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Intracerebroventricular Administration of Neural Stem Cells after Cardiac Arrest

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Abstract

Cardiac arrest (CA) is a serious disease with high rates of mortality and disability worldwide. Currently, neither pharmacological intervention nor therapeutic hypothermia can reverse the neural injury caused by CA. Neural stem cell therapy is a promising treatment for brain injury. We investigated the effects of the intracerebroventricular (ICV) administration of human neural stem cells (hNSCs) on global brain ischemia injury after CA. Twelve Long–Evans rats (4 Male and 8 female) subjected to 8-min asphyxia-CA were randomly assigned to hNSC treatment (n=7) or control group (n=5). The hNSCs were slowly infused into the left lateral ventricule 3 hours after resuscitation. An additional two rats subjected to 8-min asphyxia-CA were euthanized at 4 weeks after resuscitation to confirm the survival and function of transplanted PKH26 pre-labeled hNSCs using brain slides and whole cell patch clamp. Electrophysiological monitoring, quantitative EEG value (qEEG-IQ) and neurological deficit score (NDS) were used to evaluate the functional outcome. Immunofluorescence staining was used to investigate the survival of neurons and to track the migration of hNSCs. There was a significant improvement on the behavior tests evaluated as a subgroup of NDS ($p < 0.05$) in the NSCs group compared to the control group.

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Immunofluorescence co-staining of PKH26 and NeuN verified the neuronal differentiation from transplanted PKH26+ hNSCs in the hippocampus CA1 and cortex 4 weeks after CA. The whole-cell patch clamp technique confirmed the spontaneous firing activity that was recorded in cell-attached mode from the functionally mature neurons derived from transplanted cells. Transplanted hNSCs via ICV administration markedly improved neurologic outcomes after CA. Further studies are needed to elucidate the neuroprotective mechanism.

I. INTRODUCTION

Cardiac arrest (CA) is a serious disease with high rates of mortality and disability worldwide. About 356,500 out-of-hospital CAs occur every year in the U.S. [1]. Only 25% of patients have return of spontaneous circulation (ROSC), and fewer than 10% of patients survive to hospital discharge [2]. Numerous therapeutic interventions have been tried to reduce brain injury induced by ischemia and reperfusion. Currently, neither pharmacological intervention nor therapeutic hypothermia can reverse the neural injury caused by CA.

Neural stem cells (NSCs) are a group of self-renewing and multipotent cells. They have the potential to differentiate into three major cell types of brain and to project onto the host neurons in the brain after global ischemic injury [3]. Several studies have shown that NSCs transplantation reduces hypoxic-ischemic brain damage and brain loss in the chronic phase [4]. Further studies have indicated that in the treatment of brain hypoxic-ischemic injury, good sensorimotor function recovery can be achieved after NSCs treatment [5]. Even 2 months after brain injury, NSCs therapy can still improve the recovery of nerve function [4]. The possible mechanisms of NSCs treatment of hypoxic-ischemic brain injury include cell replacement, immunomodulation, the bystander effect, the accelerated endogenous recovery, and the enhancement of the neurogenic potential of endogenous NSCs [6]. To date, preclinical studies have provided solid evidence of the beneficial effect of NSCs in the treatment of stroke and improvement of cerebral function [7].

Intracerebroventricular (ICV) injection is a method of transplantation that directly delivers stem cells into the ventricular space. Currently, research focusing on hNSC therapy in brain injury after CA is inadequate. In this study, we investigated the neuroprotective effects of the hNSCs on a CA model after ICV administration, observed the survival of neurons and tracked the migration of hNSCs. The neurological outcomes and neurophysiological signals, including EEG, were evaluated.

II. MATERIAL AND METHODS

Twelve adult Long–Evans rats (4 Male and 8 female, average 376g), were randomly divided into the hNSC treatment group (N=7) and control group (N=5). All rats were fed with free access to water and food in a 12-hour light-dark cycle house. An additional two rats subjected to 8-min asphyxia-CA were euthanized at 4 weeks after ROSC to confirm the survival and function of transplanted PKH26 pre-labeled hNSCs using brain slides and whole cell patch clamp. The protocol was approved by the University of Maryland School of Medicine Animal Care and Use Committee.

A. Rodent Asphyxial CA model

The protocol of the CA model has been described in our previous research [8, 9]. In brief, rats were intubated following anaesthetization with 4–5% isoflurane. The rats were maintained with 1.5% isoflurane mixed in 50%N2/50%O2 via the ventilator. The femoral artery and vein were cannulated to obtain samples of analysis arterial blood gases (ABG) to monitor real-time arterial blood pressure and to inject the drug and fluid. A 5-min EEG baseline was recorded, followed by a 5-min washout period without isoflurane to exclude its effect on the EEG recording. Vecuronium (2 mg/kg) was administered to induce paralysis 2 min before the asphyxia process. Asphyxia was achieved by clamping the ventilator circuit and turning off ventilation for 8 min. CA was confirmed when pulse pressure was <10 mmHg. After 8 minutes of asphyxia, ventilation support was immediately started and then standard chest compression (200bpm) was performed. Intravenous injections of epinephrine (0.5ml/kg) and sodium bicarbonate (1ml/kg) were used to reverse CA and rectify metabolic acidosis until the return of spontaneous circulation (ROSC, pulse pressure>60 mmHg). No isoflurane was given after ROSC in order to avoid affecting qEEG.

B. Human Neural Stem Cells (hNSCs) Treatment

The hNSCs (ReNcell VM Human Neural Progenitor Cell Line, EMD Millipore) were cultured and harvested according to the protocol reported in previous studies [10]. The stem cells were labeled with PKH26 as a specific cell tracer. The rats were placed in the stereotaxic apparatus under isoflurane anesthesia as described above. An incision (1~1.5cm) was made in the skin to expose the skull. The skull was drilled by the high-speed drill bit (0.6mm in diameter), then a 24-gauge needle was inserted into the left lateral ventricle (P, 1.2mm, L 1.5mm, D 4mm) to administer 2.0×105 hNSCs in 5 ul saline solution over 1 min, 3h after ROSC. After injection, the needle stayed in place for at least 1 min to avoid efflux of the solution. The needle was slowly removed, and the skin closed with a 4–0 suture.

C. Neurological Recovery Evaluation

The recovery of the neurological function was evaluated using the neurological deficit score (NDS) [8, 9]. A part of NDS, including gait coordination, balance beam walking, righting reflex, negative geotaxis, visual placing and turning alley tests, serves as a subgroup of motor behavior and behavioral testing (ranges from 0 to 18), was assessed at 6, 24, 48 and 72hr after ROSC as we previously validated [9]. The overall NDS of 72hr was defined as the primary outcome.

D. EEG Recording and Analysis

5 min baseline EEG prior to CA followed by 180 min continuous EEG post-ROSC was recorded from two channels (Tucker-Davis Technologies, USA). All the EEG data were calculated using the core algorithm of MATLAB (MathWorks, Natick, MA). Based on modified entropy defined in our previous studies [8, 9, 11], quantitative analysis was exported in the form of qEEG Information Quantify (qEEG-IQ) value which was normalized to baseline.

E. Immunofluorescence Staining

Brains were removed and divided immediately after rats were humanely sacrificed. Samples were fixed with 4% paraformaldehyde for 24 hours. For the immunofluorescence (IF) staining, cryosections (20 μm) were incubated with Neuronal Nuclei, (NeuN); (Neuro-ChromTM Pan Neuronal Marker Antibody-Rabbit, Alexa 488 conjugate, Millipore Sigma) with the human-specific Ku86 antibody (Santa Cruz Biotechnology, Dallas, TX) to find out whether hNSCs migrate to the vulnerable region. Bright fields and fluorescence micrographs of the hippocampus were obtained with a Leica DMi8 microscope (Leica Microsystems).

F. Patch-Clamp

We performed the patch clamp on the whole cell to evaluate the function and activity of pre-labeled hNSCs. Rats were anesthetized with 5% isoflurane before decapitation. Then, samples were mounted onto the specimen disc of a VT1200S vibratome (Nussloch, Germany) after brains were rapidly dissected. Horizontal brains slices (350 μm) were cut in sucrose-based artificial CSF (aCSF). Incubation was conducted for 30min in aCSF (30°C). Slices were kept in aCSF at room temperature until electrophysiology began to record. Whole cell patch clamp signals were obtained from hippocampus neurons using a BX50WI (Olympus, Tokyo, Japan) epifluorescence microscope. Voltage and current signals were recorded with a MultiClamp 700B amplifier (Molecular Devices, Palo Alto, CA, USA) and sampled at 10 kHz with a DIGIDATA 1322A 16-bit analog-to-digital converter using Clampex, version 10.3 [12].

G. Statistical Methods

The parametric data (qEEG-IQ) were shown as mean ± S.E.M. and the non-parametric data (sub-NDS) were presented as median (25th-75th percentile). Parametric data were evaluated by univariate analysis. Nonparametric analysis of variance was used to test the NDS differences in rank order. The survival was analyzed by a Kaplan–Meier test. A level of $P < 0.05$ was considered significant. All statistics were performed using SPSS (IBM SPSS Statistics, version 22).

III. RESULTS

A. NDS assessment, survival rate, and functional recovery

The hNSCs treated animals had better recovery of neurological functional as shown by their sub-NDS scores at all time periods. The aggregate analysis showed statistically significant differences between the hNSCs treated group and control group ($p<0.05$, Figure. 1, Table 1).

Kaplan-Meier analysis revealed an improvement trend of survival in the treatment group. The 72-hour survival rate (5/7, 71.4%) of the hNSC treatment group was significantly higher than the control group (3/5, 60%), (Fig 2).

B. Quantitative EEG (qEEG) analysis

IQ rapidly decreased to 0 after CA from the baseline value of 1, and then gradually recovered over 3 hours before the ICV administration of hNSCs. The statistical results

indicated that there were no significant differences in the aggregate qEEG-IQ between the hNSCs group (0.56±0.045) and the control group (0.54± 0.054), (P=0.34).

C. Double Immunofluorescent staining

Compared to the control group, the hNSC group had higher NeuN positive cell numbers dominantly in the hippocampus, which were also Ku-86 positive 3 days after hNSCs administration (Fig.3). 28d after CA, pre-labeled hNSCs were found to be concentrated at hippocampal CA1 and cerebral cortex regions and developed into PK126+ neurons as shown by fluorescent staining with NeuN (Fig 4).

D. Patch-Clamp

The whole-cell patch clamp technique confirmed the spontaneous firing activity that was recorded from the functionally mature neurons derived from transplanted cells via ICV administration (Fig 5).

IV. DISCUSSION

CA-induced global cerebral ischemia and subsequent reperfusion injury are proposed as two primary factors of brain injury and neuron death [13]. Because of the relative lack of glucose and oxygen reserves in the brain, the physiological changes of the brain are swift. Once global cerebral ischemia and hypoxia occur, consciousness is lost within 10s after the onset of CA, and EEG activity becomes isoelectric within 20s [14]. The main pathophysiological processes include ion channel dysfunction, calcium overload, oxygen free radical damage and cell edema leading to cell death [15].

Delivery routes of cell therapy were considered as key factors affecting neurological recovery [16]. In animal models, hNSCs administration routes into the brain include parenchymal, ICV, intravascular, and intranasal routes [17–19]. Because the blood brain barrier blocks the entry of hNSCs, the ICV route may have several advantages. First, the hNSCs can be distributed to multiple sites of the brain depending on the fluidity of cerebrospinal fluid. Secondly, hNSCs potentially activate endogenous neural stem cells in the subventricular zone (SVZ), optimizing the repair effect [20]. The ICV route, which enables the administration of drugs into a lateral cerebral ventricle has been used worldwide for decades to treat pediatric and adult patients with a broad range of central nervous system (CNS) disorders [21]. While the invasive procedure has limitations, it was also proven that adequate training and strict aseptic procedures reduced the incidence of complications [22]. In this study, the effects of ICV injection with hNSCs 3h after ROSC in rats were investigated. The NSCs were found mostly in the most ischemia vulnerable areas including the hippocampus and cortex three days after transplantation. Therefore, the migration of the hNSCs in these areas is consistent with evidence of neural function recovery. The proliferation and migration of NSCs in treatment with experimental CA are very important to their therapeutic effect.

ICV administration delivered the stem cell into the left ventricular space where the distribution of stem cells circulated by the cerebrospinal fluid can achieve more viable areas of the brain compared to intracerebral administration. IF staining showed that pre-labeled

hNSCs migrated to hippocampal CA1 and cerebral cortex regions, and the whole cell patch clamp technique confirmed the existence of functionally mature neurons derived from transplanted cells at 4 weeks after ROSC. Although it is an invasive delivery, the ICV injection is a rapid and efficient route to deliver the stem cells or genes to the experimental animal, with good uptake in the contralateral hemisphere.

We have proven that early electrophysiologic markers (IQ) predict functional outcome after CA [8]. In this study, there was no significant difference in IQ within 3 hours after ROSC between the two groups, indicating that the neurological outcome predicted by electrophysiology was identical between the two groups before hNSC treatment. However, the sub-NDS of the hNSC treatment group was significantly higher than that in the control group, and the 72-hour survival rate (5/7, 71.4%) of the hNSC treatment group was higher than the control group (3/5, 60%). The results suggest that ICV transplantation of hNSCs improve functional recovery and extend survival.

There was no significant difference of overall NDS, which evaluates the level of arousal, brainstem function, motor function, sensory function, motor behavior, behavior, and seizures [11], whereas significant improvement existed with hNSC therapy in the sub-NDS assessments. The small animal number may have insufficient power to detect the difference in overall NDS. It may also be due to the higher tolerance of brainstem to hypoxic-ischemic attack, which led to no major difference of NDS scores based on impairment of arousal and brainstem function. In addition, hNSC therapy may be more effective for the recovery of advanced functional behaviors, similar to findings of hypothermia treatment [23], which will require future studies to verify. Xenotransplantation of human NSCs into animal models has already been tested in previous studies [24]. Further studies at molecular and cellular levels or other perspectives are needed to elucidate the neuroprotective mechanism and explore the pathways.

V. Conclusion

Transplanted hNSCs via ICV administration markedly improved neurologic outcomes after CA.

Acknowledgments

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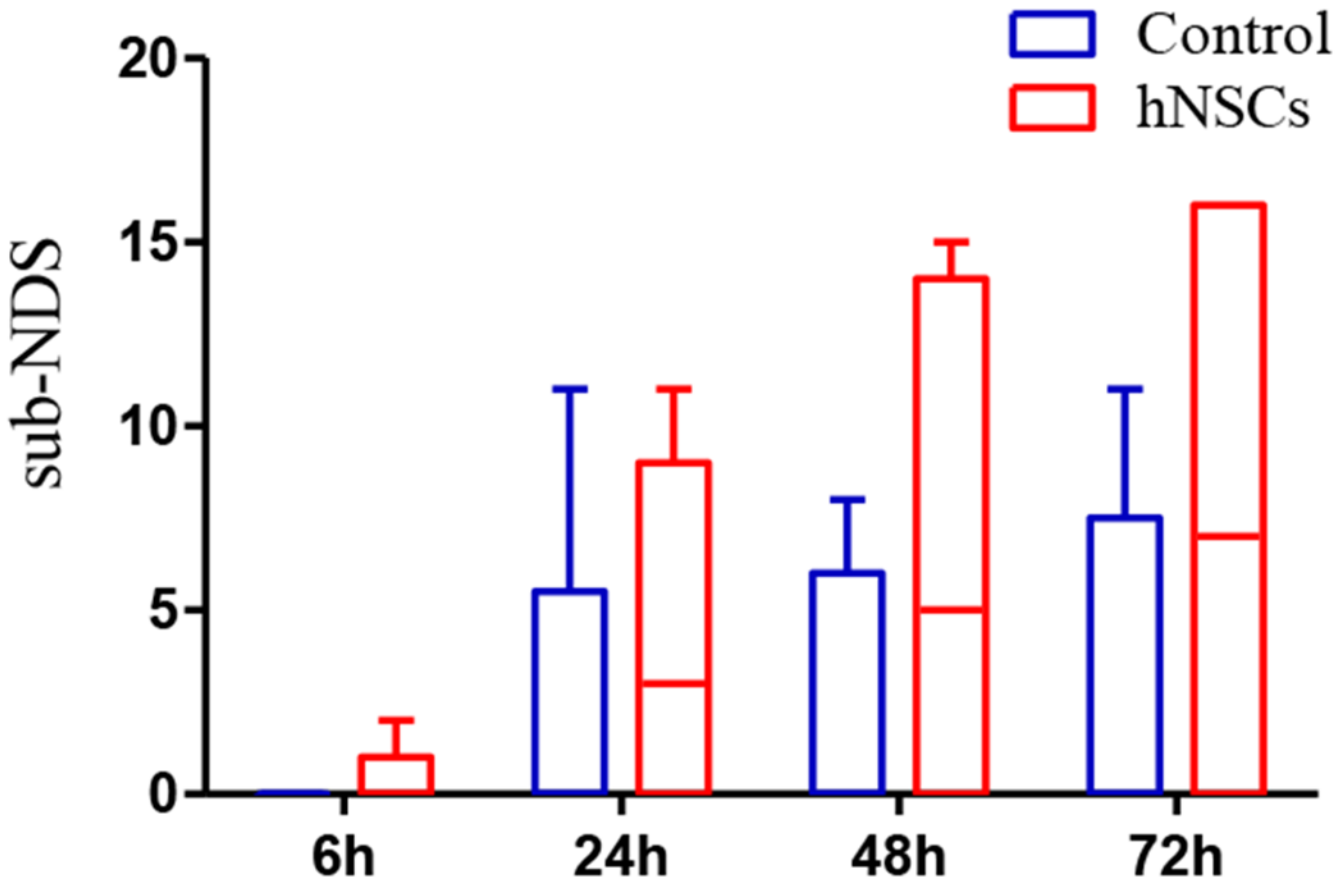

Fig 1.
The sub-NDS of hNSCs and control groups.

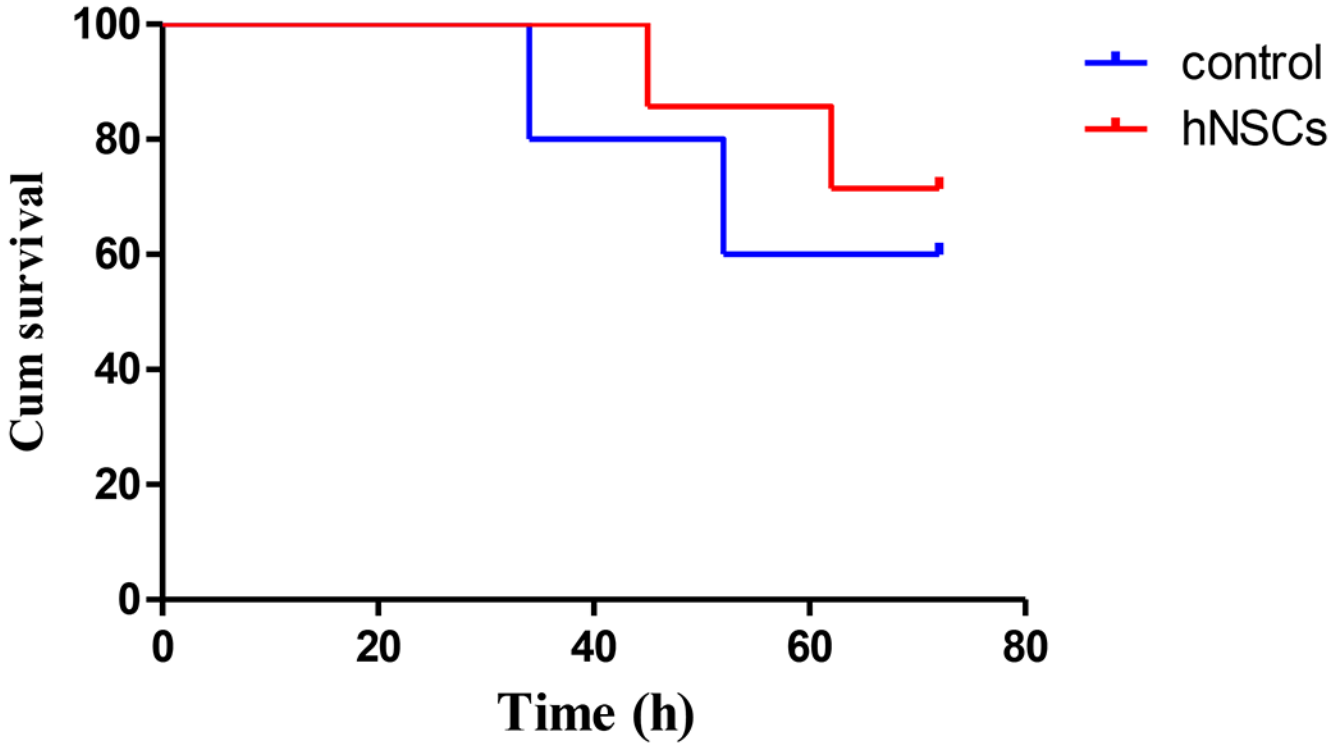

Fig 2.
Cum survival of control and hNSC treatment group

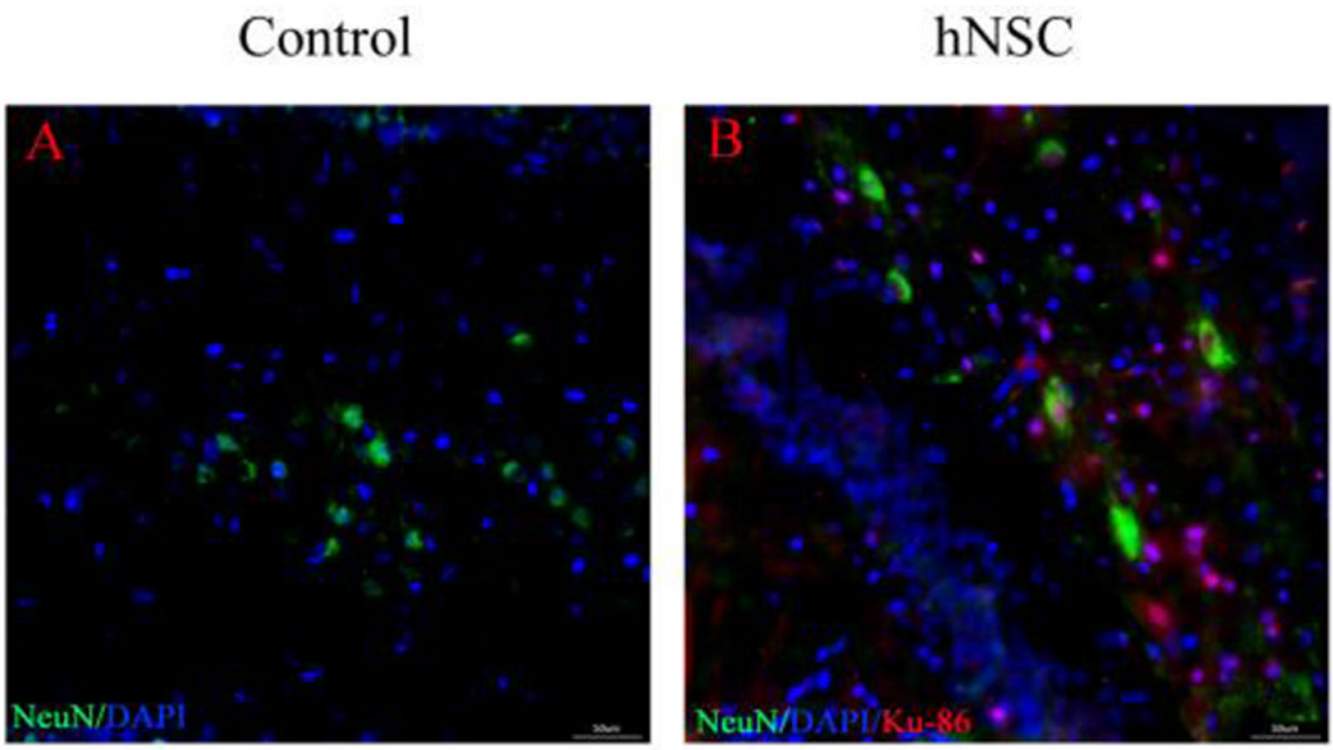

Fig 3.

Double immunofluorescent staining showed more Ku-86 (red) and NeuN (green) positive cells in the ipsilateral hippocampus 3 days after ROSC. Scale bar = 50μm.

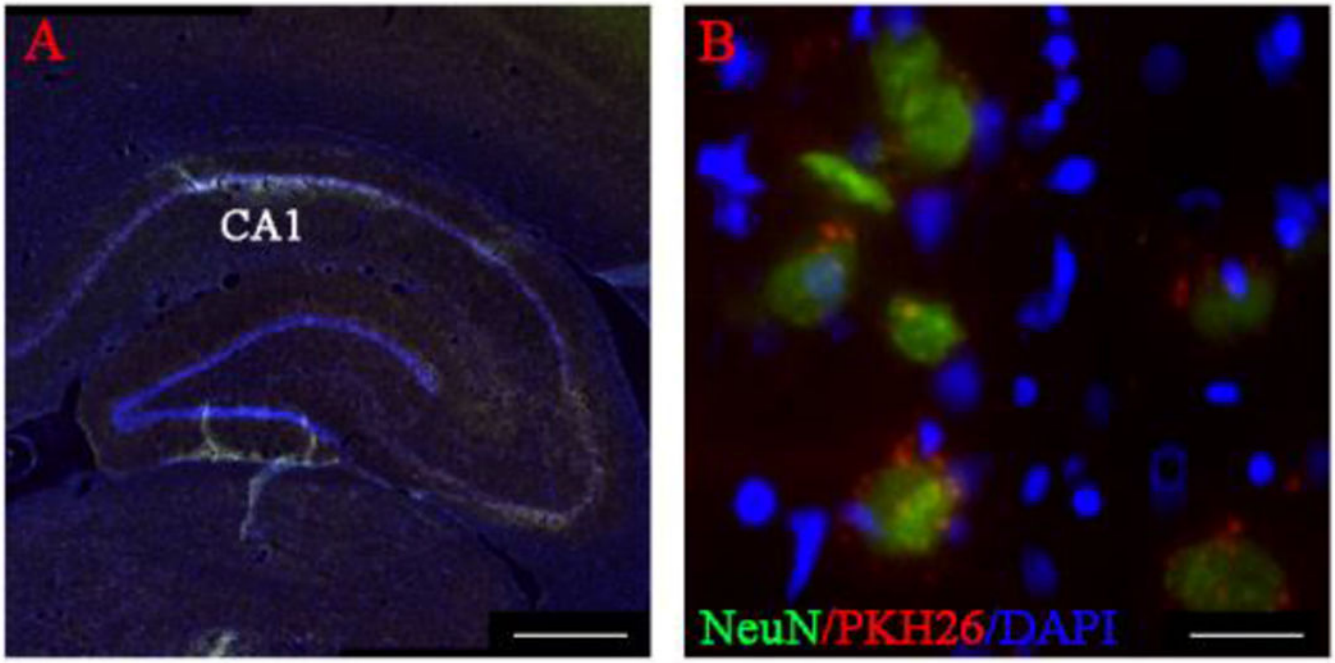

Fig 4.

The hNSCs labeled with PKH26 migrated to the CA1 of the hippocampus 4 weeks after ROSC (40X and 400X). A: Representative coronal hippocampal sections in low-magnification. B: Higher-magnification pictures shows that neuronal differentiation from PKH26–prelabeled hNSCs in CA1. Scale bar = 25μm.

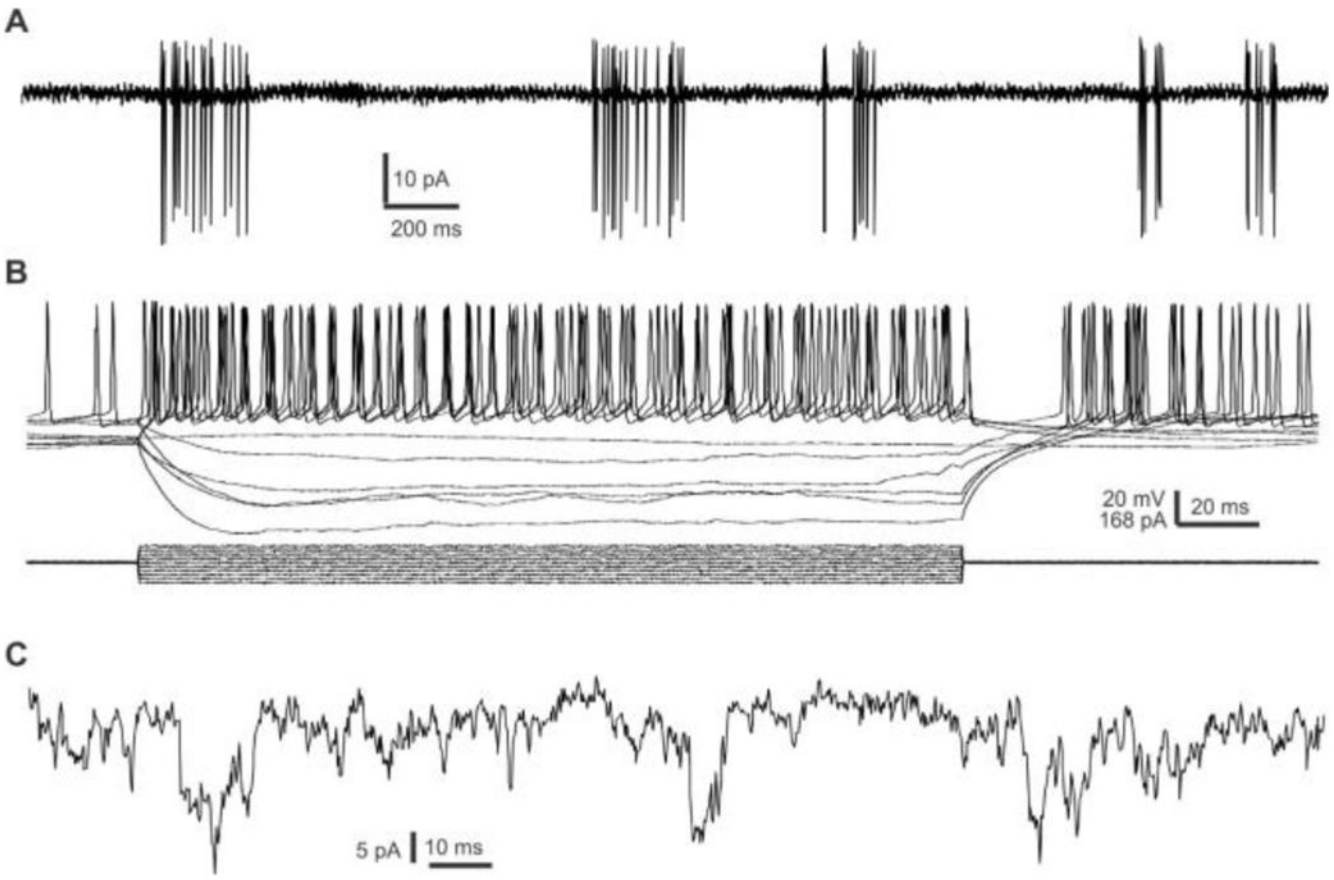

Fig 5.

Firing and synaptic characterization of hNSC-derived neurons in the motor cortical slice 4 weeks after CA. A: Spontaneous firing activity recorded in cell-attached mode. B: Input-output responses recorded in current clamp. C: Spontaneous excitatory synaptic current representing that the cell receives synaptic inputs from other neurons.

Table 1.

NDS by Control and hNSCs groups (median [25-75$_{th}$]).

GROUP	6h	24h	48h	72h
Control Sub	0 (0-0)	0(0-5.5)	0 (0-6)	0(0-7.5)
hNSCs Sub	0(0-1)	3 (0-9)	5(0-14)	7(0-16)
Control (Overall)	34.0 (14.0-44.0)	48.0 (47-57.5)	48.0 (15.0-59.5)	48 (0-66.0)
hNSCs (Overall)	42.0 (39.0-50.0)	53.0 (43.0-54.0)	56.0 (40.0-71.0)	63.0 (0-76.0)