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Transplanting GABAergic Neurons Differentiated from Neural Stem Cells into Hippocampus Inhibits Seizures and Epileptiform Discharges in Pilocarpine-Induced Temporal Lobe Epilepsy Model

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■ OBJECTIVE: **This study aimed to explore whether intrahippocampal transplantation of GABAergic neurons generated in vitro ameliorated seizures and epileptiform discharges via increasing γ-aminobutyric acid (GABA)-associated inhibition mediated by the addition of new GABAergic neurons.**

■ METHODS: **Neural stem cells (NSCs) isolated from newborn rats were induced and differentiated into GABAergic neurons. A total of 36 Pilocarpine-induced pharmacoresistant epileptic rats were divided into 3 groups: PBS (phosphate-buffered saline) group, NSCs group, and GABAergic neurons group (GABA group), with an additional 10 normal rats used (normal rat control group). The effects of grafting on spontaneous recurrent seizures (SRS) were examined and hippocampal GABA content was measured after grafting.**

■ RESULTS: **In the GABA group, the frequency of electroencephalography decreased significantly compared with the PBS group ($P < 0.001$), but there was no significant difference between the GABA group and NSCs group. Compared with the PBS group, the overall frequency and duration of SRS significantly decreased in the transplantation group, especially in the GABA group ($P < 0.01$). The number of GABAergic neurons was highest in the GABA group compared with the other groups ($P < 0.001$). Furthermore, hippocampal GABA concentrations significantly increased in the GABA group.**

■ CONCLUSIONS: **We show that GABAergic neurons generated in vitro from NSCs and grafted into the hippocampi of chronically epileptic rats can significantly reduce the frequency of electroencephalography and frequency and duration of SRS via increasing GABA-associated inhibition mediated by the addition of new GABAergic neurons.**

INTRODUCTION

Epilepsy is one of the most common and severe diseases of the central nervous system. Although most epileptic patients can be permanently relieved with regular anticonvulsant therapy, approximately 1/3 of epileptic patients fail to respond effectively to traditional antiepileptic medications. These patients are considered to be medically intractable or pharmacoresistant.[1,2] Temporal lobe epilepsy (TLE), one of the most common types of pharmacoresistant epilepsy, is characterized by multiple hippocampal abnormalities, including a substantial reduction in the number of different subclasses of inhibitory GABAergic interneurons, spontaneous recurrent seizures (SRS) originating mostly from the hippocampus, and aberrant synaptic reorganization. Hippocampal sclerosis and GABAergic interneuron loss have been observed in the hippocampus of patients TLE and animal models,[3-5] resulting in an increase in the overall

Key words
- GABAergic neurons
- Hippocampal GABA concentration
- Intrahippocampal transplantation
- Neural stem cells
- Pharmacoresistant epilepsy

Abbreviations and Acronyms
AP: Anteroposterior
BrdU: 5-Bromodeoxyuridine
CBZ: Carbamazepine
EEG: Electroencephalography
GABA: γ-Aminobutyric acid
GPCs: γ-Aminobutyric acid progenitor cells
HPLC: High-performance liquid chromatography
i.p.: Intraperitoneally
L: Lateral
NRC: Normal rat control
NSC: Neural stem cell
PB: Phenobarbital
PBS: Phosphate-buffered saline
PBST: Phosphate-buffered saline containing 0.1% Tween-100
SD: Sprague-Dawley
SE: Status epilepticus
SRS: Spontaneous recurrent seizures
TLE: Temporal lobe epilepsy
V: Ventral

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neuronal excitatory tone compared with inhibitory function.[6,7] Although hippocampus resection or removal of epileptic foci gives better seizure control, this option is associated with cognitive impairments,[8,9] loss of viable tissue during resection, and the possibility of continuing dependence on anticonvulsant drugs after resection.[10] Therefore, novel therapies that have the potential for both preventing seizures and increasing the number of GABAergic neurons in the hippocampus of TLE are needed. Cell-based therapies have emerged as a promising modality for pharmacoresistant epilepsy treatment. Intracerebral transplantation of neural stem cells (NSCs) or γ-aminobutyric acid (GABA) progenitor cells (GPCs) is evolving as an attractive therapy for promoting regeneration and repair in various brain disorders including TLE.[11]

NSCs from the fetal brain are a potential cell source for replacing interneurons in patients with severe pharmacoresistant TLE.[12] The therapeutic potential of NSCs is linked to their properties of multipotency, self-renewal, and the ease by which they can be obtained from multiple regions of the developing brain, adult brain, and human embryonic stem cells.[13,14] Studies in neurologic disease models have shown that NSCs can survive intracerebral transplantation, engraft into the injured brain areas, release a multitude of neurotrophic factors, positively influence the survival of host cells and tissues, and promote functional recovery.[11] Transplantation of NSCs in TLE may effectively restrain SRS because of their ability to give rise to many neurons synthesizing the inhibitory neurotransmitter GABA and/or astrocytes synthesizing the anticonvulsant protein glial cell line–derived neurotrophic factor.[14,15] Considering this situation, NSCs that show a propensity for differentiating into both GABAergic neurons and astrocytes may be the most ideal types for seizure suppression. A key pathologic feature of human TLE is synaptic reorganization, including neuronal loss and gliosis in CA1 and hilus, granule cell dispersion, and mossy fiber sprouting in the dentate gyrus.[16] Some studies have shown a loss of interneurons releasing inhibitory neurotransmitter GABA.[17-19] It is believed that a decrease in GABA-mediated inhibition is a critical contributing factor in epilepsy. Therefore, 1 possible therapeutic approach is to increase GABA-mediated inhibition to suppress hyperexcitable neurons during seizure initiation. Early work exploring the potential for inhibitory neural grafts in controlling epileptic activity has shown promise and has inspired further studies.[20-22] More recent experiments have shown that mouse GABAergic interneuron precursors engrafted into the TLE mouse brain reduced seizure activity.[23-27] Previous study showed that only 24% of graft-derived cells differentiated into GABAergic interneurons, and thus, NSCs from all sources do not seem to generate many GABAergic interneurons when grafted into the epileptic hippocampus.[28] However, it remains to be investigated whether grafting of GABAergic neurons differentiated from NSCs in vitro alone into the hippocampus is sufficient to increase the number of GABAergic neurons and promote suppression of SRS in pharmacoresistant TLE.

Given this background, we investigate whether using highly efficient methods for generating GABAergic neurons in vitro from NSCs and subsequent intrahippocampal transplantation into the epileptic brain could effectively suppress SRS in the pilocarpine-induced TLE in mice, a well-characterized model of human TLE.[29] We report that grafted GABAergic neurons show long-term survival and provide many GABAergic neurons around the implant sites. Moreover, grafting of GABAergic neurons differentiated from NSCs in vitro has therapeutic potential for treating TLE, particularly regarding seizure suppression. These results advance our search for an apt cell transplantation therapy for restraining chronic epilepsy.

METHODS

Experimental Materials

Dental cement was prepared by mixing superglue (Zhejiang Golden Roc Chemical Co. Ltd., Hangzhou, China) with liquid denture acrylic.

Stimulating electrodes (nichrome enameled wire with a diameter of 0.1 mm and Teflon-coated except for 0.03 mm at the tip, fabricated by the authors).

Animals and Housing

A total of 100 healthy adult male Sprague-Dawley rats ranging from 50 to 60 days old were obtained from Guizhou Laboratory Animal Engineering Technology Center. This study was approved by the institutional animal care and use committee of the Guizhou Medical University. All experiments were conducted in accordance with the protocol approved by the committee on the ethics of animal experiments of Guizhou Medical University. Animals were housed 2 per cage in ventilated cages and permitted ad libitum food and water. Lights in the vivarium were maintained on 12/12-hour light/dark cycle at room temperature (23°C–26°C). Studies took place during the light-on phase. To minimize animal pain and suffering throughout the study, all necessary precautions were performed.

Spontaneous Chronic Epileptic Model Induction

Induction of Status Epilepticus. Pilocarpine-induced TLE model was used as previously described (29 animals were infused with lithium chloride [127 mg/kg, Sigma-Aldrich, St. Louis, Missouri, USA] intraperitoneally [i.p.] 24 hours before the administration of pilocarpine). On the next day, the rats were injected with atropine methyl bromide (5 mg/kg, i.p. [Shanghai New Asiatic Pharmaceuticals Co. Ltd., Shanghai, China]) to limit the peripheral effects of pilocarpine. After 30 minutes, rats were injected with pilocarpine (30 mg/kg, i.p. [Sigma-Aldrich]) to induce status epilepticus (SE). Within 30 minutes after the pilocarpine injection, most animals developed SE, which was featured by continuous tonic-clonic seizures lasting for 2–3 hours. Diazepam (20 mg/kg, i.p. [Harbin Pharmaceutical Factory, Harbin, Dongbei, China]) was administered 90 minutes after SE to cease seizure activity and repeated when it was needed. The rats that went into SE were injected with 2.5 mL of 5% dextrose (Guizhou Pharmaceutical Factory, Guiyang, Guizhou, China) i.p. twice a day and were given a moistened rat diet during the following 2–3 days. Rats that did not develop stage 5 were excluded from this study.

For 2 weeks after SE, rats were monitored 12 hours/day, 5 days/week, for seizure severity and occurrence of spontaneous seizures using video. Rats showing SRSs were used as chronic epileptic animals. Seizure severity was assessed using the Racine scale[30]: 0, no response; stage 1, grooming, hyperactivity; stage 2, head nodding, tremor; stage 3, unilateral forelimb clonus; stage 4,

clonus with rearing; and stage 5, generalized tonic-clonic seizure with loss of righting reflex. After chronic epileptic model induction, 57 rats were chronic epileptic rats, 17 rats died of SE, 15 rats showed no epileptic seizures, and 11 rats did not develop stage 5.

Pharmacoresistance Selection. For selection of pharmacoresistance, rats were subjected to prolonged treatment with phenobarbital (PB) and carbamazepine (CBZ) (Shanghai New Asiatic Pharmaceuticals Co. Ltd., Shanghai, China).[31]

Selection of PB, CBZ Responders, and Nonresponders. Based on these preliminary experiments and a previous PB selection trial,[32,33] the following PB dosing protocol was used in the 57 rats with SRS: a bolus dose of 25 mg/kg (i.p.) in the morning of the first treatment day, followed 10 hours later by 15 mg/kg (i.p.), and then 15 mg/kg (i.p.) twice daily for 13 consecutive days. Responders were defined by complete seizure suppression during treatment or seizure suppression of $\geq$50% compared with seizure frequency in the predrug and/or postdrug control periods.[33] Eleven responders were considered pharmacosensitive, and 46 nonresponders were considered pharmacoresistant. When the epileptic rats were found pharmacoresistant to PB, the process of selection was continued using CBZ. After a 2-week postdrug period after PB, a second drug trial was performed with daily administration of CBZ. CBZ was dissolved in Tween-80 saline at a dose of 40 mg/kg and injected i.p. 3 times per day for 2 weeks. Dose and posology of CBZ were the same as previously described.[34-36] Seizures were continuously monitored (12 hours/day, 5 days/week) by video recording over 2 weeks of the experiment as described earlier. Eight responders served as pharmacosensitive epileptic rats, and 38 nonresponders served as pharmacoresistant epileptic rats.

Experiment Grouping. Six weeks after i.p. injection of pilocarpine, of the 57 induced epileptic rats, 11 were pharmacosensitive to PB, and 46 were pharmacoresistant to PB, but 8 of the 46 rats were pharmacosensitive to CBZ. Thirty-eight rats were pharmacoresistant to both PB and CBZ. A total of 36 pilocarpine-induced pharmacoresistant epileptic rats were randomly divided into 3 groups: phosphate-buffered saline (PBS) injection group (PBS group, $n = 12$), NSCs transplantation group (NSCs group, $n = 12$) and GABAergic neurons transplantation group (GABA group, $n = 12$); another 10 normal rats were used as sham (normal rat control [NRC] group, $n = 10$). Rats from the PBS group received PBS injection (10 μL PBS); NSCs and GABAergic neurons labeled with 5-bromodeoxyuridine (BrdU) were transplanted into the bilateral hippocampus of NSCs and GABA group, respectively.

Electrode Implantation. Electrodes were implanted into the right amygdala, and electroencephalography was performed to acquire seizure frequency. The methods used were the same as our previously reported studies.[37] Chronic epileptic rats were anesthetized and placed in a stereotaxic frame (Shanghai Chuansha Huamu Agricultural Machinery Factory, Shanghai, China). Then, electrodes were implanted into the right dorsal hippocampus (stereotaxic coordinates were obtained from the rat brain atlas of Paxinos and Watson[38]) as follows: −3.8 mm posterior; 2.0 mm lateral (L); −2.6 mm depth). A screw was fixed in the skull over the left frontal cortex as a reference electrode. The electrodes were connected to 3-hole wire connectors and fixed to the surface of the skull using 502 Superglue blended with denture acrylic. Penicillin (40,000 U/kg, i.p.) was injected for 3 consecutive days to prevent infection, and the rats were allowed to recover from surgery for 7 days before being subjected to the experiment.

Cell Preparation

Isolation and Culture of Neonatal Rat NSCs. Isolated and cultured neonatal rat NSCs were as described earlier.[39] Neonatal SD rats on postnatal day 0 (P0) were obtained from Guizhou Laboratory Animal Engineering Technology Center (Guiyang, China). Hippocampus from newborn SD rats was washed several times with PBS (Beijing Dingguo Changsheng Biotechnology, Beijing, China). The tissue pieces were gently titrated using a fire-polished Pasteur pipette and a homogenous suspension of individual cells with good viability was obtained (as determined by trypan blue exclusion). Cells were resuspended with Dulbecco's modified Eagle's medium/F12 (Beijing Mofei Company, Beijing, China) mixed with 20 ng/mL epidermal growth factor (Gibco, Grand Island, New York, USA), 10 ng/mL basic fibroblast growth factor (Sigma-Aldrich), B27 (2% Gibco). They were then seeded in 25-cm^2 vented culture flasks (1×10^6/L per flask) placed at 37°C in a 5% CO_2 incubator. Half of the solution was changed every 3–4 days. Subculture started on day 7 and was repeated once every 6–7 days. For identification of NSCs, immunocytochemistry was performed using antibodies specific for nestin, a marker for NSCs (Abcam, Shanghai, China).

Differentiation of NSCs. The methods were the same as previous studies.[40] Passage3 NSCs were mechanically dissociated and plated on laminin-coated (30 μL/mL of PBS [Roche Molecular Biochemicals, Shanghai, China]) 24-well plates in differentiating medium (Dulbecco's modified Eagle's medium/F-12, B_{27} supplement, all-*trans*-retinoic acid, 1 μM [Sigma-Aldrich]) and dibutyryl cyclic adenosine monophosphate (1 mM [Sigma-Aldrich]). S-Oligonucleotides (Sigma-Aldrich) with the following sequences were used: anti-hairy and enhancer of split 1 (anti-Hes1 [Takara, Beijing, China])-1, 5'-ACC GGG GAC GAG GAA TTT TTC-3'; anti-Hes1-2, 5'-CAC GGA GGT GCC GCT GTT GCT GGT GTA GAC GGG GAT GAC-3'; control S-oligonucleotide contained a scrambled sequence 5'-TCG GAG ACT TTC TGT CGG GCT GAT CGG TCG GGC TGG GGA G-3'. Oligonucleotides were added to the growth or differentiation culture media at a final concentration of 5 μM. The medium with the oligonucleotides was replaced every day during the experiment. For antisense oligonucleotide treatment of neurospheres were transferred into 24-well plates in growth media containing antisense or scrambled oligonucleotides for 4 days. For further analyses, the cells were either plated onto poly-D-lysine coated 24-well plates or centrifuged onto glass slides and fixed in cold acetone/methanol for 2 minutes.

Immunocytochemistry for Cells Markers. Cells were washed with PBS and fixed for 2 minutes in cold acetone/methanol. After washing in PBS, they were blocked for 30 minutes in 4% donkey serum (Abcam). Primary antibodies against nestin (1:100, (Abcam)); rabbit anti-GABA (1:5000; Sigma-Aldrich) were applied for 90 minutes. Then, the cells were washed in PBS and incubated in Cy3-conjugated secondary antibodies for 30 minutes, washed in PBS, and examined under a fluorescent microscope. The cells were counterstained with

4, 6-diamino-2 phenylindole dihydrochloride (Boster Biological Technology Co. Ltd.,Wuhan, Hubei, China) to identify nuclei.[40]

Transplantation

At 2 weeks after pharmacoresistant selection, NSCs and GABAergic neurons taken at between passage3 (P3) and P5 were selected and prepared. Cells were labeled with bromodeoxyuridine (BrdU; 3 μM (Abcam)) for 5 days before grafting. For BrdU labeling, preparing 1-mM stock solution by dissolving 3 mg of BrdU in 10 mL proliferation medium. 2.5 μL of this stock solution was added to each culture flask containing 10 mL of the proliferation medium to obtain a final concentration of 2.5 μM. The neurospheres were allowed to grow for 5–6 days. On day 6 or 7, the medium containing the NSC-derived neurospheres was transferred from the flasks into 15-mL centrifuge tubes and the neurospheres were left to settle. The supernatant medium in each tube was removed, and neurospheres from all tubes were pooled in a single 15-mL centrifuge tube. A Pasteur pipette was used to obtain a clear cell suspension. Titrated cells were washed 3 times to get rid of most of the dead cells in the suspension, each time by centrifuging for 5 minutes at 1500 r/minute, at room temperature, removing the supernatant, resuspending the cells in 3 mL fresh proliferation medium, and then, centrifuging again as before and removing the supernatant. The final pellet was reconstituted in 10-μL PBS and the viable cells were counted using a trypan blue exclusion test. The density of viable cells was adjusted to 1×10^6 cells/10 μL using PBS. For GABAergic neurons, cells were harvested by trypsinization (Boster Biological Technology Co. Ltd.). The cells were centrifuged (1500 r/minute, 3 minutes), the pellet was washed 3 times with PBS, and the entire cell pellet was then resuspended in PBS at a density of 1.0×10^6 cells/10 μL PBS. The concentrated NSCs and GABAergic neurons in a sterile freezing tube (Nunc [Boster Biological Technology Co. Ltd.]) were then delivered to the animal operation room. Pharmacoresistant epileptic rats were anesthetized, 10 μL vehicle (PBS), 1.0×10^6 cells/10 μL NSCs or GABAergic neurons suspension was injected into each of the following 3 locations in the bilateral hippocampus using the following stereotaxic coordinates: 1) anteroposterior (AP) = 3.0 mm posterior to bregma, (L) = 2.2 mm right L to midline, and ventral (V) = 3.5 mm from the surface of brain; (2) AP = 3.8 mm, L = 3.0 mm right L, and V = 3.5 mm; and (3) AP = 4.6 mm, L = 4.0 mm right L, and V = 3.5 mm.[41] Vehicle or NSCs suspension was infused at a flow rate of 1 μL/minute using a 10-μL Hamilton syringe placed on an infusion pump (KD Scientific, Holliston, Massachusetts, USA) controlled by a microprocessor. After cell injection to the site, the top of the burr holes was closed with a small amount of bone wax, the skin flaps were stapled using an autoclipping device loaded with 9-mm wound clips, and betadine was applied to the stapled skin.

Measurement of SRS

Alterations in the extent of SRS were measured rigorously after the transplantation surgery continuing for ≥3 months. The behavior of epileptic rats was observed at 1–3 months after transplantation to analyze the frequency, severity, and duration of SRS among the vehicle-injected, NSCs, and GABAergic neurons transplanted groups. Starting after transplantation, rats were video monitored for 60 hours per week (12 hours/day, 5 days/week, 2 weeks/month, and 360 hours in total). Rats were given free access to water and food in individual cages and video monitored during the period. The video recordings were analyzed by observers blinded to group allocation. Rats were scored according to the same additional Racine scale used in the establishment of the kindled model.[42] The frequency and severity of seizure and the total time spent in seizure were assessed in each rat. From the SRS metrics, the average frequencies of all SRS and stage V seizures, the average duration of individual SRS, and the total time spent in seizures each month were calculated. Postgrafting seizure scores (for different months after grafting) were compared with the pregrafting seizure scores using repeated-measures analysis of variance to ascertain the extent of seizure suppression mediated by NSCs or GABAergic neuron grafting.

Identifying Therapeutic Efficacy

Epileptic behavior was observed using the strength (μA) required to initiate epileptic sign (electronic discharge for >3 seconds). To determine the effect of GABAergic neuron grafting on seizure activity, the mean frequency of electroencephalography (EEG) across animals was calculated. The mean frequency of EEG across animals was defined as the activity appears on the screen of the EEG machine (BL-420 Biological Functional) as a waveform of varying frequency and amplitude measured in voltage. The varying frequency of each animal was calculated using the BL-420 Biological Functional software system. We obtain a total number of detected frequencies, divided by the number of animals.

Brain Harvesting and Fixation

The methods used were the same as in our previously published studies.[43] All rats were killed under deep anesthesia 3 months after cell transplantation and perfused through the ascending aorta with ice-cold normal saline. After the perfusion, the hippocampus were rapidly removed from the brain and cut into 2 parts. One was immediately fixed in 4% paraformaldehyde for 24 hours, followed by 30% sucrose in PBS until completely sunk in preparation for immunofluorescence staining. The other was dissected on an ice-cold plate as previously described for measuring GABA content.[44]

Immunofluorescence

The differentiation of graft-derived cells was typically assessed through a standard dual immunofluorescence method, by identifying both the graft cell marker (BrdU) and the GABA cell marker (GABA). Every sixth 40-μm-thick section through the entire hippocampus was chosen for immunofluorescence. Free floating sections of brain were washed 3 times for 10 minutes in PBS (Boster Biological Technology Co. Ltd.). Samples were blocked for 60 minutes at room temperature with 5% normal goat serum (Boster Biological Technology Co. Ltd.) in PBS containing 0.1% Tween-100 (PBST). Sections were then incubated overnight at 4°C with rat anti-BrdU (1:200) and rabbit anti-GABA (1:5000 [Sigma-Aldrich]) with 5% normal goat serum. The sections were washed 3 times for 10 minutes in PBST and then soaked in 5% normal goat serum in PBST containing corresponding secondary antibodies (goat anti-rat IgG with Alexa Fluor 594 [red; 1:500] [ThermoFisher, Waltham, Massachusetts, USA]) and goat anti-rabbit IgG with Alexa Fluor 488 [green; 1:500] [ThermoFisher] for 4 hours at 4°C. Sections were washed 5 times for 10 minutes in PBST and 3

times for 10 minutes in PBS, and the nuclei were labeled with 4,6-diamino-2 phenylindole dihydrochloride (DAPI). Sections were examined using a Zeiss microscope. Twelve pictures were taken for each condition and the number of target cells were counted manually using ImageJ. Pictures were taken as 2×10^5 μm^2 squares at the transplantation site in CA3 regions.[45]

Measurement of the GABA Content of Hippocampus. As previously described, hippocampi were removed, weighed, and placed in 1.5-mL microcentrifuge tubes (Beijing Mofei Company, Beijing, China) that contained 1 mL of chilled homogenization buffer (0.1 M citric acid, 0.1 M sodium dihydrogen phosphate monohydrate, 5.6 mM octane sulfonic acid,10 um EDTA in 10% [v/v] methanol solution, pH 2.8 with 4 M NaOH). Each sample was sonicated for 4 seconds, centrifuged at 14,000 rpm for 15 minutes at 4°C and the supernatant stored at −80°C until derivatization for GABA analysis.[46] The derivatization protocol was based on the method described by Nussbaum et al.[47] 100 µL of either standard mix or sample supernatant, 900 µL of borate buffer (0.1 M, pH 9.5), 200 µL of potassium cyanide (10 mM) and 200 µL of naphthalene-2,3-dicarboxaldehyde (6 mM) were added to a single reaction tube, vortex mixed, and the reaction was allowed to proceed at ambient temperatures in the absence of light. A 20-µL of the derivative was injected onto the appropriate high-performance liquid chromatography (HPLC) system. All samples were injected onto a reversed-phase Luna 5 µm C18(2) 250 mm × 4.6 mm column (Phenomenex, Tianjing, China) and a reversed-phase Luna 3 µm C18(2) 150 mm × 2 mm column (Phenomenex, Tianjing, China). The mobile phase was used on the electrochemical detection system and HPLC grade methanol (35:65). The mobile phase was filtered through Millipore 0.45 µm HV Durapore membrane filters (Abcam) and vacuum degassed before use. Compounds were eluted isocratically over a 20-minute run time at a flow rate of 0.65 mL/minute after a 20-µL injection. The column was maintained at 30°C and samples/standards were kept at 4°C in the cooled autoinjector before analysis unless otherwise stated. The fluorescent detector was set at an excitation wavelength of 420 nm, an emission wavelength of 480 nm and a photomultiplier tube gain of 5. GABA was identified by its characteristic retention times as determined by standard injections that were run at regular intervals during sample analysis. Sample peak heights were measured and compared with standard injections to quantify the amino acids.

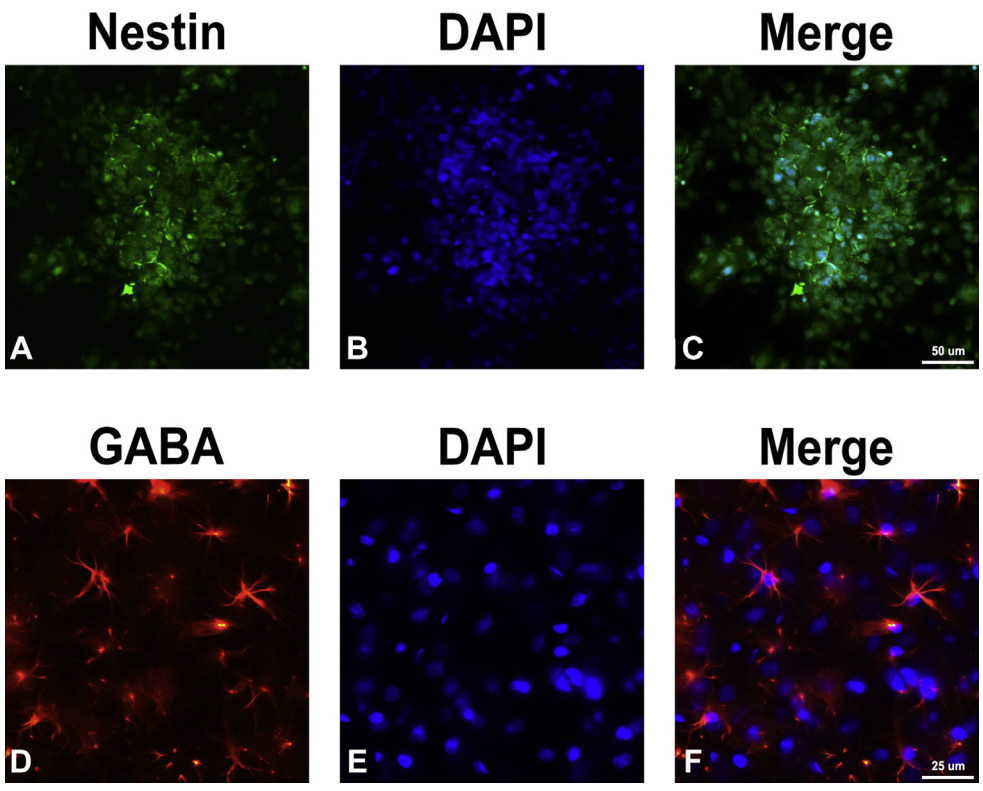

Figure 1. Neural stem cells and differentiate into GABAergic neurons in culture. (**A**–**C**) Shown under proliferation conditions, the cell derived from the hippocampus expressed nestin (*green*), a marker for neural stem cells (**A**). Nuclei were counterstained with 4, 6-diamino-2 phenylindole dihydrochloride (DAPI) (*blue*) (**B**). (**C**) is merged. Induced via the differentiation medium, neural stem cells were able to differentiate into GABAergic neurons expressing γ-aminobutyric acid (*red*) (**D**). Nuclei were counterstained with 4, 6-diamino-2 phenylindole dihydrochloride (*blue*) (**E**), (**F**) is merged. Scale bar, **A**–**C,** 50 µm; **D**–**F,** 25 µm.

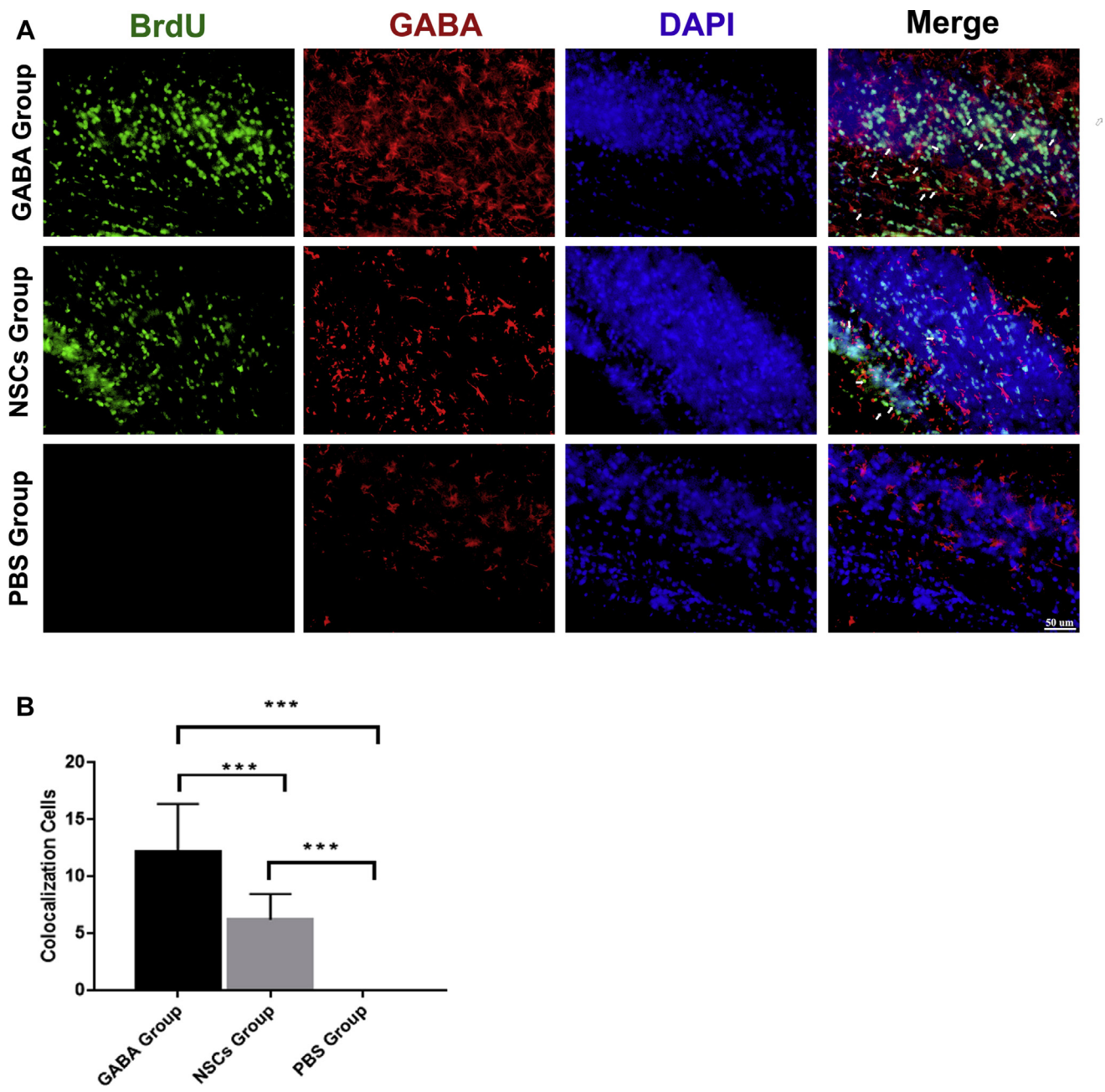

Figure 2. Differentiation of neural stem cells (NSCs) and survival of GABAergic neurons generated in culture after transplanting into the hippocampus of epileptic rats. Dual labeling analyses with 5-bromodeoxyuridine (BrdU) and γ-aminobutyric acid (GABA) antibodies showed that many BrdU-positive cells were colabeled with the GABAergic neurons marker (GABA) in the hippocampus in the GABA group. Fewer colocalization cells were detected in the NSCs group compared with in the GABA group. In the PBS (phosphate-buffered saline) group, there were many GABA-positive cells in the hippocampus, but no BrdU-positive cell (**A**). Comparing the NSCs group with the GABA group, the number of colocalization cells in the hippocampus of the GABA group was significantly higher than that of the NSCs group ($P < 0.0001$). DAPI, 4, 6-diamino-2 phenylindole dihydrochloride. (**B**) Compared with the PBS group, the number of colocalization cells in the hippocampus of the NSCs group and GABA group was significantly increased ($P < 0.0001$). Values are represented as mean ± standard error of the mean. (***$P < 0.001$), Scale bar = 50 μm.

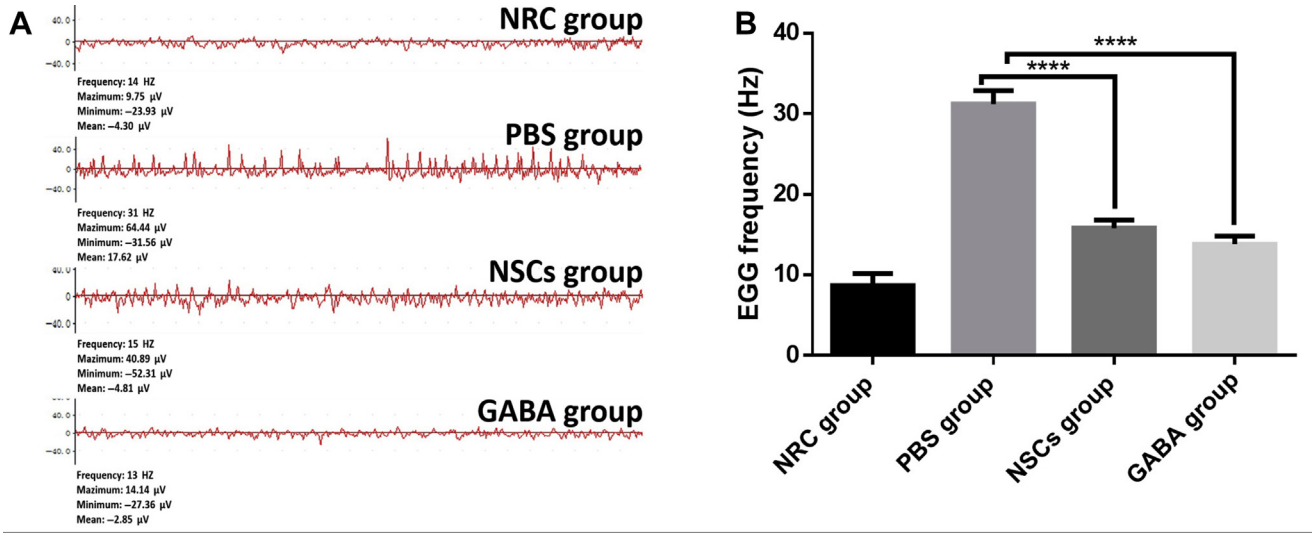

Figure 3. Changes of electroencephalograms (EEGs) in different groups. (**A**) Amygdala electroencephalogram in the NRC group showing waves with low frequency and amplitude epileptiform discharges such as interictal spikes and single peak with high frequency and high amplitude were observed in the PBS (phosphate-buffered saline) group. All animals showed a decrease in mean spike frequency after transplantation. (**B**) Bar chart showing the seizure frequency of the PBS group was the highest, compared with the other 3 groups (**B**) ($P < 0.001$). The seizure frequency of the transplantation group decreased remarkably after transplantation, but there was no significant difference between the GABA (γ-aminobutyric acid) group and NSCs (neural stem cells) group.

Statistical Analysis

For data analyses, SPSS software version 13.0 (SPSS Inc., Chicago, Illinois, USA) was used to test for data normality and homogeneity of variance. All data are presented as mean values ± standard. Comparisons between 2 sample means were made using a t test. One-way analysis of variance was carried out for detecting differences among the groups. $P < 0.05$ was considered statistically significant. Statistical analysis was performed in consultation with the Department of Biostatistics of Guizhou Medical University.

RESULTS

NSCs and GABAergic Neurons Generated in Culture

To confirm the identity of cells cultured in vitro as NSCs, antibodies against nestin were used. Expression of nestin was detected in NSCs derived from hippocampus (Figure 1A–C). Immunohistochemical analyses using antibodies against GABA (marker for GABAergic neurons) showed that most neural cells were GABA-positive cells after induction, suggesting that NSCs were induced to a GABAergic phenotype via inducing medium (Figure 1D–F).

Differentiation of NSCs and Survival of GABAergic Neurons Generated in Culture After Transplantation (GABA+Brdu-Positive Cells)

Dual labeling analyses with BrdU and GABA antibodies showed that many BrdU-positive cells were colabeled with the GABAergic neurons marker (GABA) in the hippocampus in the GABA group. Fewer colocalization cells were detected in the NSCs group compared with in the GABA group. In the PBS group, there were many GABA-positive cells in the hippocampus, but no BrdU-positive cell (Figure 2A). Comparing the NSCs group with the GABA group, the number of the colocalization cells in the hippocampus of the GABA group was significantly higher than that in the NSCs group ($P < 0.0001$) (Figure 2B). Compared with the PBS group, the number of colocalization cells in the hippocampus of the NSCs group and GABA group was significantly increased ($P < 0.0001$). These data show that GABAergic neurons differentiated in vitro from NSCs can survive in the hippocampus of epileptic rats for an extended period and NSCs are capable of differentiating into a few GABAergic neurons after grafting into the hippocampus.

Changes of EEGs in Different Groups

To determine the effect of cell transplantation on seizure activity, the mean EEG frequency across animals was calculated. Spontaneous seizures were frequently observed after the pilocarpine model of epilepsy was successfully established. The normal EEGs showed a lower frequency and a lower amplitude base wave with no interictal spikes. The most high-voltage synchronized polyspikes, high-frequency, and high-amplitude single peak of epileptic discharge were seen in the PBS group (Figure 3A). All rats in the NSCs group and GABA group showed a decrease in mean spike frequency. The EEG frequency in the PBS group (31.20 ± 1.686 Hz) was significantly higher than that in the other groups ($P < 0.001$): NRC (8.700 ± 1.494 Hz), NSCs group (15.800 ± 1.033 Hz), and GABA group (13.80 ± 1.033 Hz). In the GABA group, the frequency of EEG (13.80 ± 1.033 Hz) decreased significantly compared with the PBS group, but there were no significant differences between the GABA group and NSCs group. The seizure frequency of the PBS group was the highest compared with the other 3 groups ($P < 0.001$) (Figure 3B). These results indicated that transplantation NSCs and

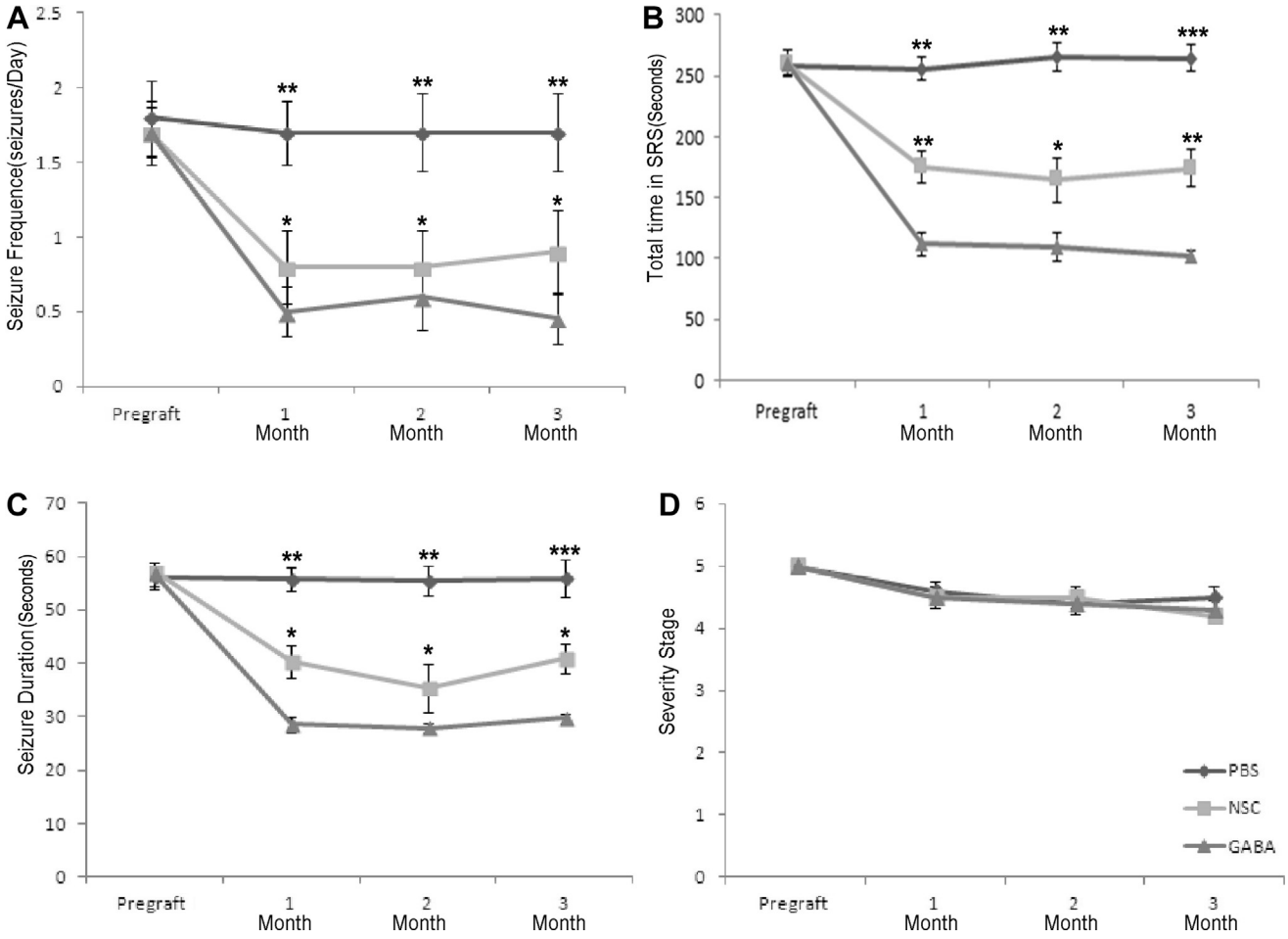

Figure 4. Efficacy of GABAergic neurons grafts for restraining spontaneous recurrent seizures (SRS). The graph shown the mean seizure frequencies (**A**), total time spent in seizures (**B**), duration of individual seizures (**C**), and seizure severity stage (**D**) were calculated during 1, 2, and 3 months after transplantation in the PBS (phosphate-buffered saline) group and cell-transplanted groups. PBS grafting surgery did not alter the seizure frequency or the seizure duration in chronically epileptic rats. The mean seizure stage of SRS was not different among the groups; seizure severity was usually stage 4 or 5 ($P > 0.05$). Compared with the PBS group, the transplanted group showed substantial decreases in all 3 seizure parameters, especially in the GABA (γ-aminobutyric acid) group. (**A**–**D**) ($P < 0.01$). The 3 seizure parameters decreased in the GABA group more than in the NSC group ($P < 0.05$). (***$P < 0.001$, **$P < 0.01$,*$P < 0.05$). NSC. neural stem cells.

GABAergic neurons could generate a significant decrease in the frequency of seizure.

Efficacy of GABAergic Neurons Grafts for Restraining SRSs

To ascertain whether GABAergic neurons restrain SRS on a long-term basis, we quantified the frequency of SRS in grafted rats for 3 months at different time points after SE. Rats were video monitored to document the emergence of SRS. During the video monitoring period, we measured the seizure frequency, the duration of individual seizures, the severity of seizures, and the total time spent in seizures. PBS grafting surgery did not alter the seizure frequency or the seizure duration in chronically epileptic rats. The mean seizure stage of SRS was not different among the groups, with seizure severity usually at stage 4 or 5 ($P > 0.05$). Compared with the PBS group, the transplanted group showed substantial decreases in all 3 seizure parameters, especially in the GABA group (Figure 4A–D) ($P < 0.01$). The 3 seizure parameters decreased in the GABA group more than in the NSCs group ($P < 0.05$). Thus, epileptic rats that received GABA grafts had significantly lower seizure frequencies compared with the other 2 groups at different time points after transplantation. Seizure severity was usually stage 4 or 5. The mean seizure stage of SRS was not significantly different among the 3 groups. There was a significant reduction in the average total time spent in SRS in transplanted groups compared with the PBS group. The average total time spent in seizures in the GABA group was the lowest. The data suggested that transplantation of GABAergic neurons considerably reduced SRS frequency, duration, and severity over the observed time frame of 3 months.

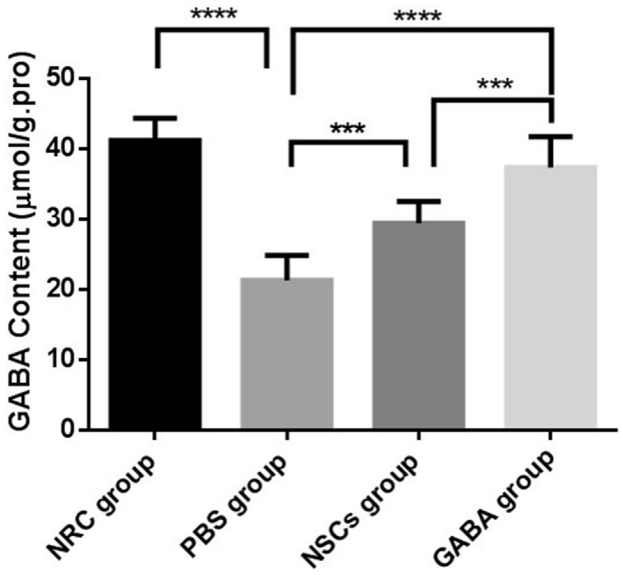

Figure 5. Effects of grafting on γ-aminobutyric acid (GABA) content in the hippocampus. The mean hippocampal GABA concentration was measured using high-performance liquid chromatography. In the PBS (phosphate-buffered saline) group, GABA content of the hippocampus was the lowest (21.36 ± 1.956 μmol/g.pro). After transplantation, GABA content of the hippocampus increased notably in the NSCs (neural stem cells) (29.48 ± 3.454 μmol/g.pro) group and remarkably in the GABA group (37.45 ± 4.344 μmol/g.pro). The level of GABA significantly increased in the GABA group compared with the PBS group ($P < 0.0001$) and NSCs group ($P < 0.001$). However, the GABA group was still lower than the NRC group (41.26 ± 4.837 μmol/g.pro). (**** $P < 0.0001$, *** $P < 0.001$).

Effects of Grafting on GABA Content in Hippocampus

After finding evidence that NSCs could differentiate into GABAergic neurons, we examined whether NSCs and GABAergic neurons could increase GABA concentration in the hippocampus of the epileptic rats using HPLC. In the PBS group, the GABA content of the hippocampus was the lowest (21.36 ± 1.956 μmol/g.pro). After transplantation, the GABA content of the hippocampus increased notably in the NSCs group (29.48 ± 3.454 μmol/g.pro) and remarkably in GABA group (37.45 ± 4.344 μmol/g.pro). However, the GABA group was still lower than the NRC group (41.26 ± 4.837 μmol/g.pro). The level of GABA was significantly increased in the GABA group compared with the PBS group ($P < 0.0001$) and NSCs group ($P < 0.001$) (Figure 5). Those results suggested that seizures resulted in decrease of the GABA content of the hippocampus. Moreover, in the NSCs group, grafted NSCs could differentiate smaller GABAergic neurons, which release GABA. GABA content increase did not reach statistical significance, because the total number of GABAergic neurons from grafted NSCs was not excessive. In contrast, transplantation of GABAergic neurons differentiated from NSCs in vitro generated many GABAergic neurons that were capable of increasing the GABA content of the hippocampus in the epileptic rats.

DISCUSSION

The present study shows that GABAergic neurons differentiated from NSCs in vitro were able to survive for a long time after intrahippocampal transplantation. Furthermore, bilateral grafting GABAergic neurons into hippocampus after SE exerted a therapeutic effect in suppressing SRSs in axpilocarpine-induced TLE model. After transplantation, the GABA level increased in the NSCs group and GABA group compared with in the PBS group. However, the GABA content of the hippocampus significantly increased in the GABA group, although it did not reach the levels seen in the NRC group. In the PBS group, increased seizure severity and frequency were observed. After grafting NSCs and GABAergic neurons into the hippocampus, the seizures were inhibited, as shown by decreased total time in seizures and frequency as well as shortened duration of seizures, especially in the GABA group. Furthermore, significantly reduced frequency of SRS in TLE rats that received GABAergic neuron grafts was coupled with significant increase of the hippocampal GABA content, in contrast to its dramatic loss in rats that underwent PBS injection alone. These results have implications for developing an effective cell transplantation therapy for restraining chronic epilepsy after SE.

A variety of cells have been noted in preclinical models of TLE for their proficiency to suppress seizures after grafting into the brain. NSCs are attractive for use as donor graft cells in TLE because these cells can be expanded in culture for extended periods from diverse sources, such as the fetal, postnatal, adult brain, human embryonic stem cells, and human-induced pluripotent stem cells.[48-53] Previous studies have shown that NSCs grafting is able to modify the disease pathology through introduction of both new astrocytes, which secrete a multitude of beneficial neurotrophic factors and new GABAergic interneurons.[13,15,28] GABAergic cell therapy is capable of replacing lost GABAergic interneurons, which in turn increase the inhibitory synaptic neurotransmission in the epileptic area of the brain.[24,25,54,55] Studies have shown pervasive migration of NSCs graft-derived cells into different regions of the hippocampus, with the overall yield of graft-derived cells equivalent to approximately 50% of the injected cells. Additional analyses showed the ability of hippocampal NSCs to give rise to GABAergic neurons (equivalent to approximately 24% of graft-derived cells) after grafting into the epileptic hippocampus.[28] However, the number of GABAergic neurons differentiated from NSCs after transplantation into the hippocampus is low. Building on these findings, we hypothesized whether transplantation of GABAergic neurons differentiated from NSCs in vitro can improve the number of GABAergic neurons so as to better suppress epileptic activity. We show that NSCs could be induced to differentiate into GABAergic neurons in vitro and successfully transplanted into the hippocampus. Thereafter, numerous GABAergic neurons were detected in the GABA group compared with the PBS group, whereas only a few GABAergic neurons were observed in the hippocampus of the NSCs group. The present results suggest that NSCs are able to differentiate into a few GABAergic neurons after grafting into the hippocampus of the epileptic rats. This finding is consistent with previous findings.[28] The novelest finding in this study is the discovery that transplanted

GABAergic neurons differentiated from NSCs in vitro can survive for a long time and improved the number of GABAergic neurons, effectively attenuating epileptic activity.

The pilocarpine-induced model is a well-established model of TLE. Kindling models show many of the characteristic features of TLE, but the disadvantages of kindling are the deficiency of spontaneous seizures and physiologic similarities of hippocampal lesions compared with human mesial TLE. However, the pilocarpine-induced model shows prominent features of TLE that spontaneous seizures acquired after a brain insult, behavioral alterations, and poor responses to antiepileptic medications. It is similar to human TLE in adults.[6,56-58] Therefore, we grafted NSCs and GABAergic neurons into the bilateral hippocampus of pilocarpine-induced epileptic rats after SRS emerged. The data showed a significant decrease in seizure frequency and total time spent in seizure at 3 months after cell grafting in GABA group.

The principal inhibitory neurotransmitter, GABA, in the cerebral cortex maintains the inhibitory tone that counterbalances neuronal excitation. When this balance is perturbed, seizures may ensue. Hippocampal GABA concentration decrease has been found in epileptic rats. Because the occurrence of seizures in TLE is believed to be linked partially to reduced numbers of hippocampal GABergic interneurons[17,59] and loss of functional inhibition,[60,61] the idea of restraining SRS via grafting of cells that release GABA has received increasing attention.[62] The data showed that hippocampal GABA concentration increased significantly in the GABA group. Seizure suppression was effectively achieved with transplanted GABAergic neurons in this study, but their effects on cognitive metrics such as reverse learning, memory, and mood impairments after transplantation may be a desirable target for future studies.

CONCLUSIONS

We show that GABAergic neurons differentiated in vitro from NSCs and grafted into the hippocampi of chronically epileptic rats have the ability to significantly reduce the frequency of EEG and frequency, duration, and severity of SRS via increased GABA-associated inhibition mediated by the addition of new GABAergic neurons. Describing biodistribution of GABAergic neurons generated in vitro after transplantation furthers our understanding of how these cells offer their therapeutic effects as well as enhancing our knowledge of GABAergic neurons generated in in vitro implications in epilepsy pathology.

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