radiation Center of Air Force Military Medical University, Xi'an, China) at a dose rate of 1.59 Gy/min and a total dose of 30 Gy. Animals were placed in perforated plastic bottles before receiving a single dose of 30 Gy by stereotactic irradiation (at a dose rate of 1.59 Gy/min and a distance of 65 cm from the source). The animals' movements were restricted by anaesthetization. The head of each rat was placed in the exposure field (5×2.5 cm^2) which covered the whole brain from the postaurem line to the post canthus line.

Cell Culture

Adipose-Derived Mesenchymal Stem Cells Harvest and Culture

Adult male SD rats weighing between 250 and 300 g were anesthetized by isoflurane inhalation, and subcutaneous adipose tissue in the groin was carefully dissected. Afterward, the animals were sacrificed. The adipose tissue was minced and washed with phosphate-buffered saline (PBS) twice and then digested with collagenase type I (CLS I; 2 mg/ml; Gibco, Grand Island, United States) for 60 min at 37°C with shaking. After centrifugation for 10 min at 620 × *g* and the removal of the supernatant, the precipitate was resuspended in ADMSCs culture medium consisting of DMEM/F12 (Gibco, Life Technologies, Carlsbad, CA, United States) containing 100 U/ml penicillin, 100 mg/ml streptomycin (Gibco BRL, Grand Island, NY, United States), 0.2 mM L-ascorbic acid-2-phosphate (A2P; Sigma, St. Louis, MO, United States), and 10% fetal bovine serum (Excell Bio, Shanghai, China). The cells were cultured at 37°C in a humidified incubator with 5% CO_2. For characterization of ADMSCs differentiation, Oil-Red-O, Alizarin red, and Alcian blue staining were conducted to identify adipogenic, osteogenic, and chondrogenic differentiation, respectively. After three passages, ADMSCs were cultured in adipogenesis-, osteogenesis-, or chondrogenesis-inducing medium for 2 weeks. Then, the cells were stained with Oil-Red-O, Alizarin red, and Alcian blue.

Rat Microglia Culture

Primary microglia from SD rats were obtained from the Neurological Lab of the Tangdu Hospital in China. Microglia were maintained in Dulbecco's Modified Eagle's Medium containing 5% fetal bovine serum (Excell Bio, Shanghai, China), 100 mg/l streptomycin and 100 units/ml penicillin (Gibco BRL, Grand Island, NY, United States) in a humidified 5% CO_2, 95% air atmosphere. Microglia were seeded into 24-well plates at 1×10^5 cells/well 48 h before irradiation.

Isolation and Characterization of Exosomes

Third-generation ADMSCs were cultured in DMEM/F12 supplemented with 10% exosome-depleted FBS for 48 h, and the culture supernatants were carefully collected. For exosome isolation, the cells and cell debris in the supernatants were removed by centrifugation at 3,000 × *g* for 15 min. Then, the

exosomes were isolated from culture supernatants of ADMSCs using a total exosome isolation kit (Invitrogen, Asheville, NC, United States) according to the manufacturer's instructions. Isolated exosomes were suspended in PBS and stored at −80°C. The protein content of exosome suspension was quantified using the bicinchoninic acid (BCA) protein assay kit (Beyotime, Shanghai, China). The morphology of isolated exosome was analyzed by transmission electron microscopy (TEM), and the distribution of size was analyzed by nanoparticle tracking analysis (NTA; Zeta View system). We further characterized exosomes by western blot analysis of the exosomal markers CD9, CD63, and CD81 (Abcam, Cambridge, United Kingdom).

Experimental Design

(i) Investigate whether ADMSC-Exos treatment can alleviate radiation-induced brain injury. Animals were randomly assigned to three groups as follows: (1) control group (Ctr group): SD rats were injected with 200 μl of PBS via the tail vein; (2) radiation group (RT group): SD rats were injected with 200 μl of PBS via the tail vein 15 min before irradiation; and (3) radiation + exosomes group (RT + Exo group): SD rats were injected with 100 μg of exosomes (suspended in PBS to a final volume of 200 μl) via the tail vein 15 min before irradiation. Both the RT group and RT + Exo group were irradiated with 30 Gy. Twenty-five rats per group were used in this study.

(ii) Evaluate the influences of ADMSC-Exos on the irradiated microglia and explore the role of SIRT-1 in this process. Firstly, microglia were randomly divided into three groups: (1) control group (Ctr group): cells were treated with 10 μl PBS; (2) radiation group (RT group): cells were treated with 10 μl PBS 15 min before irradiation; (3) radiation + exosomes group (Exo group): cells were preincubated with exosomes (2.0, 4.0, or 8.0 μg suspended in PBS to a final volume of 10 μl) 15 min before irradiation. Cells in the RT and Exo groups were irradiated with a single dose of 10 Gy at a rate of 3 MeV/min. The cell samples and culture supernatants were collected at 24 h after irradiation, oxidative stress and inflammatory factors were detected. Based on the results of the first step, the second step was carried out. Microglia were randomly divided into four groups: (1) control group (Ctr group): cells were treated with 10 μl PBS; (2) radiation group (RT group): cells were treated with 10 μl PBS 15 min before irradiation; (3) radiation + exosomes group (Exo group): cells were preincubated with 4.0 μg exosomes (suspended in PBS to a final volume of 10 μl) 15 min before irradiation; (4) radiation + exosomes + EX527 group (EX527 group): cells were treated with 100 nM EX527 15 min before irradiation and other interventions were the same as Exo group. Cells in the RT, Exo and EX527 groups were irradiated with a single dose of 10 Gy at a rate of 3 MeV/min. The cell samples and culture supernatants were collected at 24 h after irradiation, the expressions of CD68, SIRT1, P65, oxidative stress markers, and inflammatory factors were detected.

Western Blot Analysis

Protein samples (5 μg/lane) from exosomes, hippocampus, and microglia were loaded onto polyacrylamide gels, separated by electrophoresis, and then transferred to polyvinylidene difluoride membranes (PVDF). The membranes were blocked with 5% non-fat milk at room temperature for 3 h and incubated with rabbit monoclonal anti-rat CD63 (1:500, Abcam, Cambridge, United Kingdom), CD9 (1:500, Abcam, Cambridge, United Kingdom), CD81 (1:500, Abcam, Cambridge, United Kingdom), cleaved Caspase 3 (1:1,000, Cell Signaling Technology, Boston, United States),SIRT1 (1:1,000, Abcam, Cambridge, United Kingdom), CD68 antibodies (1:500, Abcam, Cambridge, United Kingdom), Ac-p65 (1:1,000, Cell Signaling Technology, Boston, United States) and anti-p65 (1:1,000, Cell Signaling Technology, Boston, United States) antibodies overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (1:3,000, Boster, Wuhan, China). Proteins were visualized by an enhanced chemiluminescence system (Alpha Innotech, United States).

Flow Cytometry

Adipose-derived mesenchymal stem cells at passages 3–5 were harvested. Approximately 1×10^5 ADMSCs were fixed in neutralized 2% paraformaldehyde (PFA) solution for 30 min, washed twice with PBS, and labeled with fluorescence-conjugated antibodies against CD29, CD31, CD44, and HLA-DR (BD Biosciences) at room temperature for 30 min. The primary antibodies were directly conjugated with FITC and phycoerythrin. Non-specific FITC-conjugated IgG was used to stain the cells as a control. The cells were then analyzed by a FACS Aria III (Becton-Dickinson, San Jose, CA, United States).

Fresh hippocampal tissues were harvested at various time points (0 h, 24 h, 3 days, and 7 days) from three rats in each group, and single-cell suspensions were prepared as described (Seibenhener and Wooten, 2012). For apoptosis analysis, cells were washed thrice with PBS. Then, cells were incubated with 100 μL of RNase (100 mg/L) for 30 min at 37°C and then stained with PI and annexin V-FITC at 4°C for 30 min. The cell cycle and apoptosis were monitored at 488 nm. Each sample containing 1×10^6 cells.

Histopathological Examination

The animals were sacrificed by cervical dislocation preirradiation (0 h) and at 24 h, 3 days, and 7 days after irradiation. Parts of sacrificed rats were initially perfused with 10% formalin. After fixation, a craniotomy was performed. Then, the harvested brain tissues were fixed with 10% formalin, dehydrated by graded ethanol, and embedded in paraffin. Five-micrometer-thick slices were cut for Nissl staining. The neuronal density was determined by the average number of surviving hippocampal Cornu Ammonis (CA) 1 in each 1-mm section, with three sections of bilateral hippocampal slices.

Measurement of Caspase-3 Activity

Hippocampal tissue was harvested at various time points (0 h, 24 h, 3 d, and 7 d), dissected, homogenized in chilled PBS (0.1 M, pH 7.4), and then centrifuged at 10,000 × *g* at 4°C for 10 min. The supernatants were collected, aliquoted, and stored at −80°C for subsequent analysis. The tissue protein concentration was determined by a BCA protein assay kit (Beyotime, Shanghai,

China). Caspase-3 activity in the hippocampus was determined by using a Caspase-3/CPP32 colorimetric assay kit (Beyotime, Shanghai, China) according to the manufacturer's instructions.

Measurement of Antioxidant Enzymes

The activities of superoxide dismutase (SOD) and catalase (CAT) in the hippocampus and microglia were determined by using commercial kits (Cayman Chemical Company, Ann Arbor, MI, United States) according to the manufacturer's instructions.

Measurement of Oxidative Products

The homogenates obtained as previously described were also used to measure the levels of malondialdehyde (MDA) and 8-hydroxydeoxyguanosine (8-OHdG). The levels of 8-OHdG and MDA were measured by commercial kits (Genox Corporation, Baltimore, MD, United States) according to the manufacturer's instructions.

Measurement of NO/Nitrite and Inflammatory Cytokines

NO production was indirectly assessed by measuring the nitrite levels in the culture supernatants by a commercial kit (Beyotime, Shanghai, China). The levels of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-4 (IL-4), and interleukin-10 (IL-10) in the hippocampus and the supernatant of microglia were measured by enzyme-linked immunosorbent assay (ELISA) kits (Beyotime, Shanghai, China) according to the manufacturer's instructions.

Immunohistochemistry

Immunohistochemistry was performed to detect changes of microglia in the hippocampus at 3 days after irradiation. Paraffin sections were initially soaked in xylene, deparaffinized by graded ethanol, permeabilized with 3% Triton X-100 for 10 min, blocked with 10% normal donkey serum in PBS for 60 min at room temperature and incubated overnight with primary antibodies at 4°C. Microglia were detected with rabbit anti-rat IBA-1 antibodies (1:500, Wako Pure Chemical Industries, Osaka, Japan). Primary antibodies were visualized by using Alexa Fluor 594-conjugated donkey anti-rabbit secondary antibodies (1:500, Molecular Probes, Eugene, OR, United States). After nuclear staining with DAPI, the sections were analyzed on a Nikon fluorescence microscope. The number of IBA1-positive cells were counted in six standardized fields. The specific steps were the same as we described before (Liu et al., 2015).

Immunohistochemistry was also performed to detect the expression of CD68 in Microglia at 24 h after irradiation. Microglia from 24-well were fixed with 4% PFA for 30 min, permeabilized with 0.1% Triton X-100 for 30 min, and then blocked with 1% BSA for 1 60 min. Thereafter, the cells were incubated with CD68 antibodies (1:200, Abcam, Cambridge, United Kingdom) and IBA antibodies (1:200, Molecular Probes, Eugene, OR, United States) overnight at 4°C. Then, cells were incubated with Alexa Fluor 594-conjugated donkey anti-rabbit secondary antibody (1:500, Molecular Probes, Eugene, OR, United States) at 37°C for 60 min and DAPI (10 μg/mL) for 5 min. Florescence images were acquired and processed by a Nikon fluorescence microscope.

Statistical Analysis

All statistical analyses were performed using SPSS 19.0, a statistical software package. The data generated are expressed as means ± SD. Statistical evaluation of the data was performed by one-way analysis of variance (ANOVA). A value of $p < 0.05$ was considered statistically significant.

RESULTS

After irradiation, all rats survived and showed normal daily activities, including feeding and drinking. No paralysis and convulsion were observed, and no significant difference was found in body weight gain among those groups.

Characterization of ADMSC-Exos

Under the microscope, ADMSCs presented as spindle-shaped and were aggregated in a swirled pattern. In addition, Oil-Red-O, Alizarin red, and Alcian blue staining showed that ADMSCs successfully differentiated into adipocytes, osteoblasts, and chondrocytes, respectively (**Figure 1A**). ADMSCs were identified by flow cytometry. A relatively high number of CD29- and CD44-positive cells and few CD31- and HLA-DR-positive cells were observed (**Figure 1B**). The exosome surface markers CD9, CD63, and CD81 were measured by western blotting, and the results showed that all three markers were highly expressed in the purified exosomes (**Figure 1C**). Exosomes appeared to be cup- or sphere-shaped particles as observed by TEM (**Figure 1D**). Exosomes isolated from 100 ml culture supernatants of ADMSCs were resuspended in 100 μl PBS, the results of BCA assay showed that the mean concentration of resuspended exosomes was 6.2 ug/ul. After diluting the resuspended exosomes 1000 times, NTA analysis was performed. NTA analysis identified the mean vesicle diameter was 126.5 nm and the concentration was 1.51×10^8 particles/ml (**Figure 1E**). These results in all indicated that the nanoparticles were consistent with the defined exosomes.

Adipose-Derived Mesenchymal Stem Cells Protected Hippocampal Cells From Radiation-Induced Apoptosis

Under the optical microscope, Nissl-stained sections from the Ctr group showed hippocampal neurons with regular arrangements and clearly visible nuclei. At 3 days after irradiation, chromatic agglutination and karyopyknosis were observed in both the RT and RT + Exo groups (**Figure 2A**). The numbers of neurons were significantly decreased in the CA1 region in the RT group ($96.3 \pm 23.2/mm^2$) and RT + Exo group ($136.2 \pm 27.8/mm^2$) compared with those in the Ctr group ($209.5 \pm 18.8/mm^2$) ($p < 0.05$). However, the numbers of

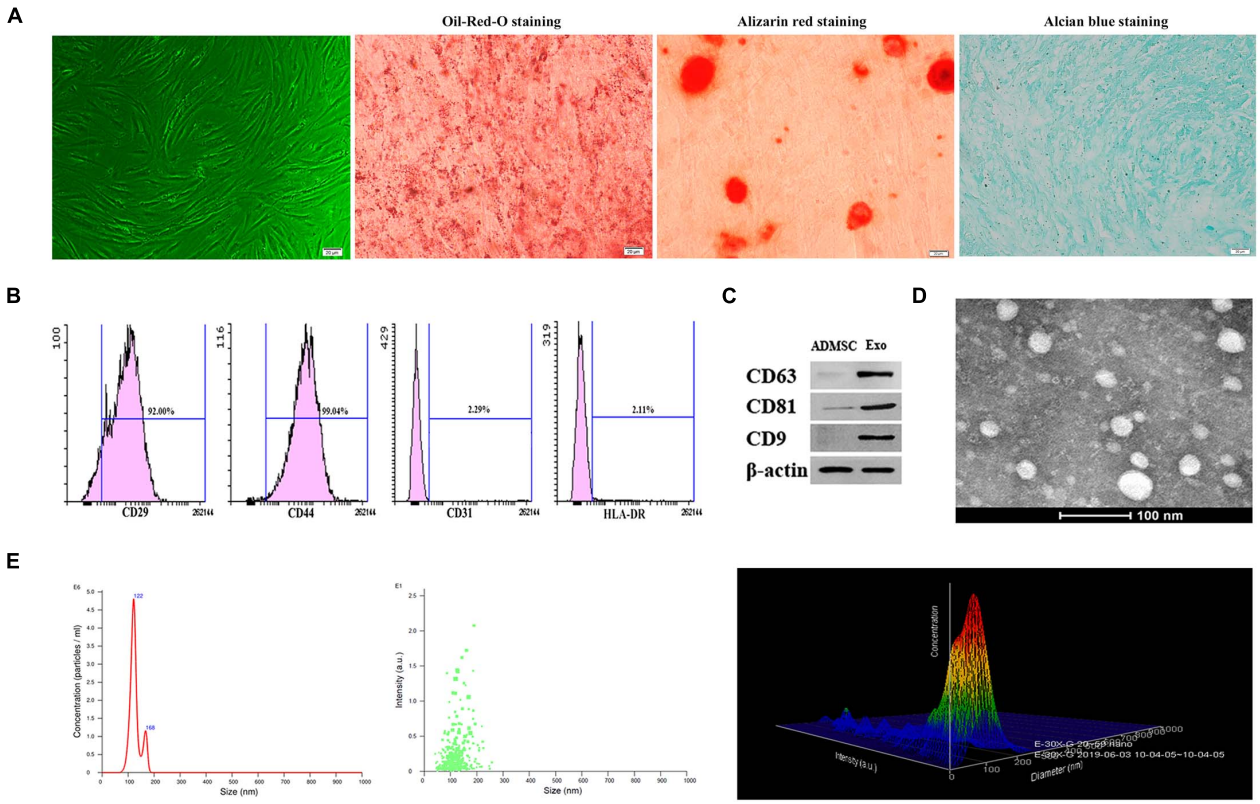

FIGURE 1 | Characterization of ADMSCs and authentication of ADMSC-derived exosomes (ADMSCs-Exos). **(A)** ADMSCs morphology observed by inverted microscope. Adipogenic, osteogenic, and chondrogenic differentiations measured by Oil-Red-O staining, Alizarin red staining and Alcian blue staining (scale bar = 20 μm). **(B)** Flow cytometry analysis showed that these cells were highly positive for CD29 (92%) and CD44 (99.04%), but negative for CD31 (2.29%), and HLA-DR (2.11%). **(C)**. Exosome specific markers (CD63, CD81, CD9) were detected by Western blot. **(D)** The morphology of ADMSCs-Exos analyzed by transmission electron microscopy images. **(E)** The particle size distribution of ADMSCs-Exos detected by nanoparticle tracking analysis (NTA).

neurons had no significant differences between RT group and RT + Exo group at 3 days after irradiation (**Figure 2B**). Flow cytometric analysis showed that the apoptosis rate in the Ctr group was significantly lower than that in the RT group or RT + Exo group at 24 h and 3 days after irradiation ($p < 0.05$). Compared with RT goup, ADMSC-Exos intervention significantly reduced the apoptosis rate in the RT + Exo group at 24 h after irradiation ($p < 0.05$). But no significant difference was found in apoptosis rate between the RT group and RT + Exo group at 3 days after irradiation (**Figures 2C,D**). Statistical analysis showed that the caspase-3 activities in the RT and RT + Exo groups was significantly higher than those in the Ctr group at 24 h and 3 days after irradiation ($p < 0.05$). At 24 h after irradiation, the caspase-3 activity in the RT group was significantly higher than that in the RT + Exo group ($p < 0.05$) (**Figure 2E**). Western blot analysis showed that at 24 h and 3 days after irradiation, the protein expression of cleaved caspase-3 in the RT + Exo and RT groups was significantly higher than that in the Ctr group ($p < 0.05$). At 24 h after irradiation, the protein expression of cleaved caspase-3 in the RT + Exo group was significantly lower than that in the RT group ($p < 0.05$) (**Figure 2F**). These results suggested that radiation could cause apoptosis of the hippocampal cells, and ADMSC-Exos intervention prevented radiation-induced apoptosis.

Adipose-Derived Mesenchymal Stem Cells Alleviated Radiation-Induced Oxidative Stress and Inflammation in the Hippocampus

The biomarkers of SOD, CAT, MDA, 8-OHdG, TNF-α, IL-1β, IL-4, and IL-10 in the hippocampus were measured preoperation (0 h) and at 24 h, 3 days, and 7 days after irradiation. SOD and CAT are the antioxidant enzymes which can counter the detrimental effects of free oxygen radicals. The results showed that the activities of SOD and CAT in the RT and RT + Exo groups were significantly lower than those in the Ctr group at all time points after irradiation ($p < 0.05$). Statistical analysis showed that the decreases in the SOD and CAT activities in the RT group were more significant than those in the RT + Exo group ($p < 0.05$) (**Figures 3A,B**).

Malondialdehyde and 8-OHdG are commonly measured indicators of free radical-induced damage. After irradiation, the levels of MDA and 8-OHdG in the RT and RT + Exo groups elevated at 24 h, reached a peak at 3 days, and decreased at 7

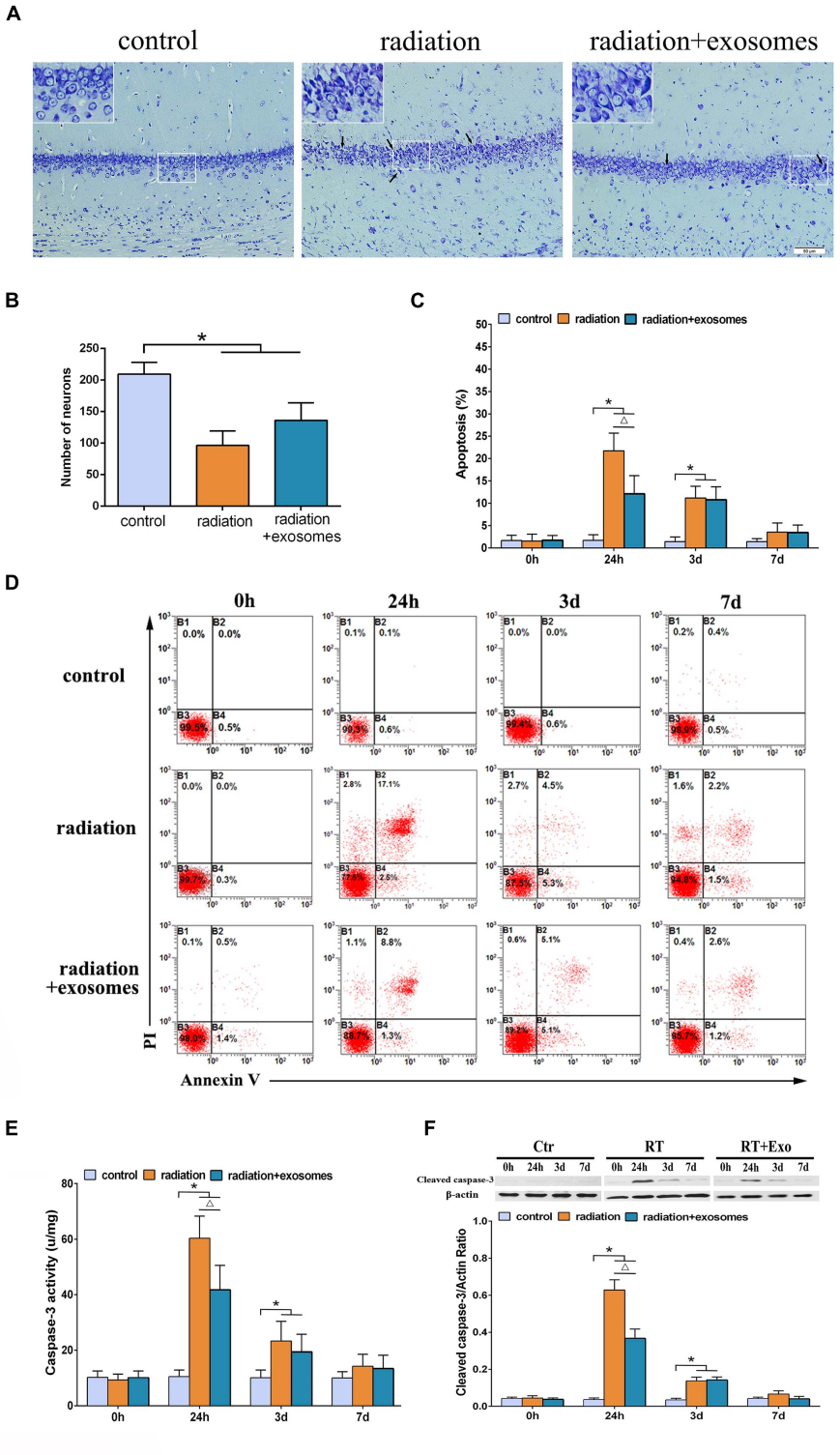

FIGURE 2 | Adipose-derived mesenchymal stem cells protected hippocampal cells from radiation-induced apoptosis. **(A)** Nissl-stained sections of the hippocampus CA1 area at 3 days after irradiation. Chromatic agglutination and karyopyknosis were observed in both the RT and RT + Exo groups. Arrows indicate the karyopyknosis (scale bar = 50 μm). **(B)** Neuron count in the hippocampal CA1 region at 3 days after irradiation ($n = 6$). **(C)** Proportions of apoptotic cells in hippocampus determined by flow cytometry ($n = 3$). **(D)** Representative flow cytometry plots of Annexin V FITC and PI stained cells. **(E)** Changes of caspase-3 activity in the hippocampus after irradiation ($n = 6$). **(F)** Expression of cleaved caspase-3 in the hippocampus after irradiation determined by Western blot ($n = 3$) (*$p < 0.05$, compared with the Ctr; $^{\triangle}$ $p < 0.05$, compared with the RT).

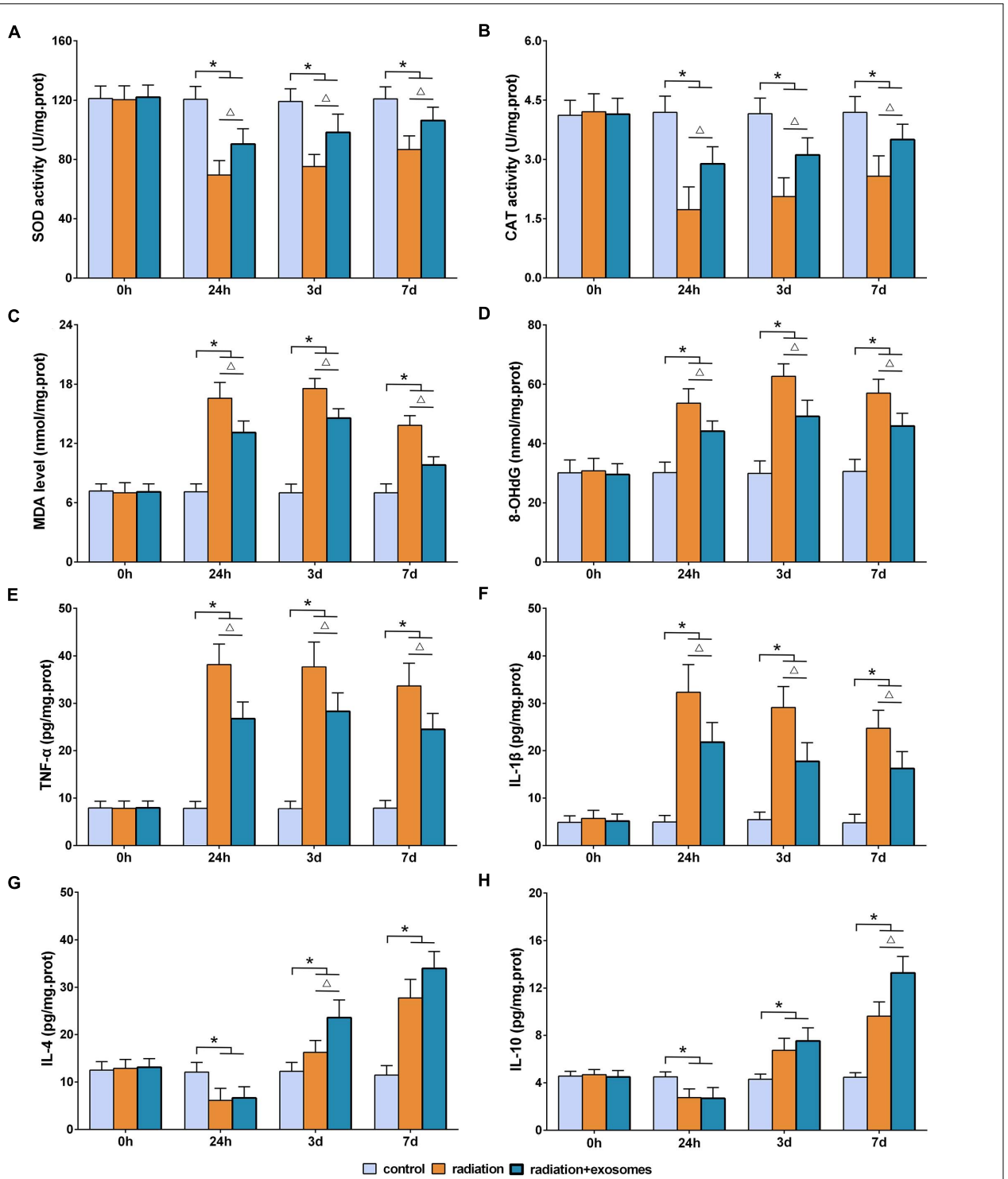

FIGURE 3 | Adipose-derived mesenchymal stem cells alleviated radiation-induced oxidative stress and inflammation in the hippocampus. Antioxidant enzymes SOD **(A)** and CAD **(B)**. Oxidative products MDA **(C)** and 8-OhdG **(D)**. Pro-inflammatory cytokines TNF-α **(E)** and IL-1β **(F)**. Anti-inflammatory cytokines IL-4 **(G)** and IL-10 **(H)**. *$p < 0.05$, compared with the Ctr; $^{\triangle}$ $p < 0.05$, compared with the RT. $n = 6$.

days. At all time points after irradiation, the levels of MDA and 8-OHdG in the RT and RT + Exo groups were significantly higher than those in the Ctr group ($p < 0.05$). Statistical analysis found significant differences in the levels of MDA and 8-OHdG between the RT group and the RT + Exo group at 24 h, 3 days, and 7 days after irradiation ($p < 0.05$) (**Figures 3C,D**).

In the RT and RT + Exo groups, the levels of TNF-α and IL-1β significantly increased at 24 h after irradiation, whereas the levels of IL-4 and IL-10 significantly decreased at 24 h after irradiation. The levels of TNF-α, IL-1β, IL-4, and IL-10 in the RT and RT + Exo groups were significantly higher than those in the Ctr group at 3 and 7 days after irradiation ($p < 0.05$). And the levels of TNF-α and IL-1β in the RT group were significantly higher than those in the RT + Exo group at all time points after irradiation ($p < 0.05$) (**Figures 3E,F**). Statistical analysis found significant differences in the levels of IL-4 at 3 days after irradiation and the levels of IL-10 at 7 days after irradiation between the RT group and the RT + Exo group ($p < 0.05$) (**Figures 3G,H**). Here, our results demonstrated that ADMSC-Exos had potent ability to alleviate radiation-induced oxidative stress and inflammation in the hippocampus.

Adipose-Derived Mesenchymal Stem Cells Inhibited Radiation-Induced Microglial Infiltration and Promoted the Expression of SIRT1 in the Hippocampus

Immunohistochemistry was performed to observe changes in microglia at 3 days after irradiation. In the RT and RT + Exo groups, the morphological changes of microglia were process retraction and cell body enlargement (**Figure 4A**). The cell count results showed that there were significantly more IBA1-positive cells in the RT and RT + Exo groups than in the Ctr group ($p < 0.05$), and there were significantly fewer IBA1-positive cells in the RT + Exo group than in the RT group ($p < 0.05$) (**Figure 4B**).

The protein expression of SIRT1 in the hippocampus was measured preoperation (0 h) and at 24 h, 3 days, and 7 days after irradiation. Western blot analysis showed that in the RT and RT + Exo groups, the protein expression of SIRT1 decreased at 24 h but significantly increased at 3 and 7 days after irradiation. Statistical analysis showed that at 7 days after irradiation, the protein expression of SIRT1 in the RT + Exo group was significantly higher than that in the RT group ($p < 0.05$) (**Figure 4C**). These results indicated that radiation-induced microglial infiltration associated with the decreased expression of SIRT1, and ADMSC-Exos might inhibit radiation-induced microglial infiltration by promoting SIRT1 expression.

Adipose-Derived Mesenchymal Stem Cells Alleviated Radiation-Induced Oxidative Stress and Inflammation, and Inhibited the Expression of CD68 in Microglia

The activities of SOD and the levels of NO, TNF-α, and IL-10 were measured at 24 h after irradiation. The levels of NO and TNF-α in the RT and RT + Exo groups were significantly higher than those in the Ctr group ($p < 0.05$) (**Figures 5A,C**), and the activities of SOD and the levels of IL-10 in the RT and RT + Exo groups were significantly lower than those in the Ctr group ($p < 0.05$) (**Figures 5B,D**). After administration of different concentrations of ADMSC-Exos, the levels of NO and TNF-α substantially decreased. In addition, ADMSC-Exos intervention significantly increased the SOD activities and IL-10 levels. These data revealed a dose-dependent effect, and the optimal concentration of exosomes was confirmed to be 0.4 μg/ul.

CD68 was one of the phenotypic gene markers of activated microglia. The immunofluorescence images revealed that microglia highly expressed CD68 after irradiation (**Figure 5E**). Results of fluorescent density and Western blotting showed that the expression of CD68 in the Exo group was significantly lower than that in the RT group at 24 h after irradiation ($p < 0.05$). However, the effect of ADMSC-Exos intervention on CD68 expression could be reversed by the SIRT1 inhibitor EX527 (**Figures 5F,G**). These results further demonstrated that the effect of ADMSC-Exos on microglia was associated with SIRT1 pathway.

Adipose-Derived Mesenchymal Stem Cells Alleviated Radiation-Induced Oxidative Stress and Inflammation in Microglia via the SIRT1 Pathway

Results of western blotting showed that the expression of SIRT-1 in the RT group was significantly lower than that in the Ctr group, and the expression of acetylated p65 in the RT group was significantly higher than that in the Ctr group ($p < 0.05$). Statistical analysis showed that ADMSC-Exos intervention could significantly increase the expression of SIRT-1 and decrease the expression of acetylated p65, but these effects could be reversed by EX527 (**Figures 6A,B**).

After irradiation, the activities of SOD and the levels of IL-4 and IL-10 significantly decreased, and the levels of MDA, TNF-α, and IL-1β significantly increased,compared with those in the CTR group ($p < 0.05$). ADMSC-Exos intervention significantly reversed above effects. However, the effects of ADMSC-Exos intervention could be abolished by EX527 (**Figures 6C–H**). All these results suggested that the exosome-mediated activation of SIRT1 signaling could be abolished by EX527, and the protective effects of ADMSCs-Exos on radiation-induced oxidative stress and inflammation was SIRT1 pathway dependent.

DISCUSSION

Therapeutic irradiation can cause significant injury to any part of the central or peripheral nervous system. Several studies have shown that stem cell-based interventions can effectively attenuate and repair radiation-associated injuries (Smith and Limoli, 2017; Rodgers and Jadhav, 2018). Here, we demonstrated that ADMSC-Exos exerted protective effects against radiation-induced brain injury by attenuating oxidative stress damage and reducing inflammation and microglial infiltration. Our findings suggest that ADMSC-Exos represent a promising cell-free therapy strategy for radiation-induced brain injury.

A variety of MSCs have shown their potential application prospects in the treatment of radiation-induced injury (Kiang, 2016; Xu T. et al., 2018; Qian and Cen, 2020). Among these

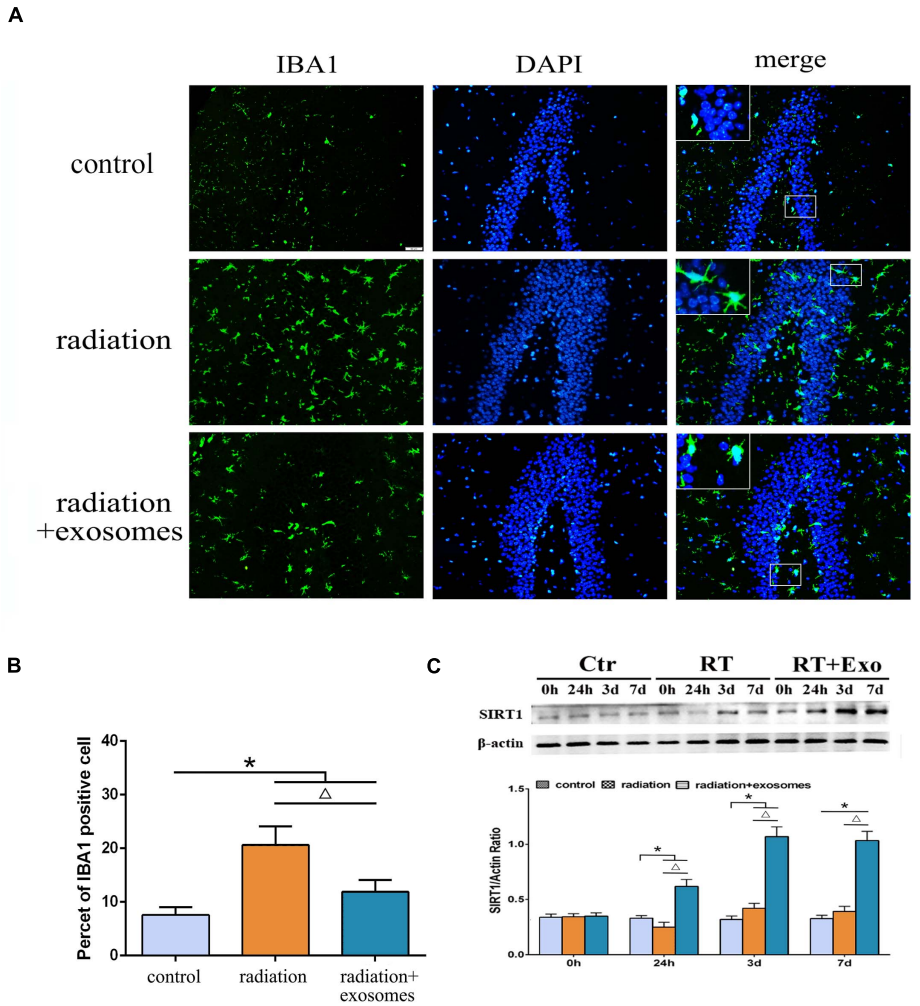

FIGURE 4 | Adipose-derived mesenchymal stem cells inhibited radiation-induced microglial infiltration and promoted the expression of SIRT1 in the hippocampus. **(A)** Microglia were stained by IBA1 (green), the nuclei were stained by DAPI (blue) (scale bar = 50 μm). **(B)** Cell count result of IBA1 positive cell in the hippocampus at 3 days after irradiation ($n = 6$). **(C)** Western blot analysis of SIRT1 expression in the hippocampus after irradiation ($n = 3$) (*$p < 0.05$, compared with the Ctr; $^{\triangle}$ $p < 0.05$, compared with the RT).

cells, MSCs derived from bone marrow, umbilical cord blood, the placenta and adipose tissue are the representative candidates for stem cell therapy (Sousa et al., 2014). However, based on the differentiation potential and immunomodulatory effects of MSCs, bone marrow derived MSCs (BMSCs) and ADMSCs are considered the optimal stem cell source for tissue engineering and regenerative medicine (Heo et al., 2016). However, BMSCs-based therapies are hindered by donor site morbidity, low stem cell concentrations, low available tissue volumes, and compromised cell number and function in elderly patients or those with skeletal diseases, such as osteoporosis. Adipose tissue provides an attractive alternative source of MSCs due to larger available tissue volumes, higher concentrations of stem cells, and reduced donor site morbidity. Moreover, studies have showed that ADMSCs are less influenced by aging and skeletal conditions than BMSCs (Holmes et al., 2021). Therefore, in this study, we chose ADMSCs as the source of exosomes.

Brain injury induced by radiation therapy has traditionally been classified according to the time of onset into acute, early delayed, and late forms (Balentova and Adamkov, 2015). While obvious tissue damage generally occurs after relatively high radiation doses, cognitive impairment can be observed after lower exposures (Tang et al., 2017). The hippocampus is well known to be in charge of study, memory and cognition, so our *in vivo* study was mainly focused on changes in the hippocampus after radiation exposure. The specific mechanisms responsible for cognitive injury are not well understood but may involve changes in the microenvironment, which could be affected by oxidative stress and inflammation. A high rate of oxidative metabolism is a hallmark of the brain. Irradiation could induce the body to generate large amounts of reactive oxygen species (ROS) (Tseng et al., 2014). When ROS generation exceeds the capacity of the cells to protect or repair themselves, excessive ROS could cause cell damage and necrosis. MDA and 8-OHdG

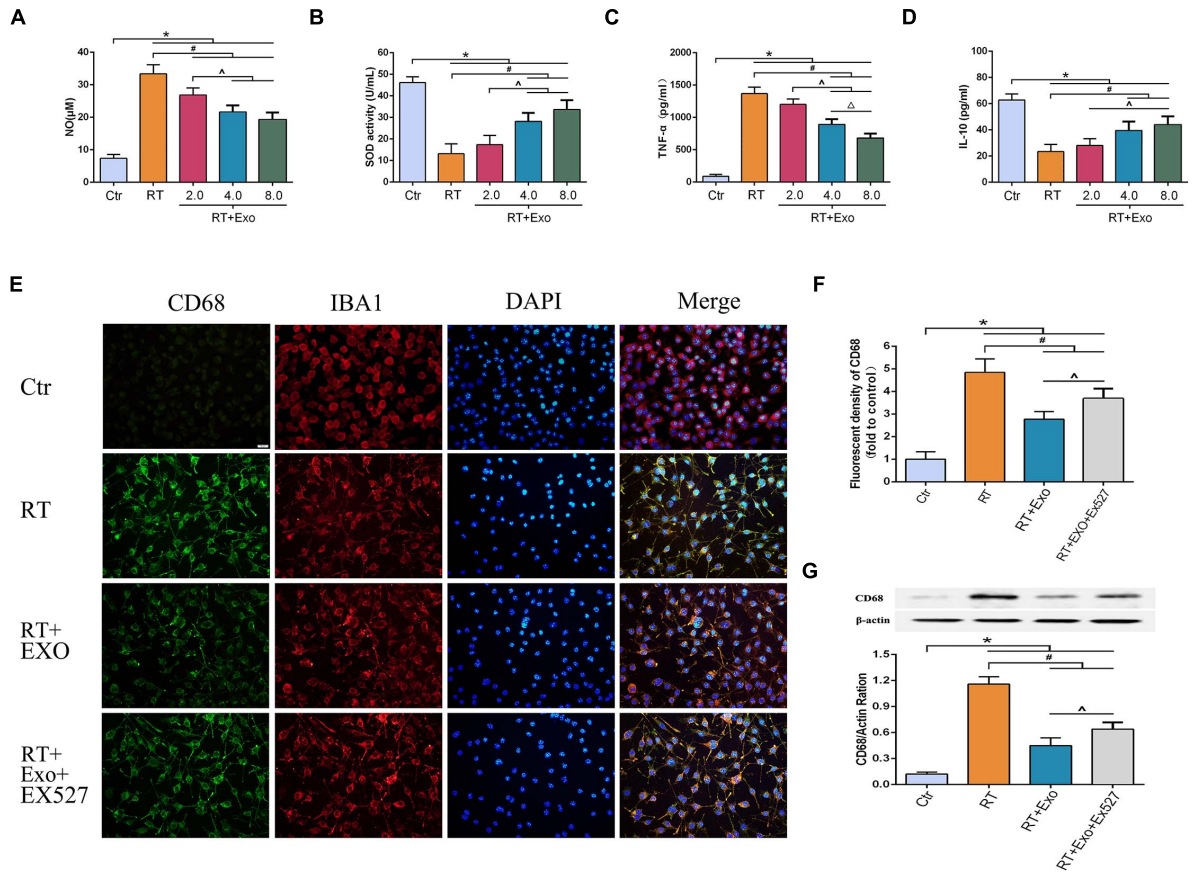

FIGURE 5 | Adipose-derived mesenchymal stem cells alleviated radiation-induced oxidative stress and inflammation, and inhibited the expression of CD68 in microglia. Microglia were pretreated with exosomes (2.0, 4.0, or 8.0 μg) 15 min before irradiation. Expressions of NO **(A)**, SOD **(B)**, TNF-α **(C)**, and IL-10 **(D)** at 24 h after irradiation measured by ELISA ($n = 6$) (*$p < 0.05$, compared with the Ctr; $^{\#}p < 0.05$, compared with the RT; $^{\wedge}p < 0.05$, compared with the Exo 2.0 μg; $^{\Delta}p < 0.05$, compared with the Exo 4.0 μg). **(E)** Microglia were stained by CD68 (green) and IBA1 (red), the nuclei were stained by DAPI (blue) (scale bar = 20 μm). EX527 abolished the effect of ADMSC-Exo on the expression of CD68 in microglia. **(F)** Fluorescent density of CD68 at 24 h after irradiation ($n = 6$). **(G)** Expression of CD68 at 24 h after irradiation determined by Western blot ($n = 3$) (*$p < 0.05$, compared with the Ctr;$^{\#}p < 0.05$, compared with the RT;$^{\wedge}p < 0.05$, compared with the Exo).

are commonly measured indicators of free radical-induced lipid peroxidation and systemic oxidative DNA damage, respectively. The detrimental effects of free oxygen radicals can be countered by the antioxidant enzymes SOD and CAT. In the *in vivo* study, we found that ADMSC-Exos could mitigate the decrease in SOD and CAT activities, as well as attenuate the increase in MDA and 8-OHdG. In addition to causing oxidative stress injury, studies have shown that radiation therapy causes inflammation, which in turn induces neuronal precursor cells in the hippocampus to differentiate into glia instead of neurons (Han et al., 2016; Zhou et al., 2017). This negative effect on neuronal progenitor cells could cause cognitive decline after radiation therapy (Makale et al., 2017; Pulsifer et al., 2018). In the *in vivo* study, we found that the pro-inflammatory cytokines TNF-α and IL-1β significantly increased and the anti-inflammatory cytokines IL-4 and IL-10 significantly decreased in the hippocampus after irradiation, but those changes could be attenuated by ADMSC-Exos. Previous studies have shown that MSC transplantation could attenuate radiation-induced brain injury (Acharya et al., 2015; Liao et al., 2017; Smith and Limoli, 2017), which was similar to the results observed in our study. However, transplantation of stem cells can cause additional physical damage to brain tissue, while intravenous injection of exosomes is relatively safe.

Microglia, the resident immune macrophages of the central nervous system (CNS), are intricately branched and respond rapidly to pathological changes in the brain parenchyma such as excitotoxicity, neurodegenerative insults, ischemia, and direct tissue damage (Ginhoux et al., 2010; Fu et al., 2012). The major findings of recent studies are that microglia can be polarized to become tumor-supportive and immunosuppressive cells by certain tumor-derived soluble factors, thereby promoting tumor maintenance and progression (Graeber et al., 2002; Wu and Watabe, 2017). Moreover, microglia also participate in tumor angiogenesis, metastasis, dormancy, and relapse (Tabouret et al., 2012; Wu and Watabe, 2017). In the present study, radiation-induced microglial infiltration and activation were observed *in vivo* and *in vitro*. It has been proven that after intravenous injection of exosomes through the tail vein,

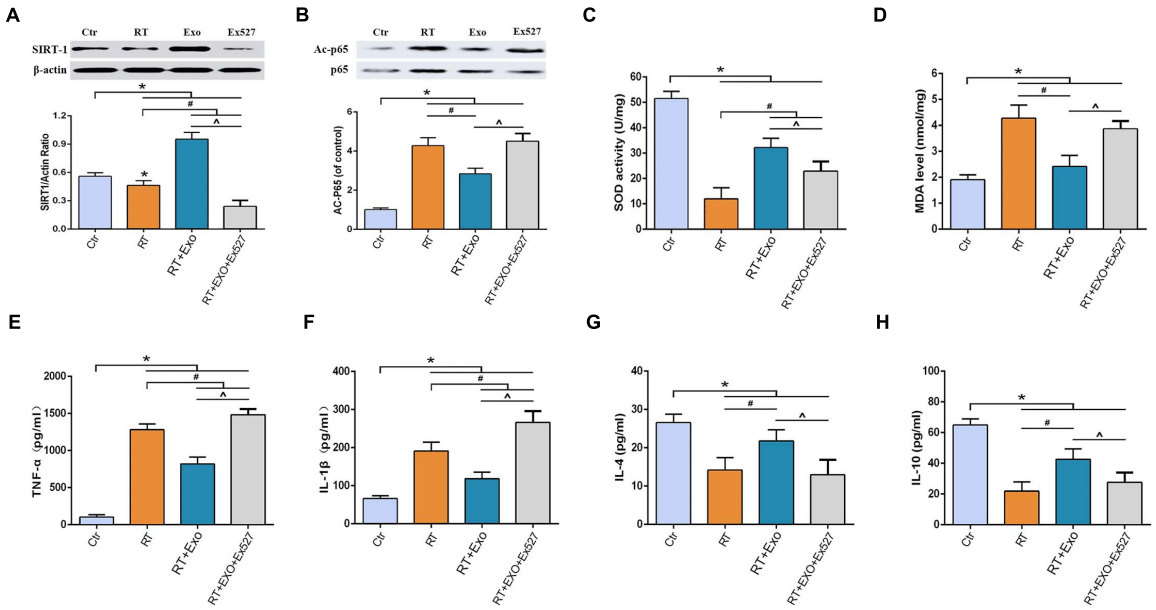

FIGURE 6 | EX527 reversed the effect of ADMSC-Exo on the expressions of SIRT-1 and acetylated p65 in microglia. Expressions of SIRT-1 **(A)** and Acetylated p65 **(B)** at 24 h after irradiation determined by Western blot ($n = 3$). And EX527 abolished the protective effects of ADMSC-Exo on radiation-induced oxidative stress and inflammation in microglia. Expressions of SOD **(C)**, MDA **(D)**, TNF-α **(E)**, IL-1β **(F)**, IL-4 **(G)**, and IL-10 **(H)** at 24 h after irradiation measured by ELISA ($n = 6$) ($^{*}p < 0.05$, compared with the Ctr; $^{\#}p < 0.05$, compared with the RT; $\hat{}p < 0.05$, compared with the Exo).

exosomes predominantly distributed in the liver, followed by the brain and then the spleens and lungs (Tian et al., 2021). In the CNS, after intranasal administration of exosomes, exosomes could incorporate robustly into neurons and microglia in rostral regions of the cerebral cortex, and predominantly into neurons in the cortex and the hippocampus at dorsal hippocampal levels. In addition, exosomes were frequently seen adjacent to processes of astrocytes and microglia throughout the frontoparietal cortex and the hippocampus (Long et al., 2017). Our results showed that ADMSC-Exos intervention could significantly reduce the number of activated microglia in the hippocampus and inhibit the expression of CD68 in microglia. Although our results confirmed the regulatory effect of ADMSC-Exos on microglia, the application of exosomes in brain tumor patients receiving radiotherapy should be extremely cautious. Because MSC-derived exosomes (MSC-Exos) are considered a double-edged sword in tumor therapy (Vakhshiteh et al., 2019). Several studies suggest that MSC-Exos perform as mediators in the tumor niche and play several roles in tumorigenesis, angiogenesis, and metastasis (Bruno et al., 2013; Yang et al., 2015; Qi et al., 2017). In contrast, there are other studies supporting the tumor-suppressing effects of MSC-Exos (Bruno et al., 2013; Wu et al., 2013; Takahara et al., 2016). More evidence is needed to evaluate the safety of MSC-Exos in radiotherapy.

SIRT1 is a highly conserved mammalian NAD+-dependent histone deacetylase. In recent years, an increasing number of studies have shown that SIRT1 not only plays a regulatory role in the inflammatory response but also has a close relationship with oxidative stress, glial cell proliferation and tumor recurrence (Singh et al., 2017; Rada et al., 2018; Yan et al., 2019). In the CNS, SIRT-1 plays an important role in promoting neurodevelopment, delaying brain senescence, maintaining homeostasis, and modulating circadian rhythm (Herskovits and Guarente, 2014; Xu J. et al., 2018). Furthermore, a growing number of studies have shown that SIRT-1 exhibits a key role in regulating neuroinflammation via inhibiting NACHT domain-, leucine-rich repeat-, and PYD-containing protein 3 (NLRP3) inflammasome activation, toll-like receptor (TLR) 4 signaling, nuclear factor- (NF-) κB pathway, and IL-1β transcription, which may relate to the modulating of microglial function (Zhang et al., 2019; Zhu et al., 2020). In the *in vivo* study, elevated levels of inflammatory cytokines, decreased expression of SIRT1 and microglial infiltration were observed. Therefore, we hypothesized that SIRT1 may be a key factor in ADMSC-Exo-mediated microglial regulation. In the *in vitro* study, we found that microglia were activated and polarized to the M1 phenotype at the acute phase after irradiation. The ADMSC-Exos intervention could significantly increase the expression of SIRT-1, thereby reducing the acetylation of the NF-κB subunit p65, which ultimately resulted in the reduction of oxidative stress and inflammation. More importantly, the effects of ADMSC-Exos intervention on microglia could be reversed by the SIRT-1 inhibitor EX527. All results verified our hypothesis that SIRT1 was a key factor in ADMSC-Exo-mediated microglial regulation, and further demonstrated that the radioprotective effects of ADMSC-Exos was SIRT-1/NF-κB signaling pathway dependent.

In this presented study, we chose the dose of 30 Gy to establish the rat model of radiation-induced brain injury. This radiation dose can induce changes in rat brains and is the minimum required to produce white matter necrosis without deaths or

gross neurologic deficits, and is a well-established animal model for the evaluation of late radiation-induced brain injury (Wang et al., 2009). Lamproglou et al. (1995) reported a radiation-induced (30 Gy) memory deficit at 1 month after irradiation in 4-month-old Wistar rats. Hodges et al. (1998) reported that low dose (20 Gy) irradiation could produce cognitive deficits in rats, but no behavioral changes were observed in the acute phase. Therefore, in this short-term study, we did not assess the effect of ADMSC-Exos on cognition. In addition, it should be noted that our results were obtained in male rats. The study from Hinkle et al. showed that there was a significant basal sex difference in microglial morphology, and irradiation-mediated alterations of microglia and dendritic spine density was sex-specific (Hinkle et al., 2019). The research on radiofrequency radiation found that male rats showed a significant increased, exposure-related DNA damage than female rats, and male mice generally displayed more exposure-related DNA damage than female mice in the hippocampus, cerebellum, and liver (Smith-Roe et al., 2020). Further long-term studies will be required to assess the effects of ADMSC-Exos on radiation-induced cognitive impairment and whether their effects are sex-specific.

In conclusion, we provided direct evidence that ADMSC-Exos exerted protective effects against radiation-induced brain injury by reducing oxidative stress, inflammation and microglial infiltration via activating the SIRT-1 signaling pathway. Compared with other strategies, we believe that the ADMSC-Exo-based approach might be a safer and more promising therapeutic strategy for radiation-induced brain injury. However, the precise mechanism by which ADMSC-Exos alleviate radiation-induced brain injury requires further investigation.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of the Air Force Military Medical University.

AUTHOR CONTRIBUTIONS

MDL, YSY, and BZ contributed to conception and design of the study. MDL, YSY, and YFY Performed the experiments. JW organized the database. KS and XKY performed the statistical analysis. MDL, GXZ, and JTH wrote the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Pharmacological Modulation of Neurite Outgrowth in Human Neural Progenitor Cells by Inhibiting Non-muscle Myosin II

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Studies on neural development and neuronal regeneration after injury are mainly based on animal models. The establishment of pluripotent stem cell (PSC) technology, however, opened new perspectives for better understanding these processes in human models by providing unlimited cell source for hard-to-obtain human tissues. Here, we aimed at identifying the molecular factors that confine and modulate an early step of neural regeneration, the formation of neurites in human neural progenitor cells (NPCs). Enhanced green fluorescent protein (eGFP) was stably expressed in NPCs differentiated from human embryonic and induced PSC lines, and the neurite outgrowth was investigated under normal and injury-related conditions using a high-content screening system. We found that inhibitors of the non-muscle myosin II (NMII), blebbistatin and its novel, non-toxic derivatives, initiated extensive neurite outgrowth in human NPCs. The extracellular matrix components strongly influenced the rate of neurite formation but NMII inhibitors were able to override the inhibitory effect of a restrictive environment. Non-additive stimulatory effect on neurite generation was also detected by the inhibition of Rho-associated, coiled-coil-containing protein kinase 1 (ROCK1), the upstream regulator of NMII. In contrast, inhibition of c-Jun N-terminal kinases (JNKs) had only a negligible effect, suggesting that the ROCK1 signal is dominantly manifested by actomyosin activity. In addition to providing a reliable cell-based *in vitro* model for identifying intrinsic mechanisms and environmental factors responsible for impeded axonal regeneration in humans, our results demonstrate that NMII and ROCK1 are important pharmacological targets for the augmentation of neural regeneration at the progenitor level. These studies may open novel perspectives for development of more effective pharmacological treatments and cell therapies for various neurodegenerative disorders.

Keywords: human neural progenitor cells (hNPCs), blebbistatin, neurite, non-muscle myosin II, extracellular matrix (ECM)

INTRODUCTION

Neurodegenerative disease conditions, such as Huntington's disease, Alzheimer's disease, and Parkinson's disease are characterized by a progressive loss of various types of neurons. Regenerative mechanisms, although limited in adult neural tissues, may delay or even halt disease progression. A major determinant of the reduced neuronal regeneration is the diminished axonal growth capacity of mature neurons. Extensive studies have been performed to elucidate the process of axonal growth impairment (Allingham et al., 2005; Medeiros et al., 2006; Tornieri et al., 2006; Rosner et al., 2007; Kubo et al., 2008; Kollins et al., 2009; Hur et al., 2011; Pool et al., 2011; Yu et al., 2012; Roland et al., 2014; Spedden et al., 2014; Xie et al., 2014; Evans et al., 2017; Wang et al., 2017, 2020; Tanaka et al., 2018; Basso et al., 2019; Dupraz et al., 2019; Costa et al., 2020), and, in addition to the intrinsic factors, revealed the importance of environmental inhibitory mechanisms in retarded axonal growth (Ichikawa et al., 2009; Hur et al., 2011; Tan et al., 2011; Beller and Snow, 2014; Baldwin and Giger, 2015; Nakamura et al., 2019). Because of the limited availability of human neural tissues, most of these studies used rodent or avian models.

Introduction of human pluripotent stem cells (PSCs) opens a new perspective for neurobiological research. Although application of stem cells obtained from early embryos raises several ethical issues, employing induced pluripotent stem cells (iPSCs) reprogrammed from adult tissues dispels these concerns. Differentiation of either embryonic stem cells (ESCs) or iPSCs into neural progenitor cells (NPCs), and subsequently into mature neurons, allows us to study human neural development and regeneration, as well as to explore the pharmacological modulation of neural cells of human origin.

Human neural progenitor cells, which represent *in vivo* a reservoir for neural regeneration, can be generated from human PSCs by directed differentiation. These cells resemble the usual cell lines in several aspects, as they can be passaged numerous times, cryopreserved, and transfected with various transgenes. At basal state, NPCs have a low number of short processes (1–2 per cell) with no substantial branching. However, when they start to differentiate into neurons, as an initial step, NPCs protrude projections, which subsequently elongate and branch. One of these projections differentiates into the extended axon, while the others become dendrites. Although the processes of NPCs greatly differ from those of mature neurons in both length and complexity, they can be considered as precursors of axons and dendrites. Therefore, we subsequently use the word "neurite" also for the projections of the neural progenitor cells.

Neurite outgrowth is a dynamic process; the elongation of these protrusions is not unidirectional but rather is a result of repeated growth and retraction. At the tip of these projections, actin dynamics has a crucial role. One of the factors governing actin dynamics is the non-muscle myosin II (NMII), which is an ATP-driven molecular motor protein responsible for the retrograde actin flow (recently reviewed in Costa and Sousa, 2020). Blebbistatin (BS) is a well-characterized, selective and potent inhibitor of NMII (Straight et al., 2003), and this compound blocks myosin heads in a low affinity state for actin, thus preventing formation of actomyosin complexes (Kovacs et al., 2004; Zhao and Overstreet-Wadiche, 2008). Despite that BS is widely used in studies on actomyosin network function, it holds a number of unfavorable properties, such as poor water-solubility, stability issues, fluorescence interference, cytotoxicity, and even phototoxicity (Roman et al., 2018). To overcome these negative properties of blebbistatin, several BS-derivatives have recently been developed. The most promising derivatives, which are non-fluorescent, highly soluble and non-toxic compounds, include para-nitroblebbistatin (NBS) and para-aminoblebbistatin (AmBS) (Kepiro et al., 2014; Varkuti et al., 2016; Rauscher et al., 2018; Gyimesi et al., 2021).

In the present work, we investigated the dynamics of neurite outgrowth in human stem cell-derived NPCs and examined the effect of BS and its derivatives on this process in permissive and inhibitory environments.

MATERIALS AND METHODS

Generation of Pluripotent Stem Cell-Derived Neural Progenitor Cells Expressing Enhanced Green Fluorescent Protein

A human iPSC line, previously generated from fibroblasts of a healthy male individual by Sendai virus reprogramming (Vofely et al., 2018), was kindly provided by Fred H. Gage (Salk Institute), whereas the human ESC line, HUES9 was a kind gift from Douglas A. Melton (Howard Hughes Medical Institute). These cell lines were differentiated into NPCs by a directed differentiation protocol, which is based on a previously published, multistep procedure (Yu et al., 2014), somewhat modified in our laboratory (**Supplementary Figure 1**). Briefly, PSCs were cultured in mTeSR media (Stem Cell Technologies) on Matrigel-coated dishes (Corning), and passaged by ReLSR (Invitrogen). Embryoid bodies (EBs) were generated from human PSCs on low-adherence plates; then the cells were differentiated toward the neural lineage using DKK1 (PeproTech), SB431542 (Sigma), Noggin (Thermo Fisher Scientific), and cyclopamine (Sigma). After 21 days, the EBs were seeded onto plates previously coated with poly-ornithine/laminin (Sigma and Thermo Fisher Scientific), then cultured for an additional 7 days. Appearing rosettes were collected and dissociated, and then the cells were plated onto plates previously coated with

Abbreviations: Aggr, aggrecan; AmBS, para-aminoblebbistatin; AU, arbitrary unit; BS, blebbistatin; CNS, central nervous system; CS, chondroitin-sulfate; CSPG, chondroitin-sulfate proteoglycan; EB, embryoid body; ECM, extracellular matrix; eGFP, enhanced green fluorescent protein; eGFP-NPC, enhanced green fluorescent protein-expressing neural progenitor cell; ESC, embryonic stem cell; HUES-NPC, human embryonic stem cell-derived neural progenitor cells; iPSC, induced pluripotent stem cell; iPSC-NPC, induced pluripotent stem cell-derived neural progenitor cells; JNK, c-Jun N-terminal kinase; MAP2, microtubule-associated protein 2; MOG, myelin oligodendrocyte glycoprotein; NBS, para-nitroblebbistatin; NMII, non-muscle myosin II; NPC, neural progenitor cell; PBS, phosphate-buffered saline; PSC, pluripotent stem cell; ROCK1, Rho-associated, coiled-coil-containing protein kinase 1; RT, room temperature; SCG10, superior cervical ganglion 10.

poly-ornithine and laminin (see below). When attached NPCs became super-confluent, they were transferred onto new poly-ornithine/laminin-coated plates.

To obtain stable expression of enhanced green fluorescent protein (eGFP) in human NPCs, a Sleeping Beauty transposon-based gene delivery method was applied (Kolacsek et al., 2014). NPCs were co-transfected with a plasmid harboring a CAG promoter-driven eGFP and puromycin resistance gene (**Supplementary Figure 2A**), as well as with another vector containing CMV promoter-driven Sleeping Beauty transposase. Transfection was carried out using Fugene HD reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Eight days following transfection, the cells were subjected to 1.6 μg/ml puromycin for 24 h. To obtain NPCs expressing eGFP at a high level (eGFP-NPCs), the cells were sorted by a FACS Aria cell sorter (**Supplementary Figure 2B**).

Surface Coating and Cell Culturing

Six-well plates (Greiner) were coated with 2 ml of 10 μg/ml poly-ornithine solution for 24 h at room temperature (RT). The wells were then washed three times with phosphate-buffered saline (PBS; Thermo Fisher Scientific), and 2 ml of 5 μg/ml laminin solution was added for an additional 16 h at 4°C. A total of 3–5 × 10^5 NPCs were seeded into each well, and cultured in DMEM/F-12, Glutamax medium supplemented with N2 Supplement-A, B27 (all from Thermo Fisher Scientific), basic fibroblast growth factor (Invitrogen), Antibiotic-Antimycotic (Gibco) and laminin (1 μg/ml). The culture medium was replaced every other day. Confluent wells were washed with PBS, and the cells were detached with a 5-min Accutase (Stem Cell Technologies) treatment.

For experiments, 96-well plates (Greiner) were coated with various extracellular matrices (ECMs) as follows: Matrigel, previously thawed on ice and dissolved in cold media (50 μg/ml), was distributed into pre-chilled 96-well plates (100 μl per well), which were then incubated for 24 h at 4°C. Alternatively, the plates were treated with 100 μl of poly-ornithine (10 μg/ml) or poly-lysine (100 μg/ml, Sigma) for 24 h at RT, followed by overlaying with 200 μl laminin (5 μg/ml), where indicated. Other surface treatments included coating with 100 μl of aggrecan (Aggr, 50 μg/ml, Sigma), chondroitin-sulfate (CS; 10 μg/ml, Merck), myelin oligodendrocyte glycoprotein (MOG, 50 μg/ml, Sigma), and chondroitin-sulfate proteoglycan (CSPG; 10 μg/ml, Merck) solutions for 24 h at 4°C.

Neurite Outgrowth Measurements and Analysis

Two hours after seeding eGFP-NPCs onto 96-well plates previously coated with various ECM components (3–5 × 10^3/well), the cells were subjected to NMII, Rho-associated, coiled-coil-containing protein kinase 1 (ROCK1), or c-Jun N-terminal kinase (JNK) inhibitors at various concentrations, as indicated. While NBS and AmBS were produced as described previously (Gyimesi et al., 2021), the ROCK1-selective inhibitor Y27632 and the pan-JNK inhibitor SP600125 were purchased from Sigma. Green fluorescence images were acquired by an ImageXpress Micro XLS instrument (Molecular Devices) equipped with environment control unit providing 37°C temperature and humidified atmosphere containing 5% CO_2, 20% O_2, and 75% N_2. Six fields of view, covering approximately 40% of the total well surface (see **Supplementary Figure 2C**), were imaged for 4 h in 15 min intervals using an FITC filter cube (ex. 482/35 nm, em. 536/40 nm) and a 10× Nikon objective (Plan Fluor, NA = 0.3). All conditions were measured in three technical parallels.

For quantitative analysis, the total neurite lengths were assessed in each field of view using the Neurite Outgrowth module of MetaXpress software (Molecular Devices). The maximum width for cell bodies was defined as 150 μm not to exclude tight groups of cells from the analysis. The minimum fluorescence intensity of cell bodies was set to 3000 arbitrary units (AU) over the background on the 16-bit images (dynamic range 0–65,535). The criterion parameters for neurites were as follows: maximum width 10 μm, minimum fluorescence intensity 500 AU, and minimum extension from the cell body 20 μm (see **Supplementary Figure 2D**). For all analyses, the total neurite lengths were determined. To eliminate errors originating from initial cell number variations, the kinetic curves were normalized to initial cell numbers, and background was subtracted. To analyze the initial neurite growth rates, the first three points of the kinetic curves, covering a 30-min period, were linearly fitted, and the slope was used as an output parameter. For statistical analyses, non-parametric Kruskal–Wallis test was performed to compare a series of various experimental conditions, whereas Mann–Whitney tests were used to identify particular differences between individual sample pairs. The results are expressed as mean ± SEM obtained from at least three independent experiments.

RESULTS

Stimulation of Neurite Outgrowth in Human Neural Progenitor Cells by Blebbistatin and Its Derivatives

To study neurite dynamics in human neural progenitor cells, stem cell-derived NPCs expressing eGFP at high level were examined using a high-content screening and analysis system. Insertion of eGFP cDNA into the NPC genome caused no alteration in the cell morphology or the proliferative capacity (**Figures 1A,B**). High eGFP expression levels resulted in high-contrast images allowing visualization of even thin cellular projections (see **Supplementary Figure 2D**). Negligible phototoxic effect was observed even during long-term experiments with frequent illuminations (data not shown). As a poly-ornithine/laminin-coated surface was proposed as optimal condition for NPC culturing (Turney and Bridgman, 2005; Yu et al., 2014), the cells were first studied on this coating. Two hours after seeding the cells fully adhered to the surface, and started to protrude 1–3 projections per cell (2.1 ± 0.09 as an average). The steady state length of these neurites was approximately 20 μm (17.8 ± 0.3 μm) each with negligible branching (0.055 ± 0.008 branches per neurite) (**Figure 2A**).

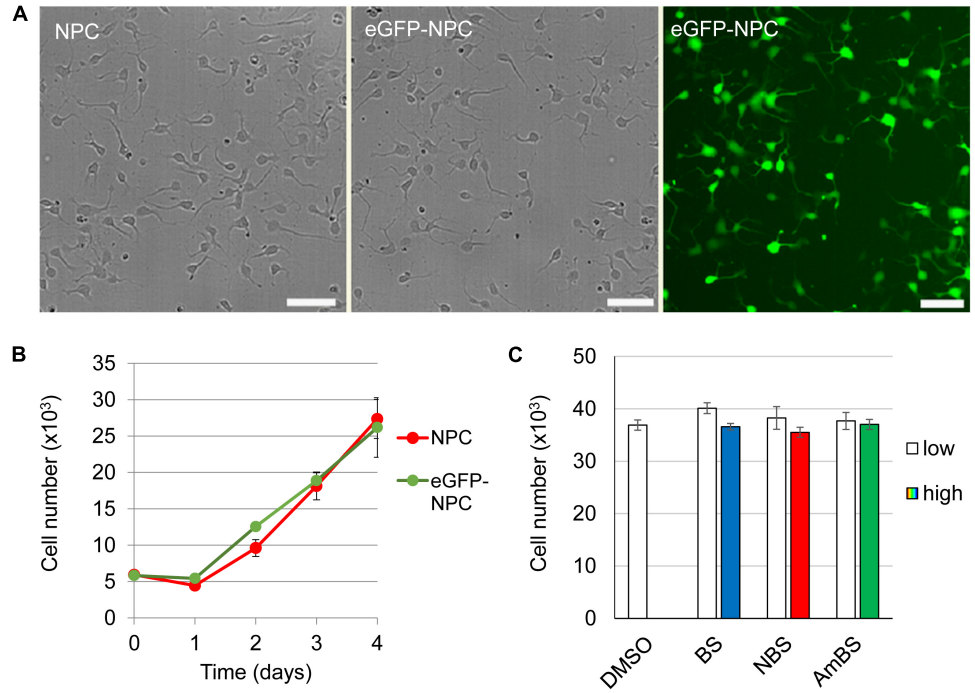

FIGURE 1 | Characterization of human neural progenitor cells stably expressing eGFP. eGFF was stably expressed in human NPCs using a Sleeping Beauty transposon-based expression system. **(A)** No morphological difference between the parental (NPC) and the GFP-expressing NPCs (eGFP-NPC) can be observed. However, the transfectants exhibit uniform green fluorescence, making not only the cell body but also the neurites visible. **(B)** There is no difference in the proliferation capacity of NPCs and eGFP-NPCs. **(C)** The cytotoxic effects of blebbistatin (BS), para-nitroblebbistatin (NBS), and para-aminoblebbistatin (AmBS) was investigated by subjecting eGFP-NPCs (3×10^4) to these compounds 2 h after seeding. BS and NBS were applied at 10 or 20 μM concentrations marked as low and high, respectively; whereas AmBS was used at 20 or 40 μM concentrations. The cell numbers were determined 4 h after the treatments and compared to the cell number of the vehicle (DMSO) treated sample. The mean ± SEM values from three independent experiments are shown in **(B,C)**. No significant differences were found.

These steady state values were a result of recurrent elongation and retractions of the projections (**Supplementary Video 1**).

When NPCs were subjected to blebbistatin, rapid outgrowth of neurites was observed (**Figure 2B** and **Supplementary Video 2**). As shown in **Figure 2C**, BS stimulated neurite protrusion at a concentration as low as 0.25 μM, but its effect was more pronounced above 2 μM. The morphological changes observed in NPC treated with 20 μM BS may suggest toxic effect of BS at these higher concentrations, and can explain why less stimulatory effect was seen at 20 μM than at lower concentrations (5 and 10 μM). Less toxic and more water-soluble derivatives of BS, NBS, and AmBS also promoted neurite outgrowth in human NPCs (**Figures 2D,E**). Contrary to BS, these derivatives were fully effective even at high concentrations. It should also be noted that AmBS appeared to be less potent than BS and NBS. The maximal stimulatory effect of AmBS was achieved only at 40 μM concentration. The cytotoxicity of BS, NBS, and AmBS was also examined, but no significant reduction in the cell numbers were observed in the given time period (**Figure 1C**), although a slight decrease was seen, when BS or NBS was applied at higher concentration (20 μM).

The stimulatory effect of NBS on neurite outgrowth in NPCs differentiated from HUES9 cells was similar to that seen in iPSC-derived progenitor cells (**Supplementary Figure 3**). The initial rate of NBS-stimulated neurite outgrowth was determined in each kinetic curve, and dose-response relationships for both HUES- and iPSC-derived NPCs were generated. No substantial differences between these two types of NPC lines were observed (**Figure 2F**). The EC_{50} values for NBS stimulation in HUES- and iPSC-derived NPCs were 3.97 ± 0.34 and 3.11 ± 0.25 μM, respectively.

In addition to the total neurite lengths in the studied fields of view, we analyzed cell morphology changes in response to BS compounds. At basal state, 2 h after seeding, NPCs possessed ∼2 neurites per cell with the total length of 30.0 ± 1.9 μm on average. After an additional 3-h period, the number of processes remained unaltered, but the length slowly elevated to 34.4 ± 1.9 μm per cell. However, when NPCs were treated with BS, NBS, or AmBS, the neurite lengths per cell extensively increased in a dose dependent manner reaching 73.8 ± 2.4, 87.9 ± 4.1, and 94.8 ± 7.0 μm per cell, respectively (**Figures 3A,B**). Interestingly, the number of processes also increased close to four neurites per cell (**Figure 3C**). In terms of neurite length, AmBS was the most efficacious, but less potent than the other two BS compound; whereas regarding number of processes, NBS was the most potent and most efficacious BS derivative. Under basal conditions, the neurites of NPCs exhibit hardly any branching: every 15th process has one branching. BS and BS derivatives stimulated neurite branching to some extent (**Figure 3D**). AmBS was the most effective in this regard: every third neurite showed

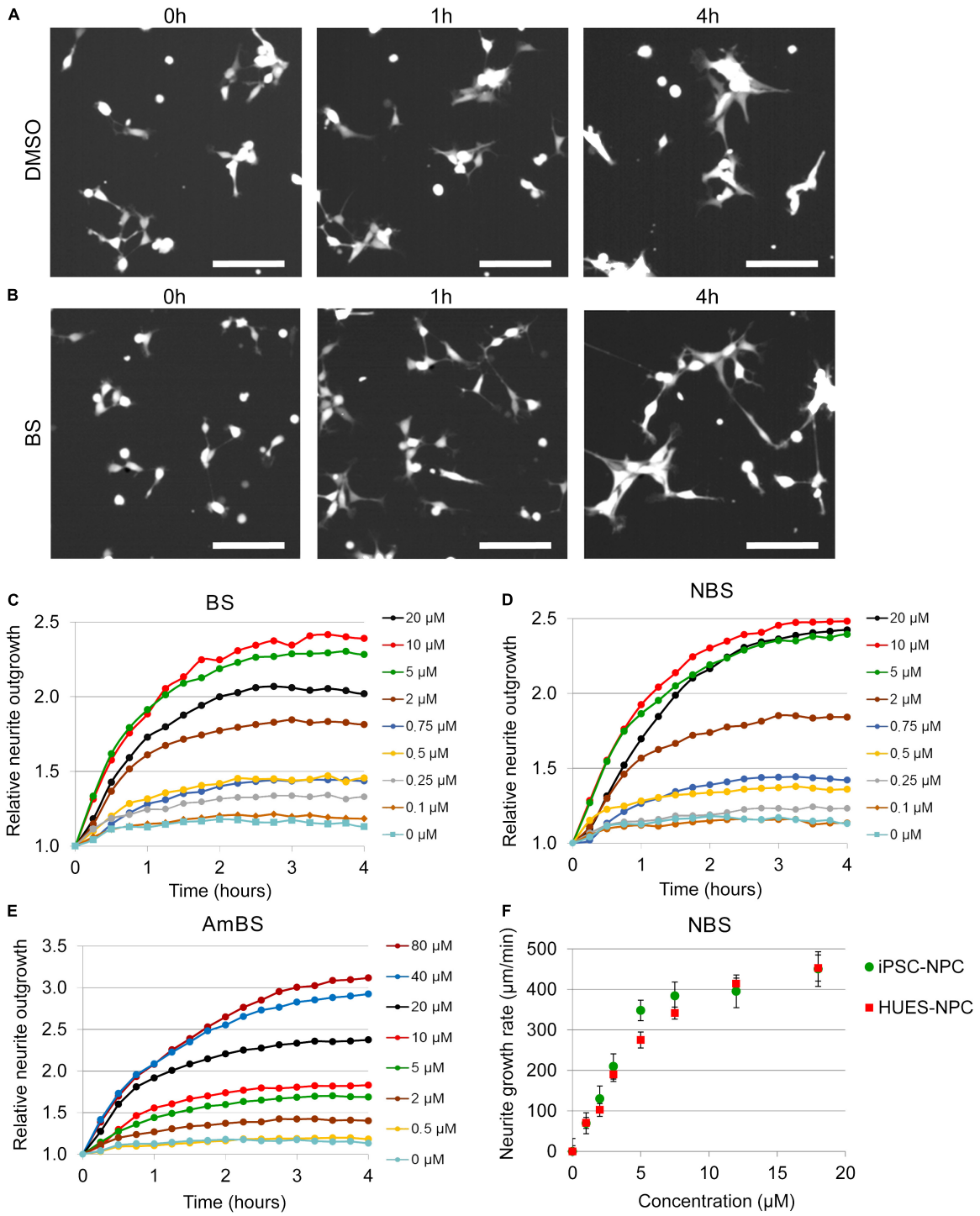

FIGURE 2 | Effect of blebbistatin and blebbistatin derivatives on the neurite outgrowth of NPCs. **(A,B)** Representative images of NPCs stably expressing eGFP treated with vehicle (DMSO) or 10 µM blebbistatin (BS). Time points indicate elapsed time after treatment. **(C–E)** Kinetics of neurite growth in eGFP-NPCs subjected to BS, para-nitroblebbistatin (NBS), or para-aminoblebbistatin (AmBS) in the indicated concentrations. The total lengths of neurites were determined in six fields of view containing 120–150 cells in each condition. The neurite lengths were normalized to the initial values. All three compounds stimulated neurite outgrowth in a dose dependent manner. The EC_{50} values for BS and NBS were about 2 µM, whereas EC_{50} value for AmBS was above 10 µM. Panels **(C–E)** depict representative experiments. **(F)** Dose-response curves for NBS-stimulated neurite outgrowth analyzed in two human NPCs of different origin, i.e., iPSC-derived neural progenitor cells (iPSC-NPC) and NPCs differentiated from the human embryonic stem cell line HUES9 (HUES-NPC). Both NPC lines stably expressed eGFP. The neurite outgrowth rates were determined from the initial growth rates. The mean ± SEM values from three independent experiments are shown. No marked difference was observed between the two cell lines.

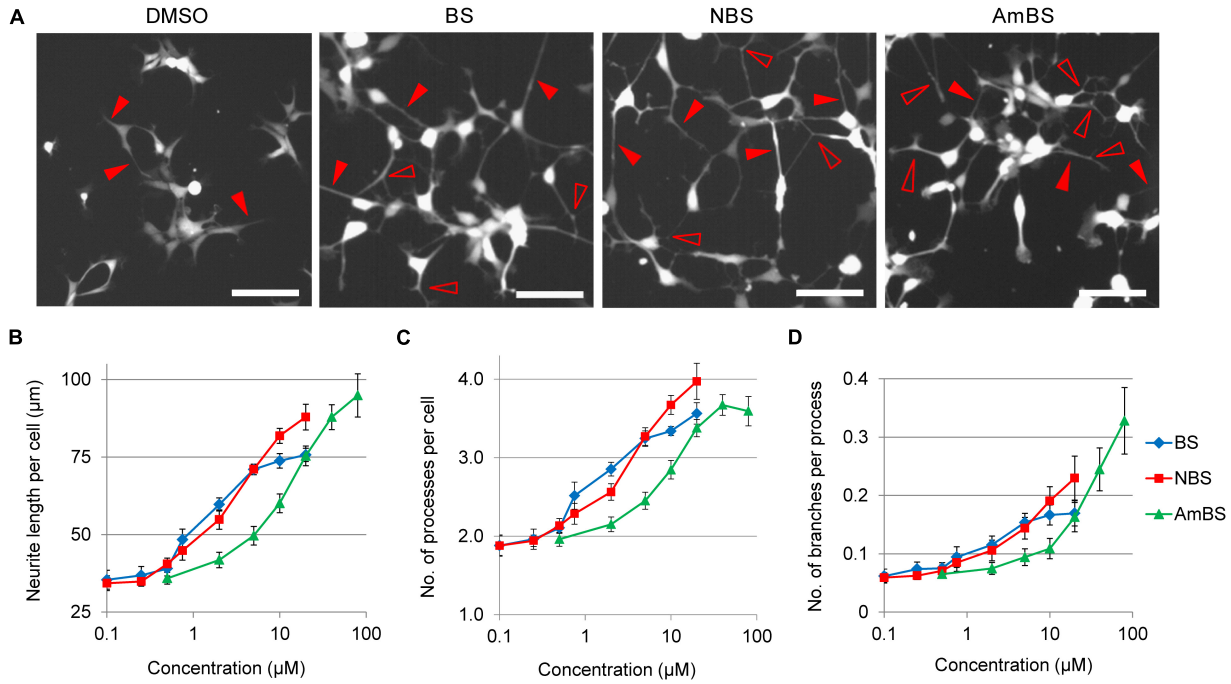

FIGURE 3 | Morphological changes in NPCs treated with blebbistatin or blebbistatin derivatives. **(A)** Representative images of eGFP-NPCs treated with vehicle (DMSO), 20 µM blebbistatin (BS), 20 µM para-nitroblebbistatin (NBS), or 40 µM para-nitroblebbistatin (AmBS) for 4 h. Filled arrowheads point to selected processes, whereas empty arrowheads indicate branching points of the processes. Scale bars indicate 10 µm. **(B–D)** Dose response curves for neurite length per cell, the number of projections per cell, and neurite branching in response to BS, NBS, or AmBS. The values were determined 3 h after treatment. The mean ± SEM from three independent experiments are indicated.

branching after the treatment with 80 µM AmBS. It is noteworthy that the potency of AmBS for branching was even less than that for neurite length or process number.

Effect of Extracellular Matrix Components on Neurite Outgrowth

To investigate how various ECM components influence neurite generation capability of human NPCs, the cells were seeded onto diverse ECMs. The frequently used, so-called growth-permissive extracellular matrices included Matrigel, laminin, poly-ornithine, poly-lysine, and combinations of poly-ornithine and laminin, as well as poly-lysine and laminin. Cell attachment and neurite outgrowth were substantially diminished, when human NPCs were seeded onto uncoated surface (data not shown). However, a steady basal neurite outgrowth was observed (2.51 ± 0.28 µm/min/cell 2 h after plating), when NPCs were plated onto poly-ornithine/laminin-coated surface. This condition was considered as a reference point for the experiments investigating the effect of various ECMs (**Figures 4A–C**). Similar neurite growth rates were seen in NPCs seeded on support coated with laminin, poly-lysine/laminin, or Matrigel, whereas poly-ornithine or poly-lysine alone provided suboptimal conditions for NPC neurite outgrowth (**Figure 4A**). It is important to note that these different ECMs markedly influenced cell morphology as demonstrated in **Supplementary Figure 4**. On poly-ornithine/laminin or Matrigel, the NPCs attached well, spread and formed small groups of cells with several outgrowths. NPCs seeded on poly-ornithine- or poly-lysine-coated support also adhered to the surface, but exhibited round-shape and remained dispersed. Despite these observations, the cell viability was not affected by these conditions (data not shown).

Next, we examined the effects of NBS and AmBS on the neurite formation of NPCs seeded on various ECMs. To achieve maximal stimulation and to avoid the toxic effects, NBS and AmBS were applied in 10 and 20 µM concentrations, respectively. We found that both blebbistatin derivatives significantly promoted neurite outgrowth (approximately threefold) regardless of the surface coating (**Figures 4A,D**). Since the basal neurite growth rates were smaller on poly-ornithine or poly-lysine, the stimulated ones were also less pronounced.

Several ECM components, mostly proteoglycans, produced primarily by oligodendrocytes in the vicinity of nervous system injury, were proposed as potential inhibitors of neuronal regeneration (Yu et al., 2012). Therefore, in the next set of experiments, we examined the effects of Aggr, CS, MOG, and CSPG on neurite outgrowth capacity of human NPCs. When laminin was replaced with these compounds in the extracellular coat, all studied ECM components strongly inhibited neurite generation (**Figures 4B,E**). Amongst them, CSPG was the most potent. When NPCs seeded on inhibitory coatings were subjected to NBS or AmBS at the maximum effective concentrations, these compounds were able to override the inhibitory effect of the ECM components. However, the stimulation of NBS did not reach

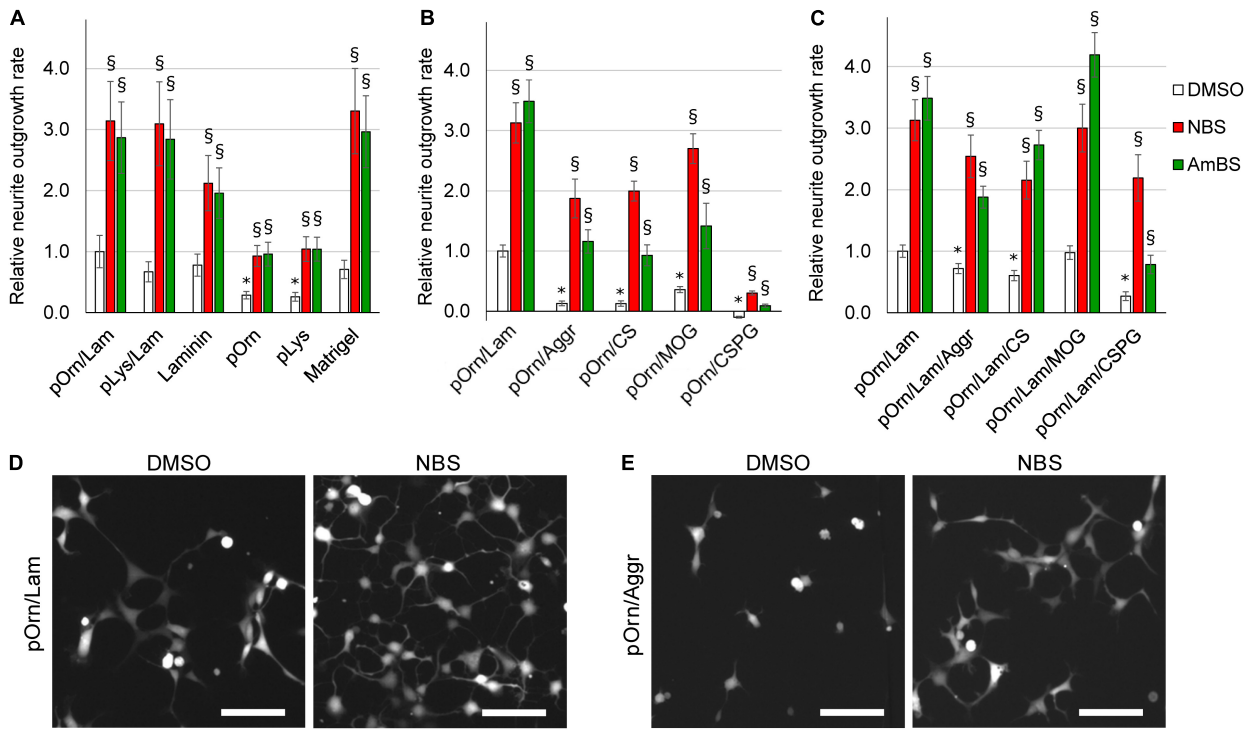

FIGURE 4 | Effect of various extracellular matrices on the neurite outgrowth. **(A)** Human NPCs were seeded onto culturing plates coated with permissive ECM components, such as laminin, poly-ornithine (pOrn), poly-lysine (pLys), Matrigel, the combination of poly-ornithine and laminin (pOrn/Lam), or the combination of poly-lysine and laminin (pLys/Lam). Neurite outgrowth was monitored in the presence and absence of 10 μM para-nitroblebbistatin (NBS) or 20 μM para-aminoblebbistatin (AmBS). The neurite outgrowth rates were determined from the initial growth rates. The neurite outgrowth rate measured in cells seeded onto pOrn/Lam-coated surface was used as a reference point, and all growth rates were normalized to this value. **(B)** Neurite outgrowth rates were determined in NPCs plated onto coatings containing poly-ornithine (pOrn) in combination with restrictive ECM components, including aggrecan (Aggr), chondroitin-sulfate (CS), myelin oligodendrocyte glycoprotein (MOG), or chondroitin-sulfate proteoglycan (CSPG). As a reference point, the neurite outgrowth rate of cells on pOrn/Lam-coated surface was used. All four ECM components inhibited neurite outgrowth of NPCs. When the cells were treated with NBS or AmBS extensive neurite outgrowth was observed except for the cells seeded onto pOrn/CSPG-coated surface. **(C)** An experiment similar to that shown in **(B)** was performed with the difference that laminin was also included in the coatings. In this combination, MOG was not detrimental to the cells, and BS derivatives were able to override the inhibitory effect of not only Aggr and CS, but also of CSPG. In all panels, the mean ± SEM from at least three independent experiments are indicated. Asterisks indicate significant differences as compared to the samples grown on pOrn/Lam-coated surface, whereas section signs mark significant differences as compared to untreated samples (vehicle controls, white columns) ($p < 0.05$). **(D)** Morphology of NPCs seeded onto pOrn/Lam-coated surface and treated with vehicle (DMSO) or 20 μM NBS for 4 h. **(E)** Representative images of NPCs seeded onto pOrn/Aggr-coated plate and treated with DMSO or 20 μM NBS for 4 h. Scale bars indicate 10 μm.

the levels achieved with NPCs on the permissive coating (poly-ornithine/laminin). AmBS was even less effective; in most of the cases, its stimulation resulted in a neurite growth rate comparable with control level, i.e., NPCs on poly-ornithine/laminin without stimulation. However, the inhibition of CSPG was so profound that either NBS, or AmBS could promote neurite outgrowth to only a small extent.

Next, we investigated the effect of laminin, which is a key component of the basal membranes and provides efficient axon guidance. In a previous experiment (**Figure 4A**), we observed beneficial effect of laminin on NPC neurite outgrowth rate. When the extracellular coat contained laminin besides poly-ornithine and the inhibitory ECM components, the inhibitory effects of Aggr, CS, and CSPG were less pronounced but remained significant (**Figure 4C**). Moreover, the inhibitory effect of MOG was reversed. Here too, NBS and AmBS were able to override the effect of inhibitory ECM components. Their stimulatory effects were comparable to the ones found in NPCs on permissive coating with only one exception. AmBS was not that effective when employed to NPCs seeded onto CSPG-containing ECM, however, its stimulatory effect was still significant (**Figure 4C**).

Role of Rho-Associated, Coiled-Coil-Containing Protein Kinase 1 and c-Jun N-Terminal Kinase in Neurite Outgrowth in Human Neural Progenitor Cells

Next, we investigated two distinct signaling pathways, which have been reported to regulate neurite outgrowth in animal model systems. ROCK1 is an upstream regulator of NMII, and has been shown to increase actin-arc contraction and translocation rates (Zhang et al., 2003). JNKs have been reported to play a key role in the stabilization and bundling of microtubules with an enhanced activity during neurite generation (Tonges et al., 2011). NPCs seeded onto a poly-ornithine/laminin coated surface

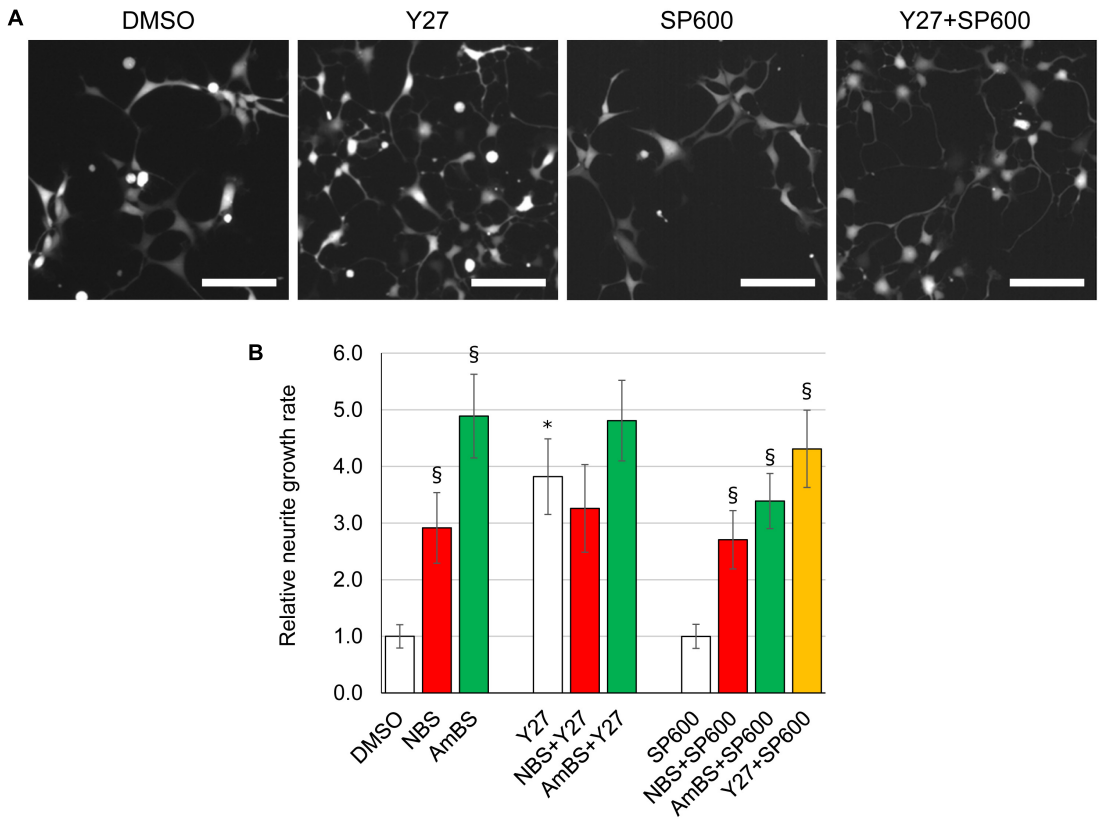

FIGURE 5 | Effect of potential upstream regulators on basal and BS derivative-stimulated neurite outgrowth. **(A)** Representative images of eGFP-NPCs treated with vehicle (DMSO), 10 μM Y27632 (Y27, ROCK1 inhibitor), 10 μM SP600125 (SP600, JNK inhibitor), or with the combination of Y27 and SP600. Images were acquired 4 h after treatment. Scale bars indicate 10 μm. **(B)** The neurite outgrowth rates were determined in NPCs treated with NBS, AmBS, Y27, SP600, or combinations of those inhibitors. Vehicle control (DMSO) was used as a reference point. The ROCK1 inhibitor stimulated neurite outgrowth to similar extent to that seen with NBS and AmBS. In contrast, JNK inhibition had no effect on neurite outgrowth. When NBS was co-administered with Y27632 or SP600125, neither additivity nor inhibition was seen. Similar results were obtained with AmBS. Similar to NBS and AmBS, SP600125 did not alter Y27632-stimulated neurite outgrowth. All panels depict the means ± SEM from 3 to 5 independent experiments. Asterisks indicate significant differences as compared to the DMSO control, whereas section signs mark significant differences as compared to samples, which are not treated with NBS or AmBS in the same group ($p < 0.05$).

were subjected to inhibitors of ROCK1 or JNK, and the neurite outgrowth rate was assessed. The ROCK1-selective inhibitor, Y27632 significantly stimulated neurite outgrowth in NPCs to an extent comparable with that the blebbistatin derivatives, NBS and AmBS elicited (**Figures 5A,B**). In contrast, SP600125, a pan-JNK inhibitor had no effect on the neurite generation. The inhibitory potential of the compound was confirmed in an *in vitro* kinase assay (data not shown). The effect of the ROCK1 inhibitor was not additive when co-administered with either NBS or AmBS (**Figure 5B**). The JNK inhibitor had no marked effect on NBS- or Y27632-elicited neurite outgrowth, but slightly reduced the neurite growth rate when the NPCs were stimulated with AmBS. Our observations suggest that NMII and ROCK1 inhibition affect the same pathway modulating neurite outgrowth in human NPCs.

In addition to neurite growth rates, the morphology changes in response to Y27632 and SP600125 have also been analyzed (**Figure 6**). The ROCK1 inhibitor stimulated not only neurite length per cell, but also increased the number of processes per cell and the number of branches per neurites. In contrast, JNK inhibitor had no effect on any of these morphological parameters. Interestingly, the potency and efficacy of Y27632 was similar to NBS in terms of neurite growth and branching (**Figures 6A,C**), but not for the stimulation of the number of processes per cell, which plateaued at around three processes per cell (**Figure 6B**).

DISCUSSION

Neural cells, especially neurons, exhibit a unique polar architecture. Polarization of neural cells is an essential part of neuronal differentiation, thus being fundamental in neural network formation and nerve regeneration. One of the very first episodes of neural polarization is the protrusion of a number of processes from NPCs, followed by the elongation and branching of the evolving neurites. Eventually, one of the projections differentiates into the axon, whereas others develop into dendrites. A fine balance between positive and negative regulatory mechanisms, including both internal and environmental factors, ultimately leads to the establishment

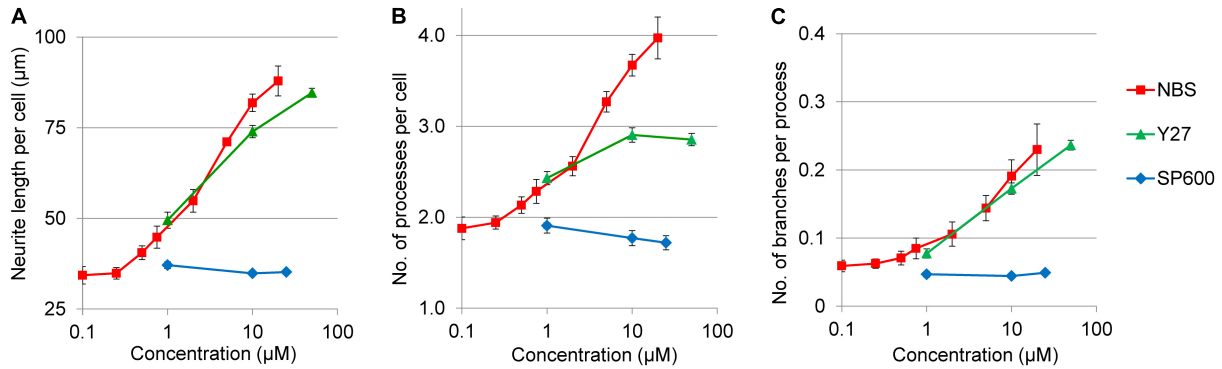

FIGURE 6 | Dose response curves for various morphological parameters of NPCs treated with ROCK1 and JNK inhibitors. The neurite length per cell **(A)**, the number of projections per cell **(B)**, as well as the neurite branching **(C)** was stimulated by Y27632 (Y27) in dose dependent manners, whereas SP600125 (SP600) had no such effects. The values were determined 3 h after treatment. The NBS stimulated dose response curves were used as references. The mean ± SEM from three independent experiments are shown.

and maintenance of the neuronal structure. Development of cell protrusions is a complex dynamic process, the result of numerous events that involve not only membrane dynamics but also rearrangement of cytoskeletal elements. The structure of neurites is established by stable microtubule bundles and a highly dynamic mesh of filamentous actin (F-actin). Critical steps of neurite development include the elongation of microtubules at the positive ends, their bundling, and the continuous remodeling of actin filaments (Lowery and Van Vactor, 2009). The ATP-driven molecular motor protein, the non-muscle myosin binds to F-actin and controls the dynamics of actomyosin locomotion, a fundamental biological process, connected to numerous cellular and physiological functions including muscle contraction, cell differentiation, migration and polarization (Eggert et al., 2006; Vicente-Manzanares et al., 2009).

Blebbistatin is a widely used, potent inhibitor of NMII (Straight et al., 2003; Kovacs et al., 2004), and has been demonstrated as a useful tool for studying neuronal differentiation. Treatment of neural cells with blebbistatin results in extensive outgrowth of neurites as a consequence of abolished retrograde actin flow, when the balance of extension and retraction of cell protrusion is shifted to extension. Several studies employed blebbistatin to demonstrate the role of actomyosin contractility using primary neurons from mice (Kollins et al., 2009; Hur et al., 2011; Yu et al., 2012; Dupraz et al., 2019; Costa et al., 2020), rats (Allingham et al., 2005; Kubo et al., 2008; Kollins et al., 2009; Pool et al., 2011; Roland et al., 2014; Spedden et al., 2014; Evans et al., 2017; Wang et al., 2017, 2020; Tanaka et al., 2018; Basso et al., 2019; Costa et al., 2020), chickens (Rosner et al., 2007; Kollins et al., 2009; Xie et al., 2014), or even gastropods, such as *Aplysia* (Medeiros et al., 2006) and *Helisoma trivolvis* (Tornieri et al., 2006). These studies were performed in non-human primary neural cells, and investigated the neurite growth in matured neurons. The role of actomyosin in cannabinoid-induced changes in neuronal morphology was also established by blebbistatin inhibition in primary rat neurons and in Neuro2a murine neuroblastoma cells (Roland et al., 2014).

Few studies employed human PSC-derived neural models to investigate the involvement of actomyosin contractility in neural polarization and differentiation. Human ESCs were treated with blebbistatin to demonstrate the role of NMII in topography-induced neuronal maturation (Ankam et al., 2015). Blebbistatin antagonized differentiation of human iPSCs into midbrain dopaminergic neurons induced by mRNAs coding for proneural transcription factors, also implying the involvement of NMII (Xue et al., 2019). A large-scale screening for bioactive small molecules regulating neurite growth identified blebbistatin as a hit compound (108 hits out of 4421) (Sherman and Bang, 2018). For the high-throughput screen, iCell neurons were used, which are human iPSC-derived, cortical-like neural cultures consisting mostly of GABAergic interneurons. Contrary to the studies above, in which differentiated neurons were investigated, we examined the role of NMII in human neural progenitor cells, a cell population, which plays a pivotal role in the development of nervous system during ontogeny, as well as in neural regeneration. The NPCs applied in our study were committed to the hippocampal dentate gyrus lineage (Yu et al., 2014; Vofely et al., 2018). As dentate gyrus neural progenitor cells are essential for learning, pattern separation, and spatial memory formation, deviations in these cells can cause several disease conditions (Zhao and Overstreet-Wadiche, 2008). Thus, the investigation of neurite polarization mechanism in this cell type can help to improve strategies for neuro-regeneration and cell-based therapies. We found that blebbistatin induces not only elongation of existing neurites but stimulates generation of new projections and branch formation of neurites in human NPCs.

Although blebbistatin is widely used, it has numerous drawbacks when employed in cell biology applications. These include chemical instability, low solubility in water, toxicity to cells, and blue light-induced phototoxicity (Rauscher et al., 2018; Roman et al., 2018). Blebbistatin also tends to form fluorescent precipitates, which interfere with many cell biology assessments. A-ring modification of blebbistatin results in higher water solubility, but comes at the cost of lower potency for

NMII inhibition (Verhasselt et al., 2017b). D-ring-modified blebbistatin analogs, however, such as 3′-hydroxy-blebbistatin, 3′-aminoblebbistatin (Verhasselt et al., 2017a), NBS (Kepiro et al., 2014), and AmBS (Varkuti et al., 2016), have more favorable properties, like higher water solubility, diminished cytotoxicity, and preserved potency. D-ring-modified BS derivatives with the exception of 3′-hydroxyblebbistatin are non-fluorescent at the spectral range normally used for microscopy or flow cytometry. In the present study, we demonstrated that NBS effectively stimulated not only neurite growth but also new protrusion generation and neurite branching with a potency similar to that of BS, whereas AmBS was less potent but more efficacious than BS in terms of neurite growth and branching stimulation.

Non-muscle myosin II is regulated by the phosphorylation of its regulatory light chain on Ser19 and Thr18, which is carried out by a number of kinases, including myosin light chain kinase, Rho-associated, coiled coil-containing kinase, leucine-zipper-interacting protein kinase, citron kinase, Serine/Threonine-protein kinase 21, and myotonic dystrophy kinase-related CDC42-binding kinase (Costa and Sousa, 2020). An equally important part of NMII regulation is its dephosphorylation by phosphatases, such as the myosin light chain phosphatase. The precise cellular localization of these kinases and phosphatases is crucial for the site-specific regulation of NMII, which is necessary to control filopodia dynamics. ROCK is a key component of various converging signaling pathways of upstream Rho-like GTPases. The regulatory role of MLCK and ROCK in neurite outgrowth has been demonstrated by numerous studies based on animal primary neural cell models (Tornieri et al., 2006; Rosner et al., 2007; Kubo et al., 2008; Kollins et al., 2009; Al-Ali et al., 2015; Wang et al., 2017). A recent report, using wild type and RhoA knockout mice as well as primary hippocampal neuron cultures from these animals, suggested a novel mechanism for Rho/ROCK signaling control of the axonal growth, i.e., ROCK restrains protrusion of microtubules to the leading edge of the growth cone by activating NMII-mediated actin arc formation (Dupraz et al., 2019). In concert with the animal-based neural models, we found that ROCK1 inhibition also promotes neurite outgrowth in human NPCs in a non-additive manner, indicating that ROCK1 is an upstream regulator of NMII also in this human neural model. These results also suggest that ROCK1 signal is manifested mainly by the NMII activity in the regulation of neurite outgrowth. It is worth noting again that previous studies applying animal-based systems investigated the role of NMII in mature neurons, focusing mainly on the growth cone or the axon initial segment, whereas in the present study, we aim at investigating a precursory event, the initialization of neurite formation in neural progenitors.

The JNK signaling pathway is two-faced in neural cells by means of promoting either cell development/regeneration or neuronal death/degeneration depending on the cell type, subcellular localization and cellular condition. A JNK1 knockout animal model demonstrated a pivotal role for JNK1 in neuronal microtubule assembly and stabilization (Chang et al., 2003). Subsequent studies using spiral ganglion or midbrain dopaminergic neurons from rats confirmed the involvement of all three JNK isoforms, although their differential contributions have also been revealed (Atkinson et al., 2011; Tonges et al., 2011). JNK3 was found to be the most prominent in mediating neurite regeneration and cell survival (Tonges et al., 2011). JNK phosphorylation of downstream effectors, such as the dendrite-specific high-molecular-weight microtubule-associated protein 2 (MAP2) and the microtubule-destabilizing protein superior cervical ganglion 10 (SCG10), were shown to contribute to defining dendritic architecture and axodendritic length, respectively (Bjorkblom et al., 2005; Tararuk et al., 2006). Contrary to these studies above, which were performed on mature neurons isolated from rodents, Lu et al. (2017) employed mouse embryonic neural stem cells (analogous to our NPCs) to examine the involvement of JNK. Inhibition of JNK diminished valproic acid-induced neurite outgrowth and neuronal differentiation in these cells. In our hands, the specific JNK inhibitor SP600125 did not block either basal neurite outgrowth, initiation of processes, or branching in human NPCs. Similarly, blocking the JNK pathway did not affect neurite growth elicited by either NMII or ROCK1 inhibition. Several reasons can be accounted for the different effect of JNK inhibition observed in the previously described cellular models and in our system. Interspecies difference between rodents and humans is one of the plausible explanations. Also, it is known that signaling events greatly dependent on the cell type. NPCs represent a tissue-specific stem cell population, in which regulatory mechanism can be different from that seen in mature neurons. In addition, previous studied revealed that specific cellular localization of kinases in either the RhoA- or the JNK-dependent pathway is crucial for the particular cellular functions (Coffey, 2014; Costa and Sousa, 2020). It is also noteworthy that we focused only on the initialization, the first 6 h of neurite generation. Kinetic differences can also be accounted for the conflicting results. Activation of JNK is relatively slow and its kinetics is site-specific in polarized neurons (Atkinson et al., 2011).

Restricted regeneration capability of the central nervous system (CNS) is determined by both intrinsic and environmental factors. The role of ECM in neural development and regeneration has long been studied. ECM components in the CNS are produced and secreted by both neurons and glial cells. Some of them, such as laminin and fibronectin, promote neural cell growth and migration, especially in the developing CNS, while others, e.g., CSPGs, serve as a barrier and prevent axons from growing into improper regions (Baldwin and Giger, 2015). Remodeled ECM at the site of the nervous system injury constitutes a detrimental environment, imposing a major obstacle for axonal regeneration (Beller and Snow, 2014). The effect of these permissive and restrictive ECM components on the neurite outgrowth has been demonstrated *in vitro* using matured neurons (Ichikawa et al., 2009; Hur et al., 2011; Tan et al., 2011; Nakamura et al., 2019); however, their impact on neural progenitor cells is poorly studied. In the present study, we showed that laminin was essential for maximal neurite growth capacity

in human NPCs, and addition of laminin to the inhibitory ECM components weakened their detrimental effect. Moreover, BS derivatives were shown to override the ECM inhibition.

Cell therapies using stem cells or stem cell-derived transplants represent a promising and developing field of regenerative medicine. Several stem cell-based preclinical studies and also a limited number of clinical studies have been performed in connection with various CNS pathologies including neurodegenerative disorders (recently reviewed in Ford et al., 2020). Rodent models of Alzheimer's disease and Huntington's disease showed marked improvement in behavioral and cognitive deficits after transplantation of human iPSC-derived NPCs (Fujiwara et al., 2013; Jeon et al., 2014). Similarly, engraftment of human stem cell-derived NPCs or dopaminergic precursors ameliorated bradykinesia and drug-induced rotation behavior in various animal models of Parkinson's disease (Roy et al., 2006; Kriks et al., 2011; Han et al., 2015). Moreover, early clinical trials have been launched or yet been forthcoming to explore the safety and efficacy of human iPSC-derived progenitors in Parkinson's disease patients (reviewed in Parmar et al., 2020). Functional recovery was also demonstrated in stroke-damaged rodents subjected to human iPSC-derived NPC transplantation (Gomi et al., 2012; Mohamad et al., 2013). The key issue of these interventions is the functional integration of the transplanted cells. Neuronal polarization starting with protrusions of neurites is a prerequisite for NPCs to integrate. However, innate mechanisms and non-permissive nature of CNS environment for neurite growth greatly impedes this process. Our results demonstrate that targeting NMII can surmount both internal and environmental hindrance of neurite development, offering a new opportunity for improving effectiveness of integration of transplanted cells. Our *in vitro* data can serve as a base for future *in vivo* experiments to explore the potential of pharmacological augmentation of cell therapies for various CNS pathologies.

CONCLUSION

In conclusion, we developed an *in vitro* experiment tool for quantitative assessment of neurite outgrowth in human hippocampal dentate gyrus neural progenitors. Using this system, we established the role of NMII in neurite generation, as well as its upstream regulation by the RhoA/ROCK1 signaling pathway. These results may provide new perspectives in the development of stem cells therapies as NMII might be considered as a novel drug target for integrating transplanted cells in neurodegenerative disorders, such as Parkinson's and Alzheimer's diseases, as well as in traumatic brain and spinal cord injuries (Ford et al., 2020; Lengel et al., 2020; Liu et al., 2020; Parmar et al., 2020; Bagheri-Mohammadi, 2021; Hu et al., 2021).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

JL and ZH performed the experiments, analyzed the data, and prepared the original draft. ES and EH performed the experiments to generate NPCs stably expressing eGFP. AM-C, JR, and ÁA contributed to conceptualization and design of the study, and reviewing and editing of the manuscript. LH contributed to study conceptualization and design, supervision of experiments, data analysis, and writing and editing of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.719636/full#supplementary-material

Supplementary Figure 1 | Generation of GFP-expressing NPCs. This scheme summarizes the multistage procedure how human neural progenitor cells stably expressing eGFP were generated from pluripotent stem cells. A human induced pluripotent cell line (iPSC) and a human embryonic stem cell line (HUES9) were seeded onto low-adherence plates to form embryoid bodies, which were subjected to DKK1, SB431542, Noggin, and cyclopamine for 21 days. Embryoid bodies were transferred to poly-ornithine/laminin-coated plates and cultured for an additional 7 days. Appearing rosettes were isolated, dissociated, and seeded again onto poly-ornithine/laminin-coated plates. In the stabilizing neural progenitor cells, eGFP was expressed using a Sleeping Beauty transposon-based gene delivery system (see **Supplementary Figure 2**). After selection with puromycin, NPCs expressing eGFP at high levels were sorted out by FACS.

Supplementary Figure 2 | Assessing neurite outgrowth in NPCs expressing eGFP at a high level. **(A)** CAG promoter-driven eGFP sequence was inserted into Sleeping Beauty transposon vector, which also contained a puromycin resistance gene. The NPCs differentiated from pluripotent stem cells were co-transfected with this plasmid and another one harboring the sequence of the active transposase, resulting in insertion of the transgene into the genome of the NPCs. **(B)** After puromycin selection, the cells exhibited inhomogeneous eGFP expression levels (left panel). This cell culture was sorted for low and high (right panel) GFP-expressing populations. **(C)** GFP-expressing NPCs, seeded onto 96-well plates, were treated with various compounds. Six fields of view of each well were imaged in the indicated configuration. Image acquisition was performed for 4 h in 15-min intervals using a high-content screening equipment (ImageXpress Micro XLS, Molecular Devices). **(D)** During subsequent image analysis, on the basis of eGFP fluorescence and morphological parameters, the somas and neurites were identified at each timepoint using the Neurite Outgrowth module of MetaXpress software. Scale bars indicate 10 μm.

Supplementary Figure 3 | Effect of para-nitroblebbistatin on neurite outgrowth in hESC-derived NPCs. An experiment similar to that shown in **Figure 2D** with iPSC-derived NPCs was performed with NPCs differentiated from the human embryonic stem cell line, HUES9. The NPCs were subjected to para-nitroblebbistatin in the indicated concentrations, and the total lengths of neurites were determined in six fields of view. The neurite lengths were normalized to the initial values. Initial neurite outgrowth rates were calculated from experiments similar to that depicted here, and a dose-response curve was generated (see **Figure 2F**).

Supplementary Figure 4 | Cell morphology of NPCs grown on different extracellular matrices. eGFP-expressing NPCs were seeded onto various extracellular matrices as indicated (pOrn, poly-ornithine; pLys, poly-lysine). Images were acquired before and 4 h after the addition of para-nitroblebbistatin (10 μM). Scale bars indicate 10 μm.

Supplementary Videos 1, 2 | Time-lapse images of NPCs in the presence and absence of NBS. NPCs expressing eGFP were subjected to DMSO (vehicle control, **Supplementary Video 1**) or 10 μM para-nitroblebbistatin (**Supplementary Video 2**), and the morphological changes were monitored for 4 h in 15 min intervals.

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Attractin Participates in Schizophrenia by Affecting Testosterone Levels

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Attractin (ATRN) is a widely expressed glycoprotein that is involved in energy homeostasis, neurodevelopment, and immune response. It is encoded by a gene spanning 180 kb on chromosome 20p13, a region previously implicated in schizophrenia by linkage studies. To address a possible role of *ATRN* in disorders of the central nervous system, we created an *atrn* knockout zebrafish line and performed behavioral tests. Adult $atrn^{-/-}$ zebrafish exhibited more pronounced attack behavior relative to wild-type control zebrafish in a tracking analysis. Biochemical analysis revealed elevated testosterone levels in $atrn^{-/-}$ zebrafish. At the gene expression level, we noted an upregulation of *cyp51* and *hsd17b7*, key proteins in testosterone synthesis in the brains of both adult and larvae of $atrn^{-/-}$ zebrafish. In order to further elucidate the relationship between testosterone and behavioral syndromes, we then compared testosterone levels of 9,008 psychiatric patients and 247 healthy controls from the same catchment area. Of all subjects examined, male subjects with schizophrenia exhibited lower testosterone levels compared with controls. In contrast, female subjects with a diagnosis of schizophrenia or bipolar disorder featured higher testosterone levels than did same sex controls. Purposeful sampling of extreme groups showed reduced *ATRN* expression in a subset of these subjects. Finally, we identified 14 subjects with *ATRN* mutations. All of whom displayed abnormal testosterone levels. In summary, the interplay of *ATRN* and testosterone may help to explain sexual dimorphisms in selected behavioral phenotypes.

Keywords: ATRN, schizophrenia, zebrafish, behavior, testosterone

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INTRODUCTION

Attractin is a member of the cell adhesion and guidance family of proteins containing a CUB domain, and EGF domain, and kelch repeats (Wrenger et al., 2006). It is widely expressed in humans and occurs in two isoforms of 175 and 200 kDa, encoded by the *ATRN* gene on chromosome 20p13 (Tang et al., 2000). Attractin, the membrane-bound isoform, with sequence

Abbreviations: MZ mut, maternal-zygote mutant; Dpf, days postfertilization; Mpf, months postfertilization; hATRN wt, human wild-type ATRN; HPG, hypothalamic–pituitary–gonad; LH, luteinizing hormone; PGC, primordial germ cells.

similarity to the mouse mahogany protein, forms a receptor controlling obesity (Kuwamura et al., 2002). The secretary isoform, a dipeptidyl peptidase IV/CD26-like enzyme, is expressed in human peripheral blood monocytes and has been shown to play important roles in cell-mediated immunity and neurodevelopment (Duke-Cohan et al., 1998; Paz et al., 2007).

Insight into the physiological function of Attractin has been obtained mostly from the study of rodent *Atrn* mutants. Rats with a null mutation in *Atrn* exhibit a tremor from around 3 weeks of age (Kuramoto et al., 2002; Shahrour et al., 2017) and have been named zitter rats. Mice deficient in *Atrn* feature changes in pigmentation due to disruption of the Mc1r–Agouti signaling pathway. Specifically, interference with Atrn suppresses the normal effects of Agouti on pigment production, leading to production of yellow pigment, the development of juvenile obesity, and hyperinsulinemia (Gunn et al., 2001). In addition, *Atrn* mutant mice show reduced body weight and adiposity (Kuramoto et al., 2002). ATRN is a co-receptor for Agouti regulation of pigmentation and obesity in MC1R and MC4R signaling pathways (Barsh et al., 2002). Mutant mice have also shown more nocturnal activity than wild-type mice in behavioral studies (Kuwamura et al., 2002). In 2012, Kohn studied the genetics of schizophrenia in a large inbred Arab–Israeli pedigree and found evidence for linkage on chromosome 20p13 (Teltsh et al., 2013). This locus harbors four strong candidate genes for schizophrenia: attractin (*ATRN*), pantothenate kinase 2 (*PANK2*), oxytocin (*OXT*), and arginine–vasopressin (*AVP*). Besides, He found that cDNA FLJ58441 is highly similar to attractin, and it has a high expression level in schizophrenia patients (Xu et al., 2018). Though multiple functions have been discovered, little is known about the role of attractin and the molecular mechanisms in mental disorders.

Mental disorders refer to a wide range of illnesses that affect perception, thinking, mood, and behavior due to poor mental health conditions, including schizophrenia, bipolar disorder, etc., In the psychiatric clinic, patients with mental disorders have their own behavior patterns, and often, these abnormal behaviors eventually lead to patients seeking medical treatment. Mental disorders have complex causes, among which genetic factors are triggering factors, and environmental factors are promoting factors (Piccin and Contarino, 2020). Motor behavior includes various movements from involuntary twitches to target actions, and involves every part of the body in an environment from individual actions to social interactions (Minassian et al., 2017), and motor behaviors can emerge from a mix of interacting factors (Wayner et al., 1970). Including genetic factor and hormone level. Some of the hormones are thought to directly correlate to mental disorder, but the inner mechanism is still unclear. For example, the results showed that schizophrenia patients had significantly increased testosterone levels when compared with healthy control subjects (Huang et al., 2021). Another research has found that mean total testosterone serum levels were significantly higher in bipolar disorder patients in comparison with major depressive disorder patients (Flores-Ramos et al., 2020). There is evidence that hypogonadism (lack of testosterone) is related to poor mental health outcomes and is a risk factor for depression and anxiety (Almeida et al., 2004; Shores et al., 2005). It is still unknown how neuroendocrine hormones and the phenotype of mental disorders are related, and the underlying mechanism is still unclear.

Among the multimodel animals, zebrafish has become one of the preferred model animals for studying human diseases due to its *in vitro* fertilization, early embryonic transparency, and easy genetic manipulation (Myers et al., 1986; Metcalfe and Westerfield, 1990; Sampath et al., 1998; Miklosi and Andrew, 2006; Morris, 2009; Shang et al., 2015). In addition, the genome of zebrafish is highly conserved with humans, and about 84% of human pathogenic genes have homologous genes on zebrafish (McCammon and Sive, 2015). Zebrafish exhibits complex and extensive behavioral patterns (Cachat et al., 2010), including social, anxiety, aggression, learning, and memory, cluster behavior, etc., which can be used in large-scale behavioral studies (Miklosi and Andrew, 2006; Jones and Norton, 2015; Rodrigues et al., 2016; Gupta et al., 2018; Caldwell et al., 2019).

In this study, we constructed an *atrn* mutant zebrafish line using the CRISPR/Cas9 technology (Komor et al., 2017; Liu et al., 2018). The tracking behavior analysis of adult and larvae of different genotypes with different genders was performed. The velocity of the $atrn^{-/-}$ adult male zebrafish and larvae is increased, the testosterone content is also increased, and the cholesterol synthesis pathway is activated. It is found that Atrn is involved in the cholesterol-related synthesis pathway, which affects the testosterone levels in zebrafish, changes the hormone levels, and causes changes in behavioral levels. In addition, there is a strong correlation between abnormal testosterone levels in schizophrenia patients with *ATRN* mutations.

MATERIALS AND METHODS

Ethics Statement

The breeding and experimental protocols involved in using zebrafish were approved by the IACUC of the Model Animal Research Center, Nanjing University. All methods were performed in accordance with the relevant guidelines and regulations.

The studies involving human participants were reviewed and approved by the local ethics committee of the Affiliated Nanjing Brain Hospital, Nanjing Medical University (2017-KY017). Written informed consent to participate in this study was provided by the legal guardian/next of kin of the participants.

Microinjection of Cas9 and sgRNAs Into Zebrafish Embryos

Two sgRNAs were designed according to the website.[1] The sequences of sgRNAs are **GAUAAAAUCUACAUGUACGG**GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUU (atrn-sgRNA1), **GGAGGGAAGAUUGACUCCAC**GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUUU (atrn-sgRNA2). Two sgRNAs were mixed with Cas9

[1] http://crispr.mit.edu

mRNA and microinjected into the embryos at one-cell stages. The microinjection amount was 1 nl of solution containing 0.05 ng of two sgRNAs and 0.25 ng of Cas9 mRNA. When screening the mutation, the primers were designed to identify the genotypes. atrn −4 F: 5′-CAGGATAAAATCTACATGTA-3′; atrn −4 F(34): 5′-CAGGATAAAATCTACACGGA-3′; atrn −22 F: 5′-TAAAATCTACATGTACGGAG-3′; atrn −22 F(34): 5′-TAAAATCTACATGTAGGAAA-3′; atrn R: 5′-ATATTGTACTG CTGCACTTG-3′.

Whole-Mount *in situ* Hybridization

Digoxigenin-UTP-labeled antisense RNA probes were synthesized *in vitro* using a linearized plasmid or PCR product as template. The template of atrn probe was amplified with the following primers: 5′-CCTCTCAAAGCTGGATGACATTA AAC-3′ (forward) and 5′-CACACTTCTCTCCATCTGCATAC-3′ (reverse). Whole mount *in situ* hybridization (WISH) on examining the expression patterns of atrn was performed as described previously (Yue et al., 2018). The embryos were raised to adults as founders. The F1 mutant zebrafish were screened using the method as described before (Kantari et al., 2007). Briefly, F1 generation zebrafish were genotyped by the PCR method using the genomic template prepared from the caudal fins clipped from zebrafish older than 6 weeks with a commercial kit (YSY, China). The primers used for amplifying the genomic sequences were 5′-TATGAGACTCGGTGCTGCAG-3′ and 5′-CCTAAAACATGTCCTGTACTGTATG-3′. The PCR conditions were 95°C for 2 min, 35 cycles (94°C for 30 s, 56°C for 30 s, and 72°C for 30 s), and a final extension of 10 min at 72°C. F1 mutant zebrafish carrying the atrn knockout allele were selected and inbred to get atrn null progeny (F2).

Semiquantitative PCR

Real-time RT-PCR assay (qRT-PCR) was performed to examine the relative expression levels of the genes of interest. For the coding genes in this study, we used ABI Step One Plus (Applied Biosystems), as reported previously (Yue et al., 2018). Transcript levels of the examined coding genes were normalized to the gapdh mRNA level according to standard procedures. We extracted total RNA from at least 30 embryos, three brains or testis in each group using Direct-zol RNA Mini-Prep (Zymol Research). The venous samples of six healthy people and 20 patients suffering from mental disorder with a high testosterone level were extracted total RNA by using Direct-zol RNA Mini-Prep (Zymol Research). Zebrafish and human venous cDNA at indicated stages were prepared by HiScript II 1st Strand cDNA Synthesis (Vazyme). The PCR was performed as follows: 50°C for 2 min, 95°C for 10 s, 40 cycles (95°C for 5 s, 60°C for 20 s), 95°C for 15 s, 60°C for 30 s, 95°C for 15 s. The primers used are listed as below: cyp51-rt-F1: 5′-CACACGGAGAAACACACAACCAC-3′, cyp51-rt-R1: 5′-CT AACAATGTGCAACTGTAGTG-3′; hsd17b7-rt-F1: 5′-CCGA CCAAGCAAGATGGATCTTG-3′, hsd17b7-rt-R1: 5′-CTCCAT GAGCAGTTTATAAATGACC-3′, zgapdh-qF: 5′-CGCTGGCAT CTCCCTCAA-3′, zgapdh-qR: 5′-TCAGCAACACGATGGCTG TAG-3′. hATRN-RT-F1: 5′-TCCAGACGTAGAGAGCAACT TC-3′, hATRN-RT-R1: 5′-TTGTTGCCAAAACACGGCTC-3′, hGAPDH-Q-F1: 5′-CACTAGGCGCTCACTGTTCTC-3′, hGAPDH-Q-R1: 5′-CCAATACGACCAAATCCGTTGAC-3′.

Live Imaging and Quantification

To observe the morphology, we treated embryos with 0.04 mg/ml MS-222 (Sigma) at room temperature for 5 min. Embryonic pictures were taken by an Olympus MVX10 microscope (Japan), and the photos were improved by Photoshop. The transgenic zebrafish lines Tg(*hb9*:eGFP), *atrn*^{nju70} and Tg(*olig2*:dsRed), and *atrn*^{nju70} were to confirm the development of motor neurons and oligodendrocytes. All confocal images were acquired using a Zeiss LSM880 confocal microscope. Then when measuring the weight and length of zebrafish, we treated them with 0.04 mg/ml MS-222 (Sigma), then blot the water, placed it in a Petri dish, measured it with an analytical balance, and recorded the data.

Tracking Behavioral Analysis

Live video tracking of zebrafish larvae was performed by Zebralab Video-Track system (Viewpoint, France). The mature zebrafish (4 months postfertilization) were transferred to a box (well size: 11-cm length × 7-cm width × 7-cm height) with 400 ml of E3 medium, and larvae (aged 5 dpf, 6 dpf) were transferred to a 24-well culture plate with 1 ml of E3 medium. In larvae, the detection threshold value for movement was set at 2–20 mm/s. Locomotor activity was measured per 1 min, and videos were recorded for 5 or 10 min. In adult zebrafish, the detection threshold value for movement was set at 20–75 mm/s. Locomotor activity was measured per 1 min, and videos were recorded for 1 h.

Mirror Attack Behavior

At 4 mpf, a mirror attack test was carried out using a box (well size: 11-cm length × 7-cm width × 7-cm height) with a one-sided mirror. Individual zebrafish was transferred to the apparatus with 400 ml of E3 medium. The movement was recorded for 10 min with data collection every 30 s. The detection parameter for movement was set at 20–75 mm/s, and the movement in the entire space and the movement in the mirror space were recorded.

Whole-Transcriptome Deep Sequencing

Total RNA was isolated from the single brain of a 4-months postfertilization (mpf) wild-type and atrnnju70 zebrafish using TRIzol reagent (Invitrogen). The transcriptome sequencing was performed by Novel-Bio Bio-Pharm Technology Co. (Shanghai, China). Gene expression levels were quantified by RPKM (reads per kilobase of transcript per million mapped reads) arithmetic. The MapSplice software was used for data alignment, and EB-Seq arithmetic was used for the screening of differential expression genes.

Testosterone ELISA Assay (Parameter, United States and Canada)

After the samples were separated, they were quick frozen with liquid nitrogen, 250 μl of 1 × PBS was added in the ice, the tissue was sufficiently broken with a grinder, and it was

centrifuged at 12,000 × *g* at 4°C for 1 min. The next specific steps follow the protocol. A standard curve was created using a computer software, mELISA, which is capable of generating a four-parameter logistic (4-PL) curve-fit.

Measure Concentrations of Serum Testosterone

This study was approved by the Ethics Committee of the Department of Psychiatry, Affiliated Brain Hospital of Nanjing Medical University, Jiangsu, China. From 2018 to 2020, all men and women aged 20 and above, their pathological information, including testosterone content, clinical diagnosis, etc., were all used for analysis. All individuals (including 3,588 males and 5,667 females) were required to have an empty stomach at least 8 h before venous sampling. The method of Elecsys Testosterone II, based on the principles of competition, was applied to measure concentrations of serum testosterone. Serum testosterone samples were analyzed by electrochemiluminescence using a Cobas e601 analyzer in the clinical laboratory of the Affiliated Nanjing Brain Hospital, Nanjing Medical University, and the kit was provided by Roche Diagnostics GmbH.

Statistical Analyses

Experiments were performed two or three times independently. Data are shown as mean ± SD. Statistical analysis was carried out with GraphPad Prism 7 (GraphPad Software, La Jolla, CA, United States). Data were first tested for normality using the Kolmogorov–Smirnov's test. If data sets exhibit normal distribution, we employed Student's *t*-test for equal variances or Welch's *t*-test for unequal variances. If data sets are found not to exhibit normal distribution, Mann–Whitney test was applied. Testosterone level differences between the patients with different mental disorders, and healthy controls were tested using Chi square. A value of $p < 0.05$ (*) was considered statistically significant, and $p < 0.01$ (**) and $p < 0.001$ (***) were considered very statistically significant.

RESULTS

Zebrafish *Atrn* Is Mainly Expressed in the Central Nervous System During Early Embryonic Development and Ubiquitously Most Tissues in Adulthood

Previous studies have shown that *Atrn* mutant affects motor behavior in mice and rats (Khwaja et al., 2006; Sakakibara et al., 2008; Izawa et al., 2010; Cheng et al., 2014). To better understand the function of *Atrn* in neural behavior, we use zebrafish as a model animal. Colinear analysis showed that human *ATRN*, and mouse and zebrafish *atrn* were all colinear with *GFRA4* and *SLAC4A11*, indicating that zebrafish *atrn* and mamalian *atrn* are orthologous genes (**Supplementary Figures 1A,B**). Unlike human and rat *ATRN*, the zebrafish *atrn* gene only encodes a transmembrane form of protein, but it has two transcripts, one is 1,345 amino acids (aa) and the other is 1,383 aa (Sun et al., 2017), both of them containing crucial domains.

The developmental expression of *atrn* mRNA was examined by semiquantitative PCR relative to *actb2* as an endogenous control (**Supplementary Figure 1C**). From zygote to dome stage, *atrn* mRNA was widely expressed in animal poles (Sun et al., 2017), and maternal *atrn* mRNA was decreasing at this stage. By 24 h postfertilization (hpf), the zygote *atrn* mRNA was detected extensively in the head. This result suggests that Atrn may be involved in the development of the nervous system. In adult zebrafish, a high expression of *atrn* was mainly found in the brain, eyes, testes, and ovaries (**Supplementary Figure 1D**), in line with the widespread expression pattern of *ATRN* in human tissues (Tang et al., 2000).

Knockout of *atrn* Does Not Affect Embryo Development, Maturation, and Fertility

Atrn spans a total of 29 exons and encodes a protein with a conserved CUB domain, kelch repeats, C-type lectin-like domain, and laminin EGF domain (**Figure 1A**). To further analyze the function of Atrn in zebrafish, we designed two sgRNAs to target the eighth exon (**Figure 1B**). After screening, we obtained two *atrn* mutant lines, *nju70* and *nju71*, with deletion of 22 bases and 4 bases in the genomic sequence, respectively. The protein product prediction indicates that both encode nonfunctional truncated proteins (**Figure 1C**). The whole-mount *in situ* hybridization results showed that the expression of *atrn* in the maternal zygote (MZ) $atrn^{-/-}$ is reduced (**Supplementary Figure 1B**). The mutants were raised to adulthood and found to grow normally for breeding. Since the results produced by the two mutant lines were consistent, the $atrn^{nju70}$ mutant line was chosen for the following study.

Morphology analysis of embryos produced by heterozygotes ($atrn^{nju70/+}$) in-crosses showed no developmental defects in homozygous mutant embryos. When the embryos develop to adults, the MZ mutant is not different from the wild type (**Supplementary Figure 1A**). By crossing to the transgenic Tg (*hb9*: eGFP) and Tg (*olig2*: dsRed), we found that $atrn^{-/-}$ did not affect the development of motor neurons and oligodendrocytes (**Supplementary Figure 1C**).

A Sexual Dimorphism Emerges in Locomotion, but $atrn^{-/-}$ Adult Zebrafish Exhibits Stronger Attack Behavior

Compared with wild-type (WT) littermates, $atrn^{+/-}$ and $atrn^{-/-}$ zebrafish showed reduced body weight in both genders at 4 months postfertilization (4 mpf) (**Supplementary Figures 1D,E**). This result is in line with studies in mice (Kuwamura et al., 2002). Body lengths of these mutant zebrafish were the same as WT littermates (**Supplementary Figures 1F,G**). Therefore, they appear slimmer (**Supplementary Figure 1A**).

We then analyzed their motion using ZebraLab (Viewpoint, French) during day (10:00–14:00) and night (0:00–4:00). A single adult zebrafish was placed in a box containing 400 ml of egg water and placed in Zebralab. The test time was 1 h, and the data were recorded every 1 min. The speed to stay inactive (black)/moderate motion (green) for adult fish was set

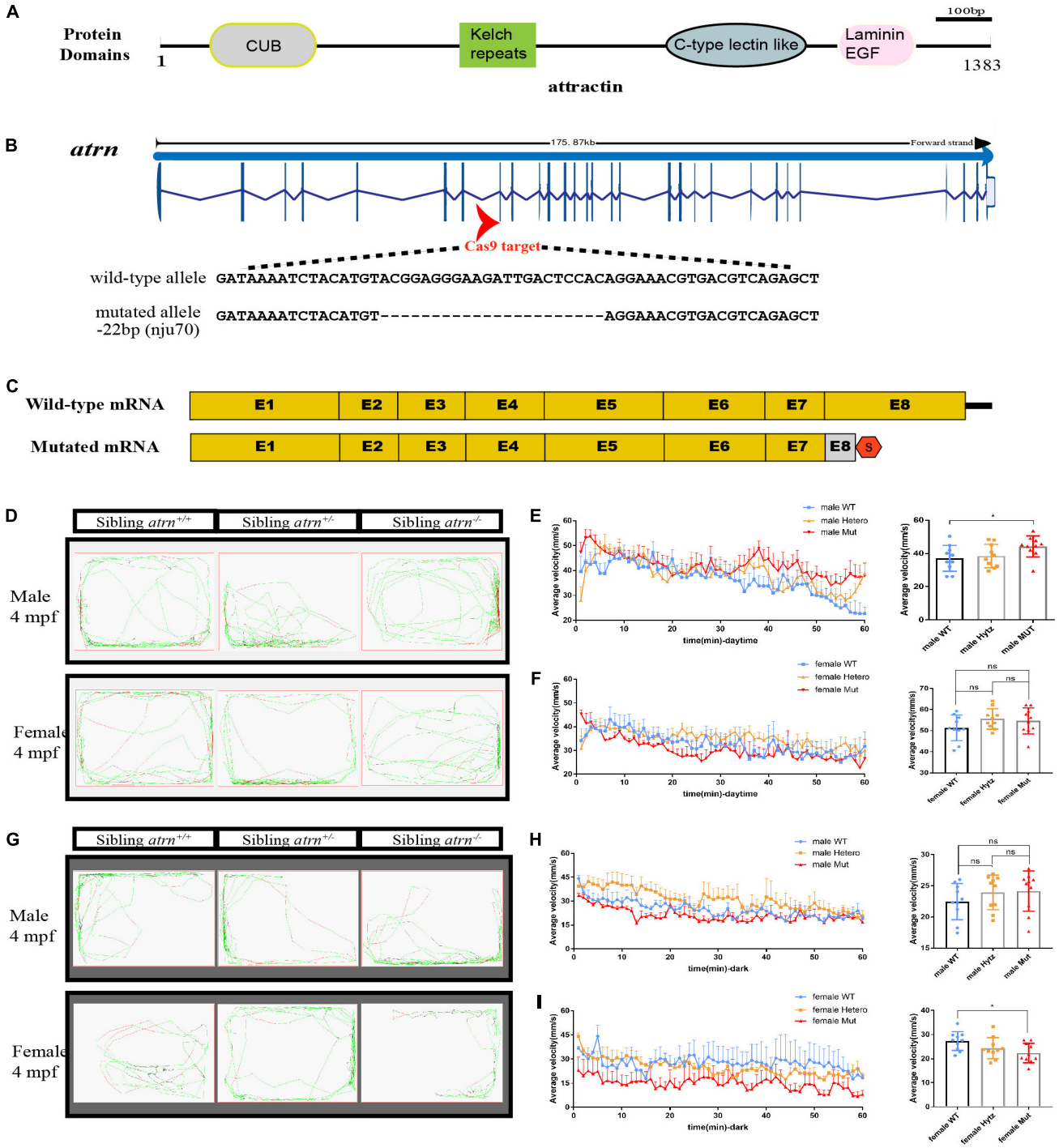

FIGURE 1 | Generation of *atrn* mutation in zebrafish by CRISPR-Cas9 strategy, and the average velocity of the male mutant is faster than the wild type during the day, but in the dark, the female mutant likes to stay quiet. **(A)** Zebrafish attractin has the CUB domain, kelch-type beta propeller, C-type lectin-like domain, and laminin EGF domain. **(B)** Exon 8 is the target for CRISPR/Cas9 gene editing in zebrafish *atrn*. The CRISPR/Cas9-induced mutation (22-basepair deletion) in *atrn* is shown in annotated *atrn* mutant sequences. **(C)** The mutated *atrn* mRNA with PTC was predicted to encode truncated protein. **(D)** Locus diagram of male and female in sibling $atrn^{+}$, sibling $atrn^{+/-}$, and sibling $atrn^{-/-}$ groups at 4 mpf during daytime. Average velocity in 1 h was recorded in three genotype groups at 4 mpf. At 10:00–14:00, we analyzed the tracking data of male **(E)** and female **(F)** three-genotype groups; the average velocity of the male mutant is faster than the male wild type, but there is no significance among the three female groups. **(G)** Locus diagram of male and female in sibling $atrn^{+}$, sibling $atrn^{+/-}$, and sibling $atrn^{-/-}$ groups at 4 mpf at night. At 00:00–4:00, we analyzed the tracking data of male and female three genotype groups. The average velocity of the female mutant is slower than the male wild type **(H)**, but there is no significance among the three male groups in the night **(I)**. $N = 10$. Data are shown as mean ± SD; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

to 25 mm/s, and the speed for moderate motion/large motion (red) is 70 mm/s. Ten males and females of different genotypes were tested each, and the analysis found that during the day, the trajectory distribution map of mutant males showed more large movements (**Figure 1D**). By counting the average velocity of each fish in each minute, and drawing the speed-time diagrams (**Figures 1E,F**), the average speed of the mutant males was significantly higher than that of the sibling wild type, and there were no differences among the three genotypes of females during the day. At night (**Figure 1G**), the average velocity of mutant females is significantly lower than that of the wild type, and they preferred to be quiet, while there is no significant difference between the three genotypes of males (**Figures 1H,I**). During the day, the behavior of mutant male zebrafish is consistent with the behavior of *Atrn* mutant mice.

In order to confirm the effect of *atrn* knockout on zebrafish's aggressive behavior, we designed a mirror attack experiment to test. A mirror was placed on the left side of the box, and ZebraLab was used to record the trajectory of the zebrafish within 10 min (**Figure 2A**). During the test, the movement distance of the female and male $atrn^{-/-}$ in the entire box was significantly higher than that of the wild type (**Figure 2C**), and the velocity–time distribution graph is also shown in **Figure 2D**. From the trajectory diagram of the zebrafish near the mirror (the attack space; the observer can determine whether it is a female or a male fish), $atrn^{-/-}$ zebrafish have more middle (velocity between 20–75 mm/s) and quicker (velocity higher than 75 mm/s) trajectories, while the wild type have more quiet trajectories (velocity lower than 20 mm/s) (**Figure 2B**). This confirms that the motility of $atrn^{-/-}$ is significantly more than that of the wild type. Finally,

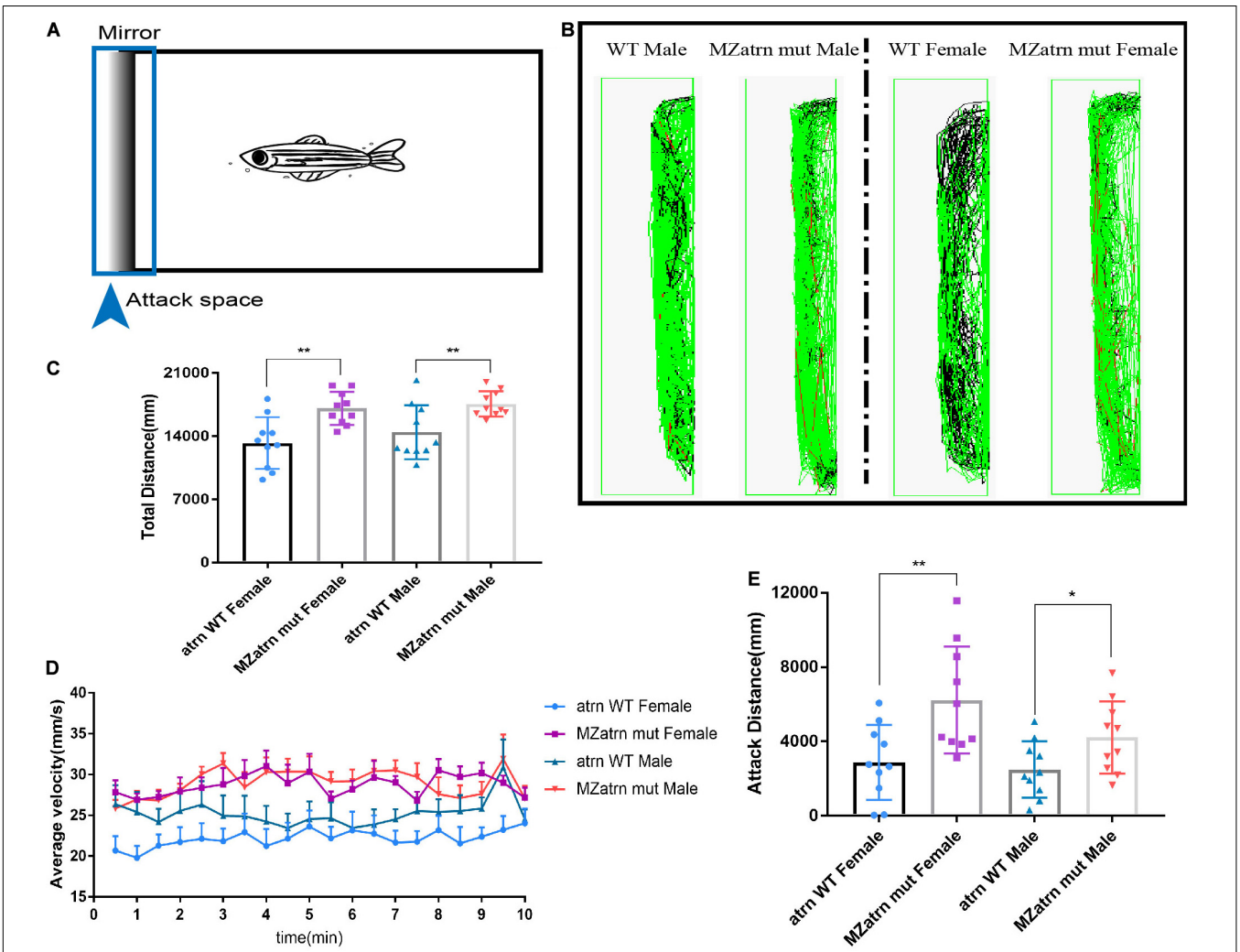

FIGURE 2 | Mirror attack analysis indicated that the aggressive behavior of the $atrn^{-/-}$ adult zebrafish was significantly higher than that of the wild type. **(A)** Schematic diagram of the experimental design of the mirroring attack. **(B)** The mirror attack tracking pattern of the wild type (WT) and MZatrn mut with different gender in 10 min. Red: the swimming speed is quicker than 70 mm/s. Green: the swimming speed is between 25 and 70 mm/s. Black: the swimming speed is slower than 25 mm/s. **(C)** The total tracking distance in whole box space of MZatrn mut is higher than that of the wild type. **(D)** The velocity–time panel of four groups of zebrafish. **(E)** The tracking distance in mirror attack space of MZatrn mut is higher than that of the wild type. $N = 10$. Data are shown as mean ± SD; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$; ns, no significance ($p > 0.05$).

by counting the movement distance in the attack space, the attack movement distance of the *atrn*$^{-/-}$ is significantly higher than that of the wild type of the same sex (**Figure 2E**).

Upregulation of Steroid Synthesis Pathway-Related Genes, Leading to Increased Levels of Testosterone in the Brain and Testis of the Mutant Males

In order to understand the underlying mechanism of the effect of Atrn on behavior, brains were isolated from 4 mpf wild-type and mutant male and female adult zebrafish. Three brains were taken from each group and sent for transcriptome sequencing analysis (**Figure 3A**). The steroid synthesis pathway was enriched in results of the male and female mutant brains (**Figures 3B,C**; Kuwamura et al., 2002). The expression levels of related genes in the steroid synthesis pathway were upregulated, including *cyp51*, encoding the key enzyme for cholesterol synthesis, and *hsd17b7*, encoding the key enzyme for testosterone synthesis (**Figure 3D**).

Testosterone is a steroid hormone synthesized from cholesterol (Hong et al., 2004). About 90% of testosterone comes from Leydig cells, and the rest is produced in the adrenal cortex and other tissues (Geniole et al., 2017; Guan et al., 2019). In the Leydig cell cytoplasm, lanosterol acts as a precursor to cholesterol synthesis, catalyzed by 14α-demethylase, which is encoded by *cyp51* (Geniole et al., 2017). Cholesterol enters the mitochondria with the help of StAR protein, produces pregnenolone under the catalysis of P450scc, and then synthesizes testosterone under the catalysis of 17β-hydroxylase, which is encoded by *hsb17b7* (Forgacs et al., 2005; Oyola and Handa, 2017).

qRT-PCR results confirmed that the expression levels of *cyp51* and *hsd17b7* were significantly increased in the brain and testis of the male mutants (**Figures 3E–H**). In the brain of female mutants, the expression levels of *cyp51* and *hsd17b7* were also increased (**Figures 3I,J**), while in the ovaries, *cyp51* was unaltered (**Figure 3K**), and *hsd17b7* was decreased (**Figure 3L**). Testosterone ELISA Assay (Parameter, United States and Canada) was then used to test the testosterone content in brain tissues and gonads of male and female zebrafish of different genotypes, 10 samples per group. The levels of testosterone in the brain tissue of heterozygous and mutant males are significantly higher than those of the wild type (**Figure 3M**). Similarly, the testosterone content of mutant testis is higher than that of the sibling wild type (**Figure 3N**).

An Increase in Average Velocity of *atrn*$^{-/-}$ Larvae on 6 dpf Matches an Increase in Overall Testosterone Content

The adult male *atrn* MZmut exhibits more pronounced active behavior when compared with the wild type and feature higher testosterone levels (**Figures 3E–H**). The increase in testosterone levels was detected in both the brain and the testis. We sought to determine which source drives the change in locomotion. To answer these questions, we collected embryos produced by the wild type and the homozygous mutant in-crossing, and raised them to 28.5°C to 6 dpf. Twenty-four larvae were randomly selected and placed in a 24-well cell culture plate. One larva was placed in each well, and 1 ml of egg water was added. A 24-well cell culture plate was placed in Zebralab, the test time was 10 min, and data were recorded every 1 min. The speed of the inactive (black)/moderate motion (green) was set to 2 mm/s, and the speed of moderate motion/large motion (red) was 10 mm/s. The results show that the mutants are significantly more active than the wild type (**Figures 4A,B**). The speed–time diagram shows that during the entire test, the *atrn* MZmut had a significantly higher rate of movement than the wild type (**Figure 4D**). The average speed of each larva within 10 min was calculated, respectively (**Figure 4C**). At a statistical level, the average velocity of the mutants was significantly higher than that of the wild type (**Figure 4D**).

Similarly, wild-type and mutant embryos were collected at the same time period, and three samples in parallel were collected from each group, with 30 larvae per sample. The qRT-PCR results showed that the expression level of *cyp51* in the mutant was not different from that of the wild type, while the expression level of *hsd17b7* was significantly higher than that of the wild type (**Figure 4E**). At the same time, 12 genotypes of larvae were collected, each of which contained 10 larvae. After removing the egg water, 250 μl of 1 × PBS was added and milled. Testosterone ELISA Assay was used to test the overall testosterone content of one larva. The testosterone content in the mutant larvae was significantly higher than that of the wild type and heterozygotes (**Figure 4F**).

Exogenous Testosterone Enhanced Larvae Motility

Does elevated testosterone have a direct effect on zebrafish locomotion? We exposed wild-type embryos to different concentrations of testosterone for treatment from the development of 24 hpf. The egg water was replaced every 24 h. The same concentration of testosterone was added and processed to 5 dpf. At this time, the zebrafish bladder was fully developed, and zebrafish were able to normally swim normally. The testosterone treatment group was divided into 1, 10, and 100 ng/ml. We then randomly selected 24 larvae from each group, placed them into a 24-well cell culture plate, and proceeded with the tracking analysis (Zebralab). The trajectory distribution chart clearly shows that the zebrafish juvenile group featured a significantly higher locus of movement than did the untreated group (**Supplementary Figures 3A–D**). Analysis of exercise rates after treatment with exogenous testosterone reveals an increase in average velocity of zebrafish juveniles. With the increase in testosterone concentration, the average velocity of larvae shows a downward trend in a dose-dependent manner (**Supplementary Figures 3E,F**). The above results indicate that the treatment with exogenous testosterone can mimic the phenotype observed in *atrn*$^{-/-}$, but with the increase in testosterone concentration, due to its drug toxicity, it inhibits the behavior of zebrafish larvae.

Human *ATRN* Rescues the Motor Phenotype in *atrn* Knockout Zebrafish

In the *atrn* MZmutant embryos, human wild-type *ATRN* (hATRN wt) was overexpressed, and we found that 25 and 50 pg of hATRN wt mRNA can efficiently reduce the swimming speed

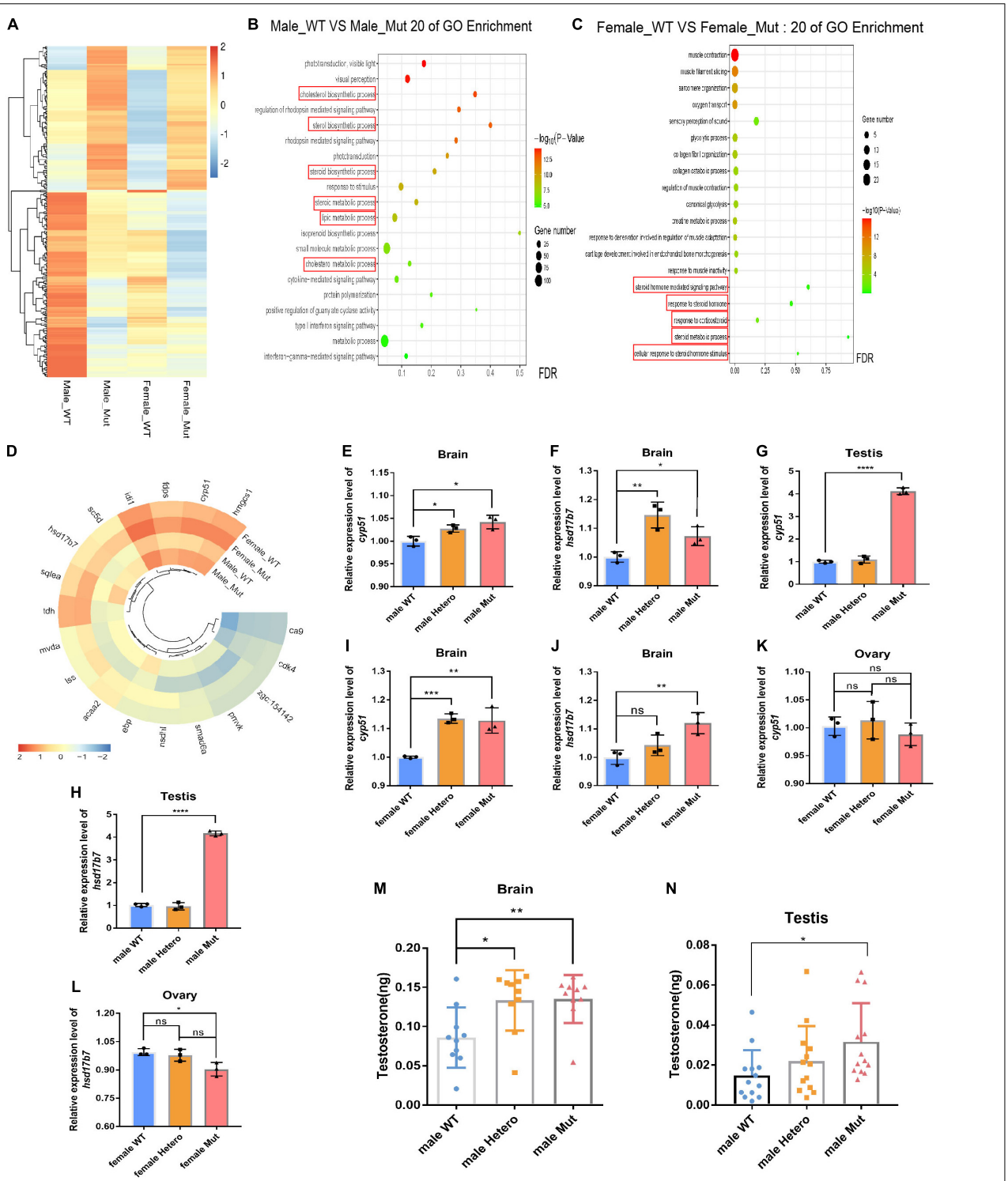

FIGURE 3 | Transcriptome sequencing of the brain in the male and female *atrn* mutants, and steroid synthesis-related genes are upregulated in the mutant. **(A)** Heat map shows genes that are differentially expressed in the brain of wild type and mutants of different sexes. $N = 3$. **(B)** GO enrichment dot bubble of male wild type and male mutants indicate that GO terms related to steroid synthesis are affected. **(C)** GO enrichment dot bubble of female WT and female Mut indicate that GO terms related to steroid synthesis are affected. **(D)** Circular heatmap shows upregulation of genes related to steroid synthesis. qRT-PCR results for *cyp51* and *hsd17b7* gene expression in the brain **(E–J)** and testis of WT, heterozygotic (Hetero) and Mut zebrafish at 4 mpf, but there is no expression difference between the brain and ovary of the female adult zebrafish **(K,L)**. $N = 9$. Testosterone ELISA results show that the content of testosterone in the brain of the male mutant **(M)** and testis is significantly increased **(N)**. $N = 10$. Data are shown as mean ± SD; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$; ns, no significance ($p > 0.05$). Heatmap was plotted by http://www.bioinformatics.com.cn, an online platform for data analysis and visualization.

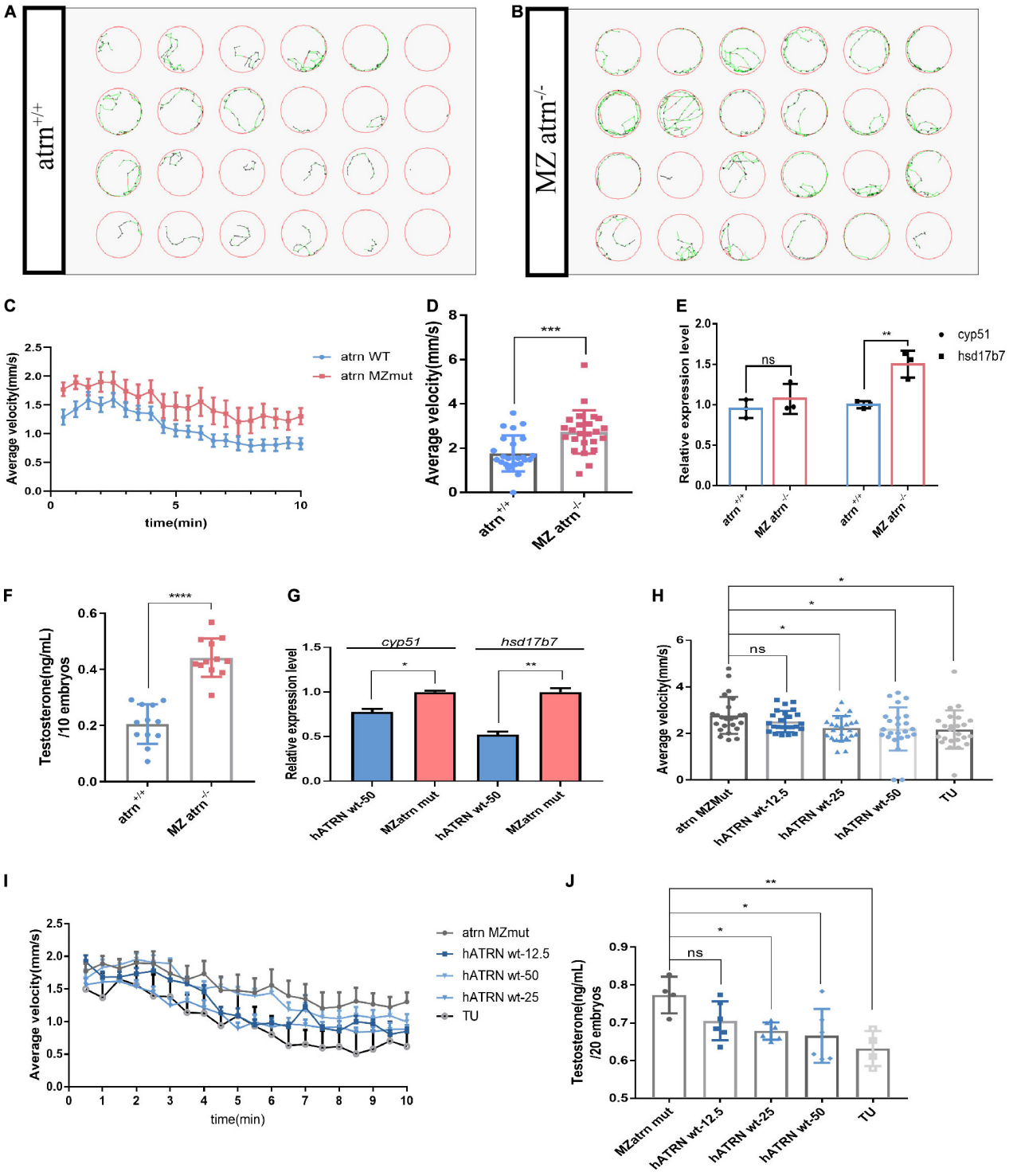

FIGURE 4 | The movements of *atrn*$^{-/-}$ larvae are faster than the wild type due to higher testosterone concentration at 6 dpf, and human *ATRN* can rescue the phenotype of zebrafish *atrn*$^{-/-}$. **(A)** The tracking pattern of the wild-type larvae. **(B)** The tracking pattern of the MZ *atrn*$^{-/-}$ larvae. **(C)** Average swimming speed–time graph of *atrn* WT and MZmut larvae of 144 hpf in 10 min. **(D)** The average velocity of 24 WT and MZ *atrn*$^{-/-}$ larvae during 10 min. Error bars, SEM. **(E)** qRT-PCR results indicate that the expression level of testosterone synthesis key gene *hsd17b7* in MZ *atrn*$^{-/-}$ larvae is increased. **(F)** Testosterone ELISA assay results show that the content of testosterone in the MZ *atrn*$^{-/-}$ is also increased. $N = 10$. **(G)** qRT-PCR results indicate that the expression level of *cyp51* and *hsd17b7* in treated MZmut larvae was rescued. **(H)** Average swimming speed–time graph of 6 dpf larvae injected with hATRN mRNA in 10 min. Error bars, SEM. **(I)** The average velocity of the control and treated larvae during 10 min. **(J)** Testosterone ELISA assay results show that the content of testosterone in larvae injected with hATRN mRNA was rescued. $N = 20$. Data are shown as mean ± SD; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; ns, no significance ($p > 0.05$).

(**Figures 4H,I**). Similarly, hATRN wt mRNA also can rescue the higher testosterone level (**Figure 4J**) and the expression level of *cyp51*, *hsd17b7* in *atrn* MZmut zebrafish embryos (**Figure 4G**). The results indicated that human wild-type *ATRN* can significantly rescue the phenotype in *atrn* MZmut. These data support the interplay of testosterone and *ATRN* in shaping behaviors in the animal model.

Clinical Impact of Circulating Testosterone Levels in Patients With Mental Disorders

Schizophrenia is characterized by disturbances in perception and thinking, in behavior, emotional, and social symptoms (including lack of motivation, lack of social interaction and apathy), and cognitive impairment (Brennan, 2011; Seibt et al., 2011). The above results have shown that *ATRN* has influence on the testosterone level and resulted to abnormal locomotion, which is similar to the positive symptoms (abnormally active), but the correlation between behavior and testosterone is still unknown. Some researchers have illustrated that more hormones can affect the development of mental disorder, for example, steroid hormones, especially testosterone, are related to emotional processing (Derntl et al., 2009; van Wingen et al., 2009).

The information of patients suffering from mental disorder in the hospital was collected to analyze the relationship between the expression level of *ATRN* and testosterone level. The clinical standard for testosterone content in the human body was determined in adults aged 20–49. The normal level for men is 8.64–29.00 nmol/L, and the normal level for women is 0.29–1.67 nmol/L. The normal testosterone level in adults aged over 50 is 6.68–25.70 nmol/L (men) and 0.10–1.42 nmol/L (women).

In light of the known testosterone effects on emotional processing, we postulated that levels of circulating hormone may serve as a surrogate marker. To put this information into perspective, both the expression level of ATRN and the testosterone level were both examined in subjects diagnosed with schizophrenia. Manufacturer data obtained from healthy adults served as a reference.

When stratified by age and gender, we counted 2,579 men aged 20–49 years, 3,926 women aged 20–49 years, 1,009 men aged over 50 years, and 1,741 women aged over 50 years. In the age group 20–49 years, men presenting with a diagnosis of schizophrenia were overrepresented relative to controls and featured lower testosterone levels (**Figure 5A** and **Supplementary Table 1**).

In contrast, women with schizophrenia were more likely to have higher testosterone levels. Likewise, women with a diagnosis of bipolar disorder also featured elevated testosterone levels (**Figure 5C** and **Supplementary Table 2**). In addition, among the test population over the age of 50, men with anxiety, insomnia, and depression tend to cluster in the category "high testosterone levels" (**Figure 5B** and **Supplementary Table 3**). Only a small group of women suffering from anxiety and insomnia exhibited higher testosterone levels (**Figure 5D** and **Supplementary Table 4**). Thus, a dimorphic pattern was observed primarily among patients with schizophrenia. Depending on gender, testosterone levels were either lower or higher than expected.

To establish the contribution made by *ATRN* expression level to testosterone levels and dimorphic effects, we then purposefully selected 14 patients with *ATRN* mutations who had been diagnosed with schizophrenia, including five men and nine women (**Supplementary Table 5**). At different points in time, testosterone levels were determined. We noted that in male patients, testosterone levels were still within the normal range or lower. In contrast, the testosterone levels of female patients were above the normal range (**Supplementary Table 5**). Moreover, male subjects with ATRN mutations were more likely to be classified as "low testosterone level." Woman with ATRN mutations were more likely to be classified as "high testosterone level" (**Figures 5E,F**).

DISCUSSION

Hormones promote the pathophysiology of schizophrenia by increasing local dopamine synthesis and metabolism (Salilew-Wondim et al., 2015; Chen et al., 2019). Besides, many hormones affect the movement behavior of organisms, such as epilepsy, depression, etc. (Bodnar et al., 2018). Some mental disorders, such as schizophrenia, usually have abnormal behaviors. Previous researches have been reported that chromosome position where *ATRN* is located is highly related to schizophrenia (Teltsh et al., 2008). In this research, we used CRISPR/Cas9 technology to construct the $atrn^{-/-}$ zebrafish lines (**Figure 1B**). The tracking analysis suggests that different genders have different behavior and more aggressive attack behavior, indicating that it may affect the hormone levels in $atrn^{-/-}$ zebrafish. Testosterone ELISA Assay was used to detect whether the content in brain of mutant males was significantly higher than that of WT (**Figures 3M,N**). At the same time, it was also found that the testosterone content of the mutant was higher than that of the wild type in the testis of male zebrafish, but the testosterone content in the testis was not as high as that in the brain tissue. It was found that the testosterone content of *atrn* MZmut larvae was significantly higher than that of the wild type (**Figure 4F**). This result suggests that testosterone produced in brain tissue plays a more important role.

Sex steroids have long been implicated in the etiology of neurodevelopment disorders and may contribute to our understanding of sexual dimorphisms. In the present study, $atrn^{-/-}$ zebrafish lines were created and served to investigate behavioral phenotypes, and address hormone states and gene/protein expression (Zhao et al., 2021). With regard to brains of zebrafish, testosterone immunoassays indicated an excess of testosterone in mutant males relative to the wild type (**Figures 3M,N**). A similar pattern was observed in the testis and in larvae (**Figure 4F**). This result confirms that testosterone synthesis is affected in multiple tissues. In the early stages of development, starting at 4 hpf, primordial germ cells (PGC) undergo specialization, migration to the genital ridge, and proliferation (Kossack and Draper, 2019). The apoptosis-dependent transition from ovary to testis starts at 21–25 dpf and may last for several weeks. Only by 35 dpf has the sex

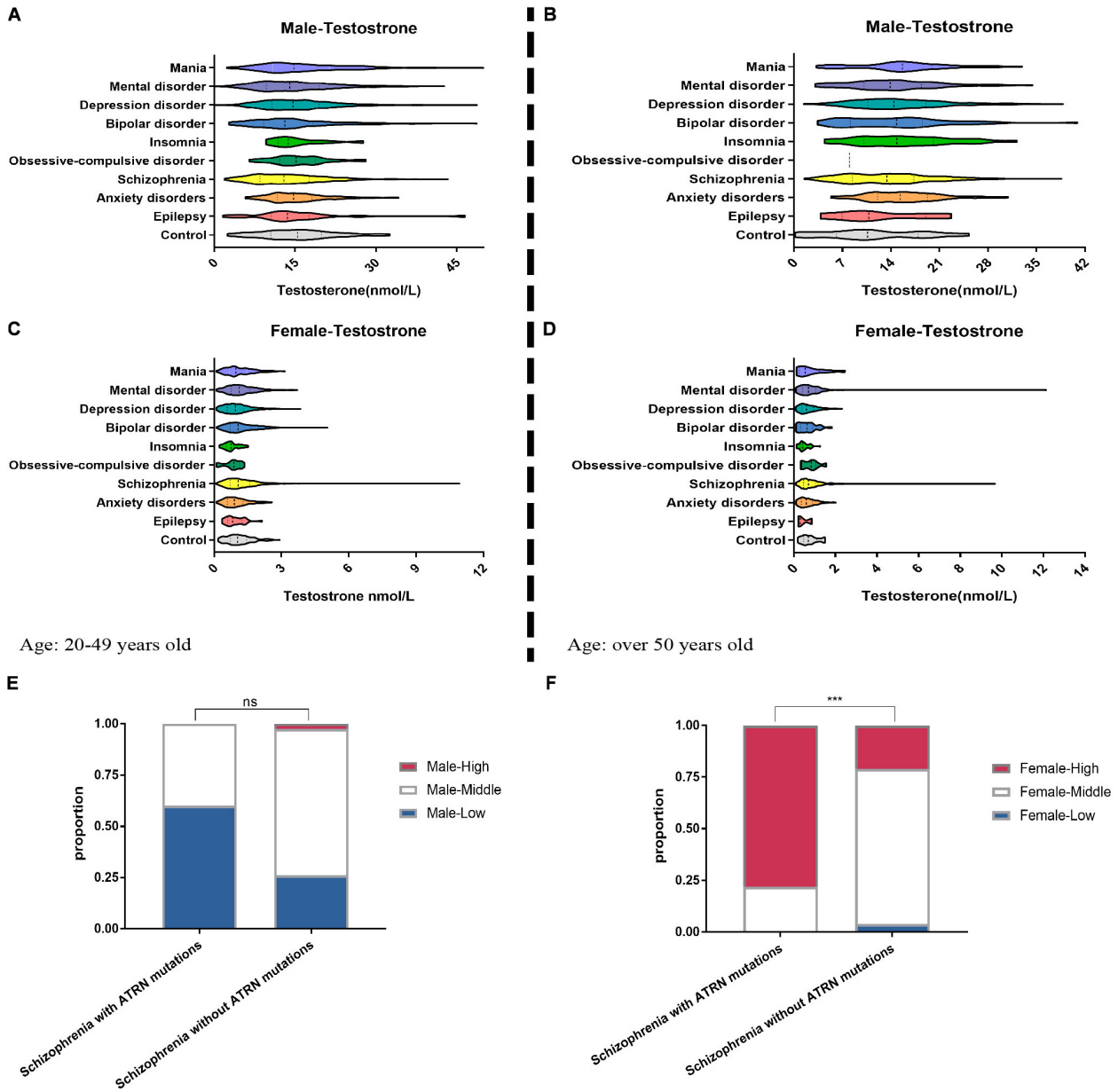

FIGURE 5 | Clinical data show the abnormal serum testosterone level of mental disorder patients. We collected the medical records of patients with mental disorder in the hospital. Among 2,579 male patients **(A)** and 3,926 female patients **(C)** aged 20–49 years, the testosterone level in their serum was tested. Among the patients over the age of 50, the proportion of abnormal testosterone levels among 1,009 men **(B)** suffering from anxiety, insomnia, and depression was significantly increased **(E–G)**, but among 1,741 women **(D)** suffering from anxiety, insomnia, and depression, the proportion of abnormal testosterone levels is significantly reduced. **(E)** For five male patients with schizophrenia who are known to have *ATRN* mutations in the clinic, their lower serum testosterone levels are more than that of schizophrenia patients without *ATRN* mutations. **(F)** For nine female schizophrenia patients with *ATRN* mutations, serum testosterone level was higher than that of the schizophrenia patients without ATRN mutations. *Chi*-test (Fisher's exact test). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; ns, no significance ($p > 0.05$).

of the gonads been determined, and sex-specific gametes are being produced in both the ovaries and testes (Wang et al., 2017; Luzio et al., 2021). As there is no mature testis in larvae, effects on locomotion of *atrn* MZmut are likely mediated by steroids originating from the brain. This observation further underscores the impact of downstream effects exerted by *atrn* on the nervous system.

There is a consensus that sex hormones play roles in anxiety-, trauma-, and stress-related disorders (Cover et al., 2014; Li and Graham, 2017). The potential for neuroactive steroids to participate in mood and cognitive regulation has fueled expectations of new treatment strategies (Sassarini, 2016). As for circulating testosterone levels, conflicting views exist on the existence of disease-specific changes in sex steroids

(Belgorosky et al., 1989). Even so, most changes reported would appear to be minor (Lichtenstein et al., 2009). It should be emphasized that in the above studies, most of the changes are still within the normal range (Hong et al., 2004).

While the magnitude of clinical effects, thus, remains to be determined, and additional regulators of testosterone levels need to be taken into account, the behavior impact of mutated *atrn* warrants further study. Large-scale investigation may help identify common functional variants in the *ATRN* gene, predicting susceptibility to candidate central nervous system phenotypes. These include major heritable conditions, e.g., schizophrenia and other traits, e.g., behaviors that tend to increase and the motivation and ability to acquire and preserve social status of an individual. Our results showed that *ATRN* plays an important role in the testosterone level to affect the behavior. Schizophrenia patients with *ATRN* mutations have more proportion in abnormal testosterone level (**Figures 5E,F**), illustrating the relationship between *ATRN* and testosterone level. Finally, the impact of *Atrn* on maladaptive reward processing and other types of behavior sensitive to testosterone will need to be part of a future agenda.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The datasets Series record GSE178506 of transcriptome sequencing results for this study can be found in the GEO repository.

ETHICS STATEMENT

The animal study was reviewed and approved by the IACUC of the Model Animal Research Center, Nanjing University. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article. The studies involving human participants were reviewed and approved by the local ethics committee of the Affiliated Nanjing Brain Hospital, Nanjing Medical University (2017-KY017). Written informed consent to participate in this study was provided by the legal guardian/next of kin of the participants.

AUTHOR CONTRIBUTIONS

XX, QZ, and NL contributed to the conception and design of the study. NL mainly performed the most experiments, organized the database, and wrote the first draft of the manuscript. SG collected the information and blood samples of patients. SW, SH, JW, LH, and DJ performed the statistical analysis and some experiments. YG, TC, and MK assisted in collecting samples. NL, YS, XX, and JZ participated in writing sections of the manuscript. All authors contributed to the manuscript revision and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.755165/full#supplementary-material

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