Stem Cell Reports

Article

Physical exercise promotes integration of grafted cells and functional recovery in an acute stroke rat model

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https://doi.org/10.1016/j.stemcr.2021.12.006

SUMMARY

Human neural progenitor cell (hNPC) transplantation holds great potential to treat neurological diseases. However, hNPC grafts take a long time to differentiate into mature neurons due to their intrinsically prolonged developmental timetable. Here, we report that post-operative physical exercise (PE), a prevailing rehabilitation intervention, promotes the neuronal commitment, maturation, and integration of engrafted hNPCs, evidenced by forming more synapses, receiving more synaptic input from host neurons, and showing higher neuronal activity levels. More important, NPC transplantation, combined with PE, shows significant improvement in both structural and behavioral outcomes in stroke-damaged rats. PE enhances ingrowth of blood vessels around the infarction region and neural tract reorganization along the ischemic boundary. The combination of NPC transplantation and postoperative PE creates both a neurotrophic/growth factor-enriched proneuronal microenvironment and an ideal condition for activity-dependent plasticity to give full play to its effects. Our study provides a potential approach to treating patients with stroke injury.

INTRODUCTION

Stroke is a leading cause of death and disability worldwide (Zorowitz et al., 2009). Clinical trials have shown that neuroprotective treatments failed to get an ideal outcome in most cases (Gladstone et al., 2002). Neural progenitor cells (NPCs) can differentiate into neurons *in vivo* and have the potential to replace lost neurons after central nervous system (CNS) injury (Blaya et al., 2015; Han et al., 2013; Kadoya et al., 2016; Lu et al., 2014). The incorporation of NPC-derived neurons into the host neural circuitry is believed to ensure long-term functional recovery. However, evidence showing good integration of transplanted human NPCs (hNPCs) is rare (Espuny-Camacho et al., 2018; Palma-Tortosa et al., 2020; Tornero et al., 2017). One of the most important reasons is that grafted hNPCs take a long time to mature and achieve functional integration with host neural circuits (Lu et al., 2017; Real et al., 2018; Semple et al., 2013), which contrasts with the relatively short experimental window and short lifespan of rodents in most studies. In the clinic, there is always a critical time window for rehabilitation intervention (Coleman et al., 2017), which may be easy to miss due to the time-consuming human neuron maturation that occurs if cell replacement therapy is used alone. Therefore, it is essential to find efficient and safe ways to promote neuronal differentiation and maturation of transplanted hNPCs, as well as to establish functional connectivity in both preclinical animal studies and clinical applications.

Physical exercise (PE) can modulate peri-infarct micro-environments by promoting angiogenesis, vasomotor reactivity, and neurotrophic factor release, reducing excitotoxicity and inflammation and thus improving neurological function (Mala and Rasmussen, 2017; Pin-Barre and Laurin, 2015b). Recently, we reported that PE can improve brain function through mitigation of autophagy and attenuation of apoptosis in stroke-damaged rats (Zhang et al., 2013). PE has been widely recommended as a standard postoperative rehabilitation regimen for brain and spinal cord injury and justified by a large number of clinical trials (Liu et al., 2019; Vanderbeken and Kerckhofs, 2017). Our group and other laboratories have also demonstrated that PE can recruit endogenous NPCs and induce neurogenesis under physiological and pathological conditions (Choi et al., 2018; Luo et al., 2014; Yau et al., 2014). However, such evidence on exogenous NPCs is virtually nonexistent.

Moreover, compared with stroke damage confined to the striatum, lesions with cortical damage showed worse recovery with cell replacement either by grafted neural stem cells or by endogenous neurogenesis in animal studies
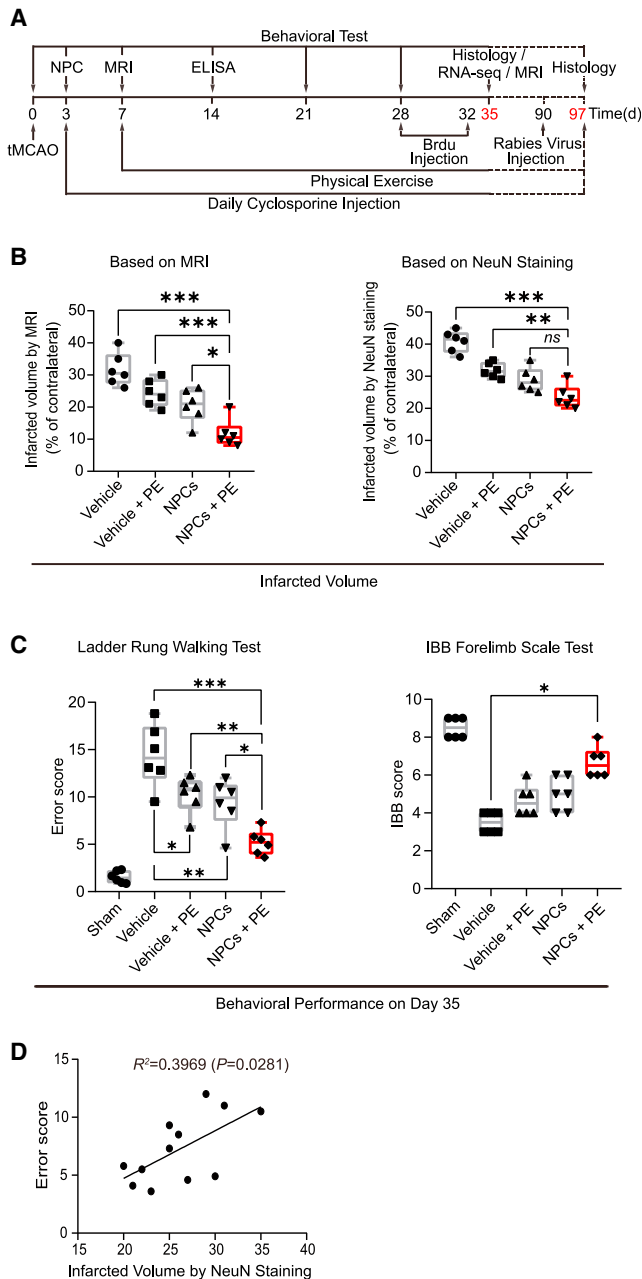
Figure 1. hNPC transplantation combined with physical exercise (PE) improves the structural outcome and neurological functions of stroke-damaged rats

(A) Experimental design.

(B) Brain infarction ameliorated at day 35 measured by T2-weighted MRI (left) and quantification of NeuN immunostaining brain sections (right). Also see Figure S2 for representative images.

(C) Neurological functions recovery at day 35 measured by the ladder rung walking test and Irvine, Beatties, and Bresnahan (IBB) forelimb scale test. Also see Figure S3A for the cylinder test and elevated body swing test. Sham: sham surgery group without tMCAO. n = 6 rats per group; *p < 0.05, **0.01 < p < 0.05, ***p < 0.01 determined by Kruskal-Wallis with Nemenyi

post hoc test (IBB) or one-way ANOVA followed by Bonferroni's *post hoc* test.

(D) Pearson's correlation analysis between infarcted volume and error score in the ladder rung walking test.

(Arvidsson et al., 2002; Smith et al., 2012). Clinical evidence from patients also shows more severe symptoms and a lesser degree of recovery when stroke lesions affect cortical areas (Delavaran et al., 2013). In this study, we employ a stroke rat model with lesions spanning most of the unilateral striatum and a large area of the overlying sensorimotor cortex and demonstrate that the combination of NPC transplantation and postoperative PE promotes functional outcomes of NPCs in stroke recovery.

RESULTS

Physical exercise after hNPC transplantation improves structural and behavioral outcomes in stroke-damaged rats

To determine whether hNPC transplantation combined with PE can be developed into a practical therapeutic strategy for treating intractable stroke, we studied the effects of poststroke exercise training on the *in vivo* cellular behavior of transplanted hNPCs and the effect of this combination on neurological recovery. We employed the transient middle cerebral artery occlusion (tMCAO) model in 8-week-old male Sprague-Dawley rats, which can simulate the clinical process of ischemic stroke well. The successful model of the rats was confirmed by the Bederson score (Bederson et al., 1986). The stroke-damaged rats were then randomly divided into four groups: sham injection (vehicle alone), PE after sham injection (vehicle plus PE), NPC transplantation alone (NPCs alone), and NPC transplantation combined with PE (NPCs plus PE). hNPCs were derived from human embryonic stem cells (ESCs) in which the CAG promoter-driven dsRed gene was targeted to the AAVS1 locus via CRISPR-Cas9 gene editing and intracerebrally transplanted into the ischemia striatum on day 3 after tMCAO (2.4×10^5 cells suspended in 3 μL DMEM/F12). Rats in the vehicle groups were injected with DMEM/F12 doses of equal volume. For PE training, an enforced wheel-running program at a moderate intensity started on day 7 after tMCAO and continued until sacrifice. We followed up the brain infarction with MRI T2-weighted imaging on days 7 and 35 after tMCAO (Figure 1A). tMCAO affected most of the ipsilateral striatum and a large area of the overlying sensorimotor cortex (Figures S1A and S1B). Rats in the NPCs plus PE group exhibited a significantly smaller infarcted volume based on both MRI and the quantification of NeuN immunostaining in brain sections (Figure 1B), as well as a higher cortical width index compared with
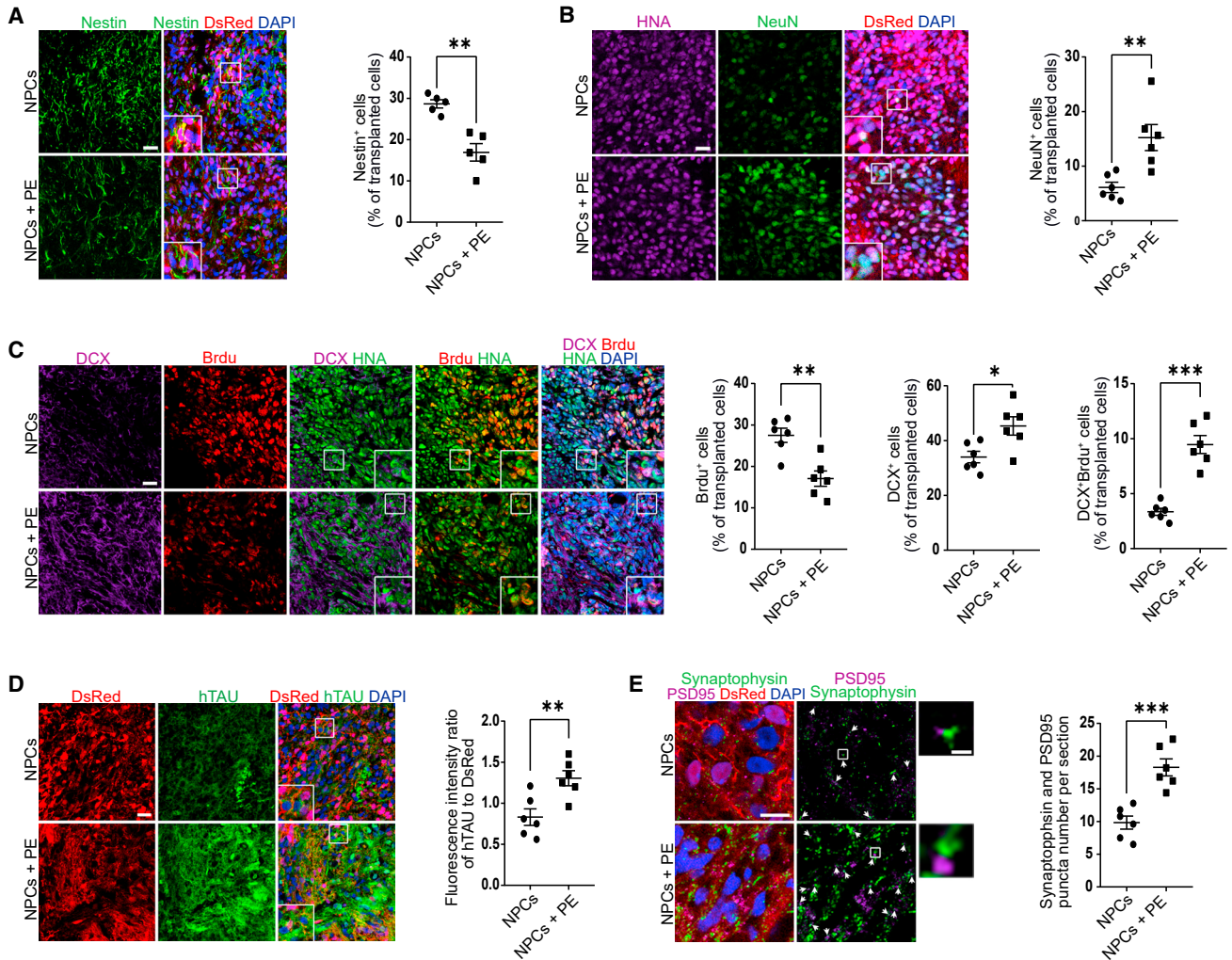
Figure 2. PE accelerates the neuronal commitment, axon outgrowth, and synapse formation of engrafted hNPCs

(A and B) Accelerated neuronal differentiation of human grafts (DsRed) measured by immunostaining of Nestin (A) and NeuN (B).

(C) Newly produced cells and/or immature neurons traced by BrdU injection and identified by immunostaining of human-specific nuclear antibody (hNA) and DCX, a marker of neuroblasts and immature neurons.

(D) Axon outgrowth of human neurons measured by human-specific TAU (hTAU) immunostaining.

(E) Synapse formation measured by colabeling of the postsynaptic marker PSD-95 and the presynaptic marker synaptophysin. n = 6 rats per group. Data are represented as mean ± SEM over scatterplots; *p < 0.05, **0.01 < p <0.05, ***p < 0.01 by Student's t test. Scale bars: 20 μm in (A, B, D, and E); 50 μm in (C).

other groups (Figure S1C). More important, they also outperformed other groups in motor function tests on day 35 after stroke (Figures 1C and S2A), including the ladder rung walking task; Irvine, Beatties, and Bresnahan (IBB) forelimb scale test; cylinder test; and elevated body swing test (for the time course, see Figures S2C–S2F). And the behavioral performance is well correlated to the infarcted volume among hNPC-transplanted individuals (Figure 1D). The NPCs plus PE rats also showed significantly better performance than the NPCs-alone rats in a long-term assessment on day 97 (Figure S2B).

Physical exercise protects hNPCs and accelerates their neuronal commitment

The engrafted human cells were identified by DsRed fluorescence and/or positive staining against human-specific nuclear antigen (hNA). The human grafts were restricted within the striatum area, with very few cells reaching the overlying cortical parenchyma. All the pictures in this paper were taken on day 35 from the rectangular area indicated in Figure S3A unless otherwise stated.

We first investigated the effect of PE on graft survival. Although apoptosis analysis of transplanted cells by

terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay at day 35 shows no significant difference resulting from PE training (Figure S3B), and Ki67 staining shows a higher proliferation in the NPCs alone rats (Figure S3C), the human graft volume in the NPCs plus PE group is significantly larger than that in the NPCs alone group (Figure S3D), implying a plausible protection role of PE on hNPCs during the early stage, supported by the enhanced release of neurotrophic factors (see below).

Thus, to gain insight into the contributions of the surviving hNPCs to the structural and behavioral recovery in the NPCs plus PE rats, we performed a detailed immunostaining of hNPCs against a variety of cell markers. Most of the grafted human cells retained the forebrain/cortical identity, expressing markers like FOXG1 and TBR1 (Figures S4A and S4B), while a small subpopulation (1%–5%) of human cells started to show the striatum marker DARPP32 (Figure S4C). A relatively high proportion of human cells (10%–30%) expressed the neural stem cell marker Nestin. Notably, PE significantly reduced Nestin-positive cells (Figure 2A) and the percentage of Ki67$^+$ human cells (Figure S3C). We also administered bromodeoxyuridine (BrdU) via intraperitoneal injection for 5 consecutive days from the 28th to the 32nd day after stroke to trace the newly produced cells and found that PE training significantly reduces BrdU$^+$ cells (Figure 2C). Conversely, PE increases the proportion of DCX$^+$ cells (a marker of neuroblasts and immature neurons) among transplanted cells, as well as that of BrdU and DCX double-positive cells (Figure 2C). We also found a 2.5-fold increase in NeuN-labeled human cells in the NPCs plus PE group (Figure 2B), while astroglia-like human cells were barely detected in both groups (Figure S4D). The majority of the human neurons were excitatory, with higher glutamate and vGluT1 levels in the NPCs plus PE group (Figures S4E and S4F). All these results demonstrated an accelerated neuronal commitment of hNPCs by PE training.

Physical exercise promotes the integration of engrafted neurons into host neural circuits

Axon regeneration and synapse reconstruction form the structural basis of functional improvement. We performed human-specific TAU (hTAU) protein immunostaining to assay human neurofilament density and unveiled that the fluorescence intensity ratio of hTAU to dsRed was much higher in the NPCs plus PE group (Figure 2D). Furthermore, the synaptophysin (presynaptic marker) and PSD-95 (postsynaptic marker) colabeled puncta surrounding the engrafted cells in the NPCs plus PE group outnumbered those in the NPCs alone rats (Figure 2E). These data indicate that human axon outgrowth and substantial synapse formation within the graft area are greatly boosted by PE training and may contribute to neurological recovery (see their correlation in Figures S5D and S5E).

The physiological maturation and integration of human neurons are also supported by the expression of the immediate-early gene c-Fos, a neuronal activity marker. The striatum receives input from the motor cortex and also projects back via the thalamic relay. The c-Fos expression in both the intrastriatal human graft (Figure 3A) and the ipsilateral host motor cortex (Figure 3B) is greatly enhanced by PE training. The upregulated activity of human neurons may represent substantial innervations from the host neurons or a more mature state. Further, the higher-level neuronal activity in the motor cortex may suggest that the intrastriatal human cells may activate the host motor cortex in a feedforward manner. However, the activating role of human cells in NPCs alone rats is marginal (p > 0.05, NPCs alone versus vehicle alone), indicating a decisive role for PE training in promoting the functional integration of human cells.

Next, we used a rabies-based retrograde monosynaptic tracing strategy (Falkner et al., 2016; Grealish et al., 2015; Tornero et al., 2017) to see the connections between donor and host neurons by using an H1-CAG-GTRqp human ESC line, which previously was generated in our lab, with constitutive expression of rabies virus glycoprotein, avian TVA receptor, and EGFP under the control of the human CAG promoter. Three months after transplantation, rabies virus for retrograde monosynaptic tracing was injected into the transplantation site and the rats were perfused 7 days later. The rabies virus specifically infected the human cells, and the grafted and infected human cells coexpressed EGFP and mCherry fluorescence (as the starter neurons, hNA$^+$/EGFP$^+$/mCherry$^+$) and received the afferent projection from the host neurons that express only mCherry (as the traced neurons, hNA$^-$/EGFP$^-$/mCherry$^+$). The traced host neurons were distributed in diverse brain areas, and mostly concentrated in the ipsilateral motor cortex (Figure 3C). Notably, the number of traced neurons as a percentage of the starter cells in the ipsilateral motor cortex of the NPCs plus PE rats was about 2-fold greater than that of NPCs alone (Figure 3C), as it was is in the contralateral motor cortex (Figure 3D).

Collectively, these data prove that PE training could promote neuronal commitment and accelerate the maturation and integration of grafted hNPCs, which are well correlated with the behavioral performance of NPC-transplanted individuals, implying that the activity of human neurons may contribute to neurological recovery (for their correlation analysis, see Figures S5C and S5G).

Physical exercise ameliorates the infarction foci and creates a proneuronal microenvironment

The density of the nerve fibers in the NPCs plus PE rats was the highest among all the groups, as indicated by the fluorescence intensity of the TAU protein (Figure 4A). PE
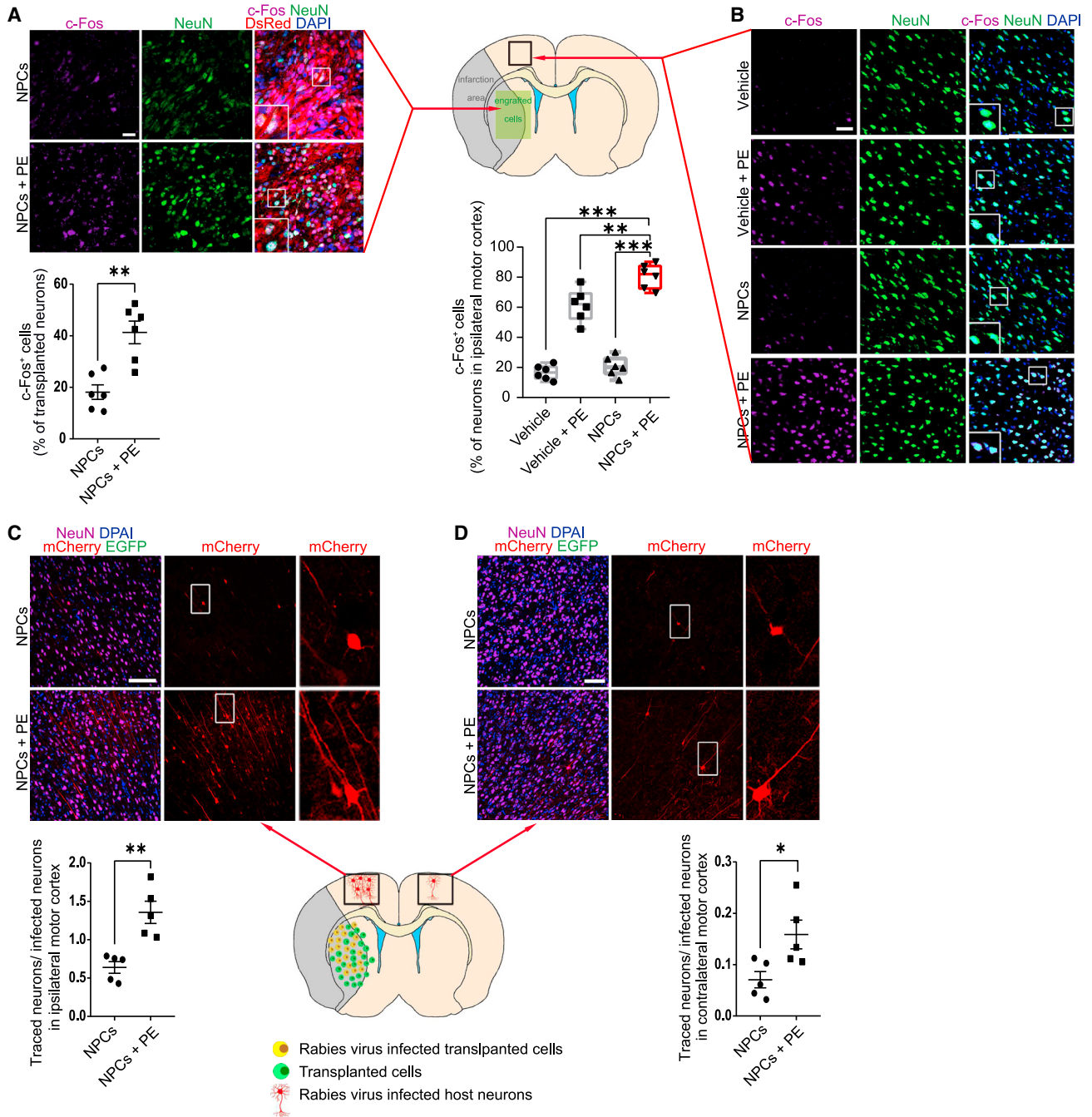
Figure 3. PE increases neuronal activity and the synaptic connection among engrafted human cells and host neurons

(A and B) Enhanced neuronal activity in both human cells (A) and the host neurons in the ipsilateral motor cortex (B) indicated by the c-Fos expression level.

(C and D) Increased synaptic projections from the host neurons to the engrafted human neurons unveiled by rabies-based retrograde monosynaptic tracing and quantified as the ratio of virus-traced host neurons/rabies virus-infected human cells (starter neurons) in the ipsilateral motor cortex (C) and in the contralateral motor cortex (D). n = 6 for (A and B); n = 5 for (C and D). *p < 0.05, **0.01 < p < 0.05, ***p < 0.01 by Student's t test (A, C, and D) and one-way ANOVA followed by Bonferroni's *post hoc* test (B). Scale bars: 50 μm in (A and B); 100 μm in (C and D).
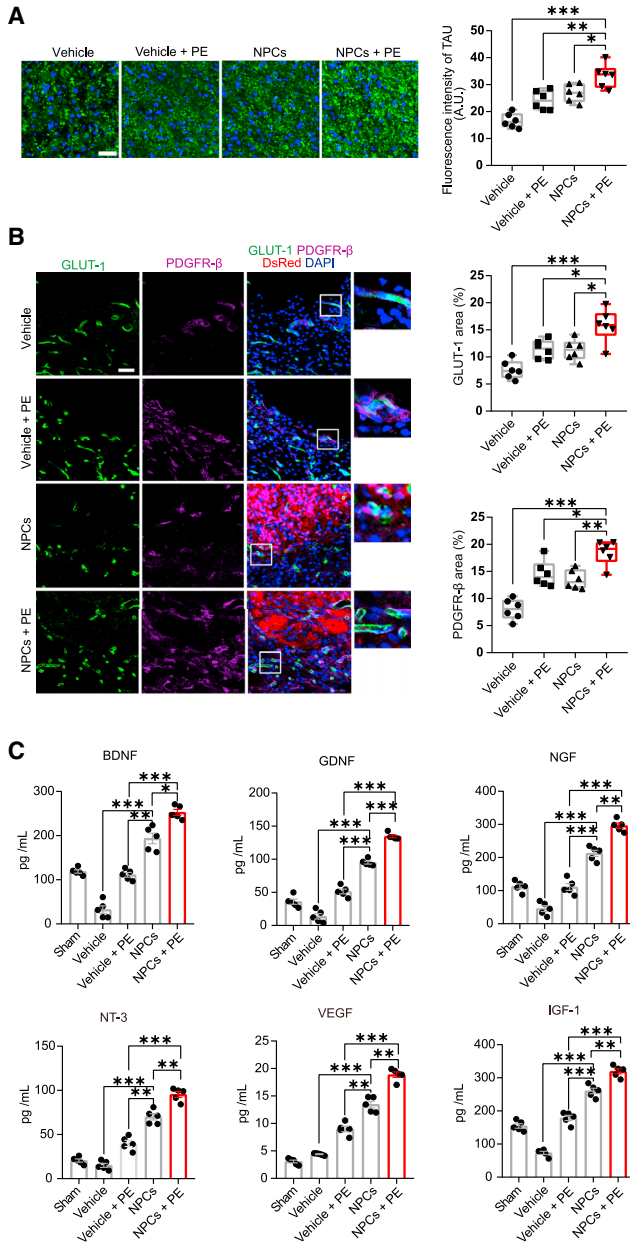
Figure 4. hNPC transplantation combined with PE ameliorates the microenvironment around the infarcted area

(A) Enhanced neural fiber regeneration along the infarction border measured by immunostaining of TAU, an axon marker.

(B) Angiogenesis within the infarcted area measured by immunostaining of vessel marker Glut-1 and pericyte/smooth muscle cell marker PDGFR-β.

(C) Upregulated secretion of neurotrophic factors and angiogenic factors on day 14 unveiled by ELISA. Sham: sham surgery group without tMCAO. n = 6 for (A and B); n = 5 for (C). *p < 0.05, **0.01 < p < 0.05, ***p < 0.01 by one-way ANOVA followed by Bonferroni's *post hoc* test. Scale bars: 20 μm in (A); 50 μm in (B).

training also promotes the ingrowth of blood vessels, as evidenced by immunostaining against glucose transporter-1 (Glut-1) and platelet-derived growth factor receptor-β (PDGFR-β) (Figure 4B). These data indicate a much ameliorated microenvironment within the infarction area in the NPCs plus PE rats that might benefit structural and functional recovery (also see Figures S5H and S5I for the correlation analysis).

To find what molecules participated in this microenvironment reconstruction, we examined the levels of candidate neurotrophic factors, angiogenesis factors, and insulin-like growth factors within the striatum as previously reported, including brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF), nerve growth factor (NGF), neurotrophin-3 (NT-3), vascular endothelial growth factor (VEGF), and insulin-like growth factor-1(IGF-1) (Pin-Barre and Laurin, 2015a; Yook et al., 2019) by using ELISA kits on the 14th day after stroke. Both NPC transplantation and PE training increase the levels of all these cytokines, with the highest being in the NPCs plus PE group (Figure 4C). To get a more thorough view of what was going on during the first recovery month, we conducted genome-wide RNA sequencing and a gene ontology (GO) enrichment analysis of differentially expressed mRNAs in each group. PE alone significantly upregulates genes in synaptic activities, especially those related to synaptic transmission (cholinergic) and acetylcholine metabolism (Figure 5A), which is very consistent with previous studies identifying a key role for acetylcholine in motor map plasticity and rehabilitation-mediated recovery from brain lesions (Conner et al., 2005; Ramanathan et al., 2009; Wang et al., 2016). NPC transplantation upregulates genes involved in neuronal differentiation, axon elongation, and angiogenesis, as well as antiapoptosis, antioxidation, and antigliosis (Figure 5B), where the NPCs plus PE rats greatly outperformed the NPCs alone rats (Figure 5C). More important, postoperative PE after NPC transplantation can further enrich the genes involved in multiple physiologically related processes and functions, such as neuronal excitability, learning and memory, the dopamine signaling pathway, and adenylate cyclase activation (Figure 5D). The last two also have long been known to play important roles in motor control and learning, as well as neural plasticity (Kandel, 2012; Pierre et al., 2009; Wooten and Trugman, 1989).

DISCUSSION

Neuronal differentiation of transplanted cells and integration into the host neural circuitry are key steps for effectual neuronal repair. Some studies have suggested that grafted hNPCs can receive synaptic input from or send projections
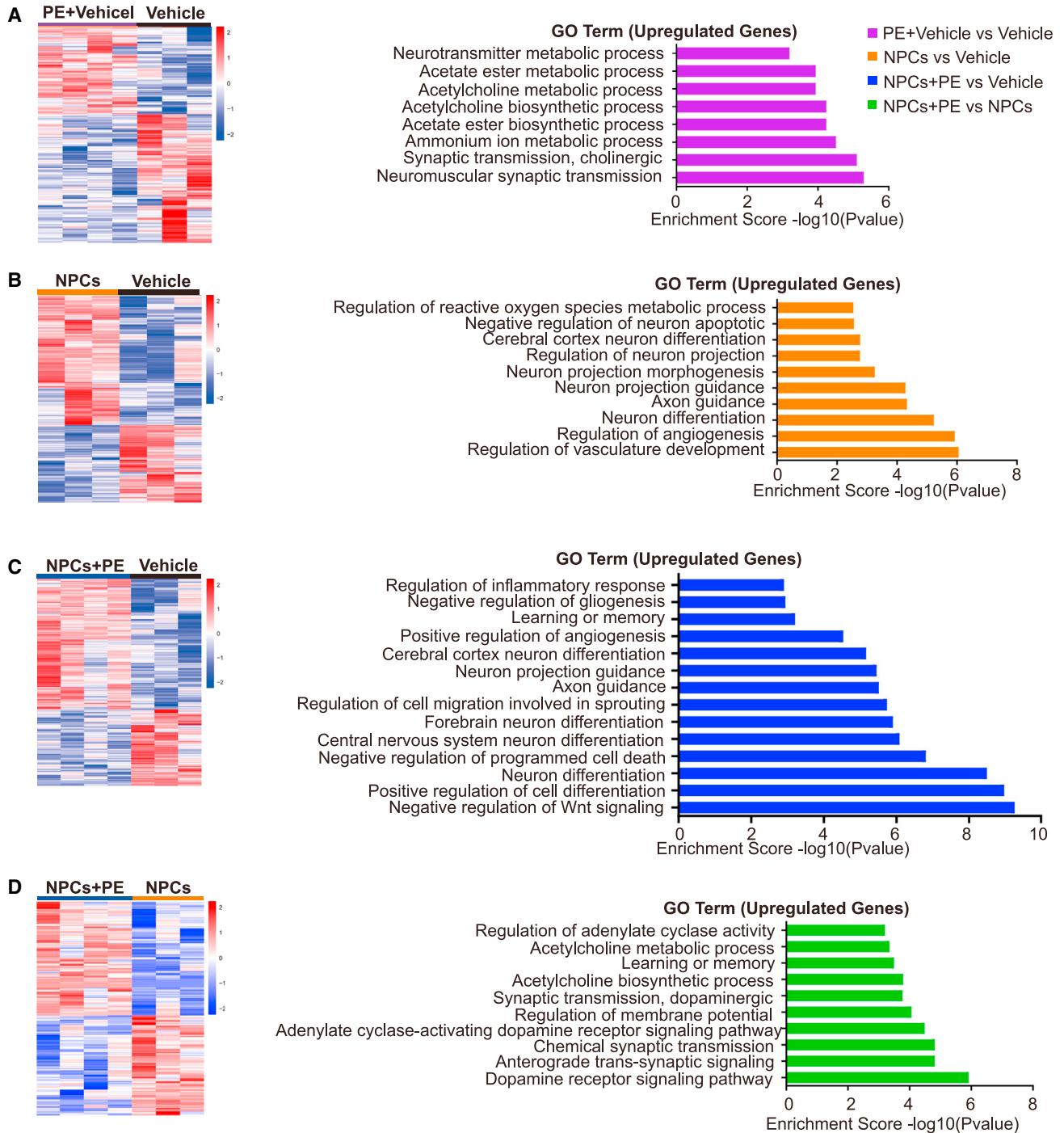
Figure 5. hNPC transplantation and PE alter the gene expression signature of stroke-damaged striatum

(A–D) Differentially expressed genes (DEGs) determined by RNA-seq and gene ontology (GO) enrichment analysis of vehicle plus PE versus vehicle (A), NPCs alone versus vehicle (B), NPCs plus PE versus vehicle (C), and NPCs plus PE versus NPCs alone (D) on day 35. Left: heatmaps depicting all significant DEGs (red is upregulated genes, blue is downregulated genes). Right: the top GO terms of upregulated genes. n = 4 rats for the vehicle plus PE and NPCs plus PE groups. n = 3 rats for the vehicle alone and NPCs alone groups. The p value indicates the significance of the enriched GO terms among different treatments.

to the host neurons by virtue of fluorescence labeling and long-time tracing techniques (Espuny-Camacho et al., 2018; Palma-Tortosa et al., 2020; Tornero et al., 2017).The activity of grafted human neurons was functionally modulated by the host sensory input (Tornero et al., 2017) and involved in maintaining normal motor function (Palma-Tortosa et al., 2020). However, hNPCs need an extended maturity timeline, which has hindered more preclinical investigations exploring functional recovery by transplanting hNPCs into animal models, thus compromising their clinical application. Active proliferation and late neuronal differentiation/maturation of human grafts have been reported recently (Lu et al., 2017; Real et al., 2018). The unpredictable or uncontrollable proliferation of transplanted hNPCs brings tumorigenic or hyperplasia risk (Chung et al., 2006; Germain et al., 2012; Guan et al., 2014). In light of the present data, we demonstrate for the first time that PE, a prevailing postoperative rehabilitation intervention, can substantially promote neuronal differentiation and accelerate the maturation of hNPCs, which might be the way to increase hNPCs curative effect. Recently, Yu and colleagues also reported that artificially selective activation of transplanted NPCs by opto-chemogenetics enhanced neuronal repair and functional recovery after ischemic stroke (Yu et al., 2019).

We further unveiled two sequentially and parallelly interacting mechanisms that could benefit neurologic recovery, where interactions between the host cells and the donor cells play a key role. The PE and NPC transplantation show a cumulative effect in this process.

First, the combination of PE and NPC transplantation can create the most proneuronal microenvironment among all groups. As previously reported (Neeper et al., 1995; Vaynman and Gomez-Pinilla, 2005), PE training alone and NPC transplantation alone increase the secretion of neurotrophic and angiogenesis factors. This was significantly augmented by the combinational intervention, which is indicative of the accumulative effect of PE training and NPC transplantation. Our genome-wide RNA sequencing results also confirmed the optimized microenvironment for neuron differentiation and maturation. The increased neurotrophic/growth factors play both neuroprotective and regenerative roles and can benefit recovery in multiple aspects. For example, VEGF can induce angiogenesis, as shown in our experiment, to increase the supply of nutrients and oxygen or directly prompt axonal outgrowth in blood-flow-independent ways, e.g., providing a neurogenic niche via neurovascular coupling or a scaffold for cell migration (Kanazawa et al., 2019). Further, the hNPCs deposited in the infarction area can provide potential targets for the striatum-projecting host neurons to innervate in a timely way, preventing them from remote degeneration (Fornito et al., 2015; Nakane et al., 1997) and unwanted sprouting (Sutula et al., 1989) due to loss of targets.

Second, the combination of PE and NPC transplantation can create an ideal condition for activity-dependent plasticity to give full play to its effects. *Brain plasticity* refers to the brain's ability to change structure and function in the face of an ever-changing environment at the levels of molecules, spines, dendrites, and axons and even the entire cell, like adult neurogenesis. PE can influence all these levels and promote the remodeling of host striatum-projecting neurons in the motor cortex and the thalamus in an activity-dependent way (Eadie et al., 2005; Redila and Christie, 2006; Wang et al., 2016). The host neurons may innervate the striatum-deposited human cells promptly *in situ* before their maturation. Previous studies have shown that immature newborn neurons in adult rats exhibit a higher plasticity capability and a lower threshold for the induction of long-term potentiation (LTP) compared with mature neurons (Ge et al., 2007; Schmidt-Hieber et al., 2004). Moreover, when PE can synchronously activate the intrastriatal human neurons and the host cortical neurons, the prerequisite for Hebbian synaptic plasticity forms (Murphy and Corbett, 2009) and the connections between the neurons would be further strengthened, whereby the c-*Fos* expression in both human grafts and the host motor cortex is specifically enhanced by PE training. One of our previous studies showed that synchronized oscillation in the corticothalamic network can readily induce c-*Fos* expression and lead to neuronal plasticity (Guo et al., 2007). Our RNA sequencing (RNA-seq) also shows that PE enriches genes involved in experience-dependent plasticity, such as neuronal excitability, adenylate cyclase activation, cholinergic and dopaminergic synaptic transmission, learning, and memory.

PE is noninvasive and safe, without side effects or risks associated with inflammation/infection, drug addiction/resistance, allergy, and stress. Now PE has been widely used in postoperative rehabilitation and home health care for a variety of brain disorders, including trauma, stoke, and neurodegenerative diseases. However, the clinical data indicate that PE training, as well as other rehabilitation interventions, is not enough. Our new results propose the combination of NPC transplantation and postoperative PE as a treatment for patients with stroke injury. However, when applied in the clinic, our study shows several limitations. The species discrepancies between rodents and humans encompassing physiological, pathophysiological, anatomical, and behavioral factors are huge, so nonhuman primates should be used as models for further preclinical and translational stroke studies (Fisher et al., 2009; Stroke Therapy Academic Industry, 1999). Moreover, we have done only an acute stroke model, and additional studies are needed in chronic stroke subjects, as many chronic stroke patients are refractory to all the prevailing

rehabilitation interventions, due to missing the critical rehabilitation time window.

In conclusion, our study demonstrates that postoperative PE can promote neuronal differentiation, maturation, and functional integration of grafted hNPCs in an acute stroke rat model. The combinational intervention of NPC transplantation and PE training outperforms both NPC transplantation alone and PE training alone in terms of structural and behavioral outcomes by creating a neurotrophic/growth factor-enriched proneuronal microenvironment and an ideal condition for activity-dependent plasticity to give full play to its effects in the stroke-damaged brain, pointing to a possible treatment strategy for patients with stroke injury. Future work should be carried out to optimize this combinational regimen by using nonhuman primates for further preclinical and translational stroke study.

EXPERIMENTAL PROCEDURES

All procedures were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health (Publication No. 80-23, revised 1996) and were approved by the ethical committee for the use of laboratory animals at the Guangzhou Institute of Biomedicine and Health, Chinese Academy of Sciences.

Cell culture
hNPCs derived from an H1 ESC line (WA01, WiCell Research Institute) were used in the experiments. ESCs were transduced with a viral construct containing red fluorescent protein and the EGFP and dsRed genes were inserted at the AAVS1 locus to generate fluorescence-expressing lines (Xing et al., 2019). Generation of NPCs from these ESC lines by using the monolayer culture method has been described previously (Xing et al., 2019). Briefly, when ESCs reached approximately 95% confluence, the culture medium was changed to N2B27 medium with 5 μM SB431542 (Selleck, S1067) and 5 μM dorsomorphin (Selleck, S7840) added for 8 days. The cells were then scraped down and replated in the N2B27 medium for another 8 days. hNPCs were obtained by manually picking rosette-like structures and maintained in suspension in the N2B27 medium with 20 ng/mL basic fibroblast growth factor (bFGF) (Peprotech, 9610018B50) and 20 ng/mL epidermal growth factor (EGF) (Peprotech, AF10015100).

Animals
Adult male (250–280 g) Sprague-Dawley rats were used in the study. The rats were purchased from Beijing Vital River Laboratory Animal Technology and raised in a specific-pathogen-free environment with a standard laboratory diet and a 12/12 h light/dark cycle. After a quick neurologic assessment by using the Bederson score (Bederson et al., 1986), the stroke-damaged animals that scored grade 2–3 were randomly grouped, and all the experiments were blinded. In total, we did three and two cell batches of transplantation experiments, respectively, for 35-day and 97-day assessments, and one batch for 14-day ELISA.

Transient middle cerebral artery occlusion
A modified tMCAO technique was used as previously described (Belayev et al., 1996). Briefly, rats were anesthetized with 2% pentobarbital sodium (50 mg/kg, i.p.) and fixed supinely on a warming pad. A vertical incision was made in the middle of the neck. The common carotid artery (CCA), internal carotid artery (ICA), and external carotid artery (ECA) were carefully separated with tweezers. The distal end of the ECA was ligated and the proximal end of the ECA and the bifurcation of the CCA were clamped. Then an arteriotomy hole was made between the ligation and the vascular clamp, and a nylon monofilament (final tip diameter, 0.38 ± 0.02 mm, Beijing Cinontech, China) was carefully inserted into the hole in the direction of the vascular clamp. The filament was gradually forwarded from the ECA to the ICA until the MCA was blocked, and then it was kept in place for 60 min. The reperfusion was performed by gently pulling out the filament. After the skin was disinfected and sewn, the rat was returned to a clean home cage.

Physical exercise
The rats in the PE group were trained on a speed-control running wheel (diameter, 21 cm; length, 60 cm) and started running exercises on day 7 (20 min per session, 2 sessions per day, 6 days per week). The initial speed was 5 rpm in the first week and gradually increased to 10, 15, and 20 rpm on days 14, 21, and 28, respectively. The rats in the control group were put in the same apparatus at the same time, but without rotating.

Immunosuppression and cell transplantation
All rats, including those in the vehicle and sham surgery (called *sham* in the results) groups, received immunosuppressant cyclosporine A (Norvartis, Sandimmune 10 mg/kg, i.p.) daily until sacrifice. At day 3 after tMCAO, the rats were anesthetized with isoflurane and fixed in a stereotactic apparatus (ASI Instruments, USA). The skulls were exposed and a hole was drilled at the appropriate position on the ischemic hemisphere. hNPCs were digested with Accutase (Sigma, A6964) into single cells and microinjected into the striatum (80,000 cells/μL, 3 μL in total, 1 μL/min) by using an infusion pump (WPI). The injection coordinates were 0.5 mm anterior to Bregma, 3.0 mm right of the sagittal suture, 5.0 mm ventral to the brain surface. The vehicle received a suspension medium injection of the same volume.

Neurobehavioral evaluation
The rats were subjected to neurologic function evaluations using the ladder rung walking task, IBB forelimb scale, cylinder test, and elevated body swing test immediately before tMCAO surgery and 7, 14, 21, 28, 35, and 97 days after MCAO. For the first three tests, each rat's performance was filmed and analyzed frame by frame by two experimenters who were blinded to the experimental groups. For more information, see the supplemental information.

The ladder rung walking task
Before the first test session, the rats were trained to cross a ladder on a regular rung pattern. In test sessions, the rats were examined on an irregular pattern (a new pattern for each trial, five trials per rat). The error score was the percentage of total steps that were in error.

The IBB score

The rats were placed in a transparent Plexiglas box in which the bottom and two adjacent side walls were mirrored. The rats were filmed while eating one entire spherical cereal piece. The score was given according to the scale described previously (Irvine et al., 2010) by checking the video. Two trials for each rat were performed, and the mean score was used in the analysis.

Cylinder test

The rats were placed in a transparent glass cylinder (diameter 20 cm, height 30 cm) and could move freely in a quiet state. Two vertical mirrors were put behind the cylinder for an all-direction observation. The number of times they reared and touched the cylinder in a weight-bearing fashion with the left, right, or both forelimbs was counted for 20 hits, and the left:right ratio was used in the analysis.

Elevated body swing test

Rats were held approximately 1 inch from the base of the tail at a height of about 1 inch. A right (left) swing was counted when the head of the animal moved more than $10°$ from the vertical axis to the right (left) side. Swings were counted for 60 s for each rat, and the percentage of hemiplegic sides (left swings) was analyzed.

MRI measurements

MRI measurements were performed using a 7 Tesla system with a Bruker console (Bruker Biospin, PharmaScan70/16, USA). Anesthesia and body temperature were maintained during the MRI measurements. T2-weighted imaging was obtained by multiple slices, multiple spins, or gradient echoes sequences (TE = 33, TR = 2,500, FOV = 35, matrix = 128 × 128, 23 slices, 0.8 mm slice thickness), respectively. Seven images were acquired for each slice, including one baseline with b = 0 and six images with b = 1,849.98 s/mm^2 at independent directions of diffusion gradients along the xyz axis.

ELISAs

Fresh ipsilateral striatum tissues were carefully isolated under stereoscopy, homogenized in an extraction buffer containing a protease inhibitor, and centrifuged at 13,000g for 30 min at 4°C. The ELISA kits used in the study were rat BDNF (Abcam, ab213899), rat VEGF (Abcam, ab100787), rat GDNF (Abcam, ab213901), rat neurotrophin-3 (Abcam, ab213905), rat IGF-1 (Abcam, ab213902), and rat NGF (ImmunoWay, KE1648). Supernatants were used to measure cytokines according to the manufacturers' protocols. Briefly, 100 μL samples and calibrators were added to the plate wells coated with an array of cytokine capture antibodies and incubated for 2 h, followed by incubations with the primary antibody (for 2 h) and the second antibody (for 1 h). An MSD read buffer was added and the plate was read immediately on a MesoQuickPlex SQ 120.

Rabies vector production and injection

A pseudotyped rabies vector was produced as previously described (Xing et al., 2019). Titration was performed using TVA-expressing HEK293T cells as defined in the protocol (1 × 10^7 TU/mL). For *in vivo* experiments, we used a dilution of 5%, and rabies virus (1 μL) was injected stereotaxically into the transplantation site (80 nL/min).

BrdU injections

BrdU (Sigma-Aldrich, KGA326) dissolved in 0.9% saline at a concentration of 10 mg/mL was injected intraperitoneally once daily at the dose of 50 μg/g body weight on days 28 to 32. For BrdU detection, see the supplemental information.

Immunofluorescence staining

At day 35 or 97, the rats were perfused transcardially and then fixed in 4% paraformaldehyde, followed by dehydration. The brains were cut into 30-μm-thick coronal slices using a microtome (Leica, CM3050S). For immunostaining, free-floating sections were permeabilized with 1% Triton X-100 in PBS for 1 h and incubated in blocking buffer (PBS containing 10% goat serum and 0.3% Triton X-100) for 1 h. The sections were then incubated with the primary antibodies on a shaking table overnight and followed by incubation with fluorescein-conjugated secondary antibodies and DAPI for 1 h at room temperature. Antibodies used in this study are provided in the supplemental information. Images were acquired by using a Carl Zeiss microscope and quantified by using NIH ImageJ software (Bethesda, MD, USA). For more information about quantification, see the supplemental information.

RNA-seq

The ipsilateral striatum was prepared with RNase-free instruments under stereoscopy and conserved in RNALater solution (Invitrogen, AM7020). The sequencing was performed by Annoroad Gene Technology. Briefly, the total RNA was extracted using the Arcturus PicoPure RNA Isolation Kit (Applied Biosystems, KIT0204) according to the manufacturer's instructions. Fragmentation was carried out using divalent cations under elevated temperature in the NEBNext First Strand Synthesis Reaction Buffer. cDNA was generated, amplified, and used for sequencing library preparation with the Illumina Nextera XT DNA Sample Preparation Kit (FC-131-1096). All libraries were sequenced by the sequencer Novaseq 6000. The fastq raw data files were generated using Illumina bcl2fastq software. Alignment and differential expression gene (DEG) analyses were performed by HISAT 2.0.5 and DEGseq 1.18.0. GO enrichment analysis was performed by using fisher.testp.adjust. Heatmaps were plotted using the gplots (heatmap.2) and ggplot2 packages.

Statistics

For all quantification procedures, observers were blinded to the experimental manipulation. Data are presented as boxplots or mean ± SEM over scatterplots. For comparison between two groups, significance was analyzed by the Student's t test. IBB scores were analyzed by nonparametric analysis (Kruskal-Wallis with Nemenyi *post hoc* test). For multiple comparisons, Bartlett's test was used to determine the variances and one-way analysis of variance (ANOVA) followed by Bonferroni's *post hoc* test using GraphPad Prism 8.0 software. A p value less than 0.05 was considered statistically significant. Pearson's correlation analysis was performed to see the correlation between the behavioral data and the histology.

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