

Valproic Acid Arrests Proliferation but Promotes Neuronal Differentiation of Adult Spinal NSPCs from SCI Rats

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Abstract Although the adult spinal cord contains a population of multipotent neural stem/precursor cells (NSPCs) exhibiting the potential to replace neurons, endogenous neurogenesis is very limited after spinal cord injury (SCI) because the activated NSPCs primarily differentiate into astrocytes rather than neurons. Valproic acid (VPA), a histone deacetylase inhibitor, exerts multiple pharmacological effects including fate regulation of stem cells. In this study, we cultured adult spinal NSPCs from chronic compressive SCI rats and treated with VPA. In spite of inhibiting the proliferation and arresting in the G0/G1 phase of NSPCs, VPA markedly promoted neuronal differentiation (β -tubulin III⁺ cells) as well as decreased astrocytic differentiation (GFAP⁺ cells). Cell cycle regulator p21^{Cip/WAF1} and proneural genes Ngn2 and NeuroD1 were increased in the two processes respectively. In vivo, to minimize the possible inhibitory effects of VPA to the proliferation of NSPCs as well as avoid other neuroprotections of VPA in acute phase of SCI, we carried out a delayed intraperitoneal injection of VPA (150 mg/kg/12 h) to SCI rats from day 15 to day 22 after injury. Both of the newborn neuron marker doublecortin

and the mature neuron marker neuron-specific nuclear protein were significantly enhanced after VPA treatment in the epicenter and adjacent segments of the injured spinal cord. Although the impaired corticospinal tracks had not significantly improved, Basso–Beattie–Bresnahan scores in VPA treatment group were better than control. Our study provide the first evidence that administration of VPA enhances the neurogenic potential of NSPCs after SCI and reveal the therapeutic value of delayed treatment of VPA to SCI.

Keywords Valproic acid · Neural stem/precursor cells · Proliferation · Differentiation · Spinal cord injury

Abbreviations

BBB	Basso–Beattie–Bresnahan scale
BDA	Biotin dextran amine
bFGF	Basic fibroblast growth factor
CCK-8	Cell counting kit-8
DAPI	4–6–Diamidino-2-phenylindole dihydrochloride
DCX	Doublecortin
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FCM	Flow cytometry
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
LDH	Lactate dehydrogenase
NeuN	Neuron-specific nuclear protein
NSPCs	Neural stem/precursor cells
OD	Optical density
qPCR	Quantitative PCR
RT	Reverse transcription
SCI	Spinal cord injury
VPA	Valproic acid

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Introduction

Traumatic spinal cord injury (SCI) causes massive cell loss due to necrosis during the primary injury and prolonged apoptosis or autophagy resulting from the secondary injury, often leading to permanent neurological disabilities. Although considerable advances have been achieved in SCI therapy, to date, no therapeutic strategy has been proven effective in restoring the motor function of patients [1–3].

Recently, several studies have identified neural stem/progenitor cells (NSPCs) in the adult mammalian spinal cord and revealed the potential of these endogenous NSPCs to mobilize and replace lost neurons after SCI [4–6]. Indeed, increasing evidence demonstrates that SCI elicits NSPCs proliferation in the ependymal region [7–10]. But inconsistent with expectation, endogenous neurogenesis and remyelination is very limited after SCI because activated NSPCs primarily differentiate into astrocytes rather than neurons or oligodendrocytes [10–13]. In addition, after SCI, newly formed astrocytes migrate to the site of injury and contribute to astrogliosis, which is considered as an obstacle to neurogenesis [10, 12, 13]. These studies suggest that the cell replacement of NSPCs is negatively influenced by the post-SCI environment and optimizing the activity of NSPCs after SCI may represent a therapeutic strategy, which not only promote endogenous neuron and oligodendrocyte replacement but also reverse the unconstructive effects of astrocyte activation, such as glial scarring [13, 14].

Valproic acid (VPA) has been used as an anticonvulsant and mood-stabilizing drug for decades. Recently, VPA was used as an anti-cancer therapy in several clinical trials due to its activity as a histone deacetylase inhibitor [15–17]. The epigenetic effects of VPA are also reflected in the fate regulation of some stem cells; e.g., VPA promotes the differentiation of mesenchymal stem cells and hematopoietic stem cells [18–20]. In view of its fate-regulatory effects on stem cells, we hypothesized that VPA treatment optimizes the activity of NSPCs and contributes to recovery after SCI.

In this study, we show that administration of VPA arrested proliferation but promoted neuronal differentiation of spinal NSPCs from a chronic compressive rat SCI model. *In vivo*, a delayed VPA treatment (15 days after injury) increased the expressions of the newborn neuron marker doublecortin (DCX) and the mature neuron marker neuron-specific nuclear protein (NeuN) in injuring spinal cord, meanwhile, facilitated motor function recovery assessed by Basso–Beattie–Bresnahan (BBB) scale. These results suggest that optimizing activity of spinal NSPCs represents a promising regenerative strategy following SCI,

thus providing a potential alternative or complementary approach to cell transplantation.

Materials and Methods

SCI Model

All animal experiments were performed in accordance with the national regulations regarding the use of laboratory animals and were approved by the institutional animal care and use committee of the Third Military Medical University, Chongqing, China. Every effort was made to minimize the number of animals used and their suffering, and all surgical procedures were performed using aseptic technique. One hundred and thirty-five (135) adult male Sprague–Dawley rats (Experimental Animal Center of the Third Military Medical University, Chongqing, China) weighing 180–220 g were used in this study. Compressive SCI was performed as reported previously [21–23], with minor modifications. Briefly, before the operations, all animals were anesthetized using chloral hydrate (300 mg/kg, intraperitoneal injection). A longitudinal incision was made on the midline of the back to expose the vertebrae, and the spinal cord was exposed via a two-level T9–T10 laminectomy. SCI was produced via transient (1 min) extradural compression of the spinal cord at level T10 using an aneurysm clip at a closing force of 30 g. The skin incision was closed after removal of the clip and was smeared with erythromycin ointment. The identical surgical procedure was performed during the sham control surgery, except that clip compression was not performed. The rectal temperature was maintained at 36.5–37.5 °C using a thermostatically regulated heating pad during surgery. During the recovery phase, the animals were placed in a room with an alternating 12-h light/dark cycle at a temperature of 22–25 °C and humidity of 50–60 %, and manual urinary bladder massage was performed thrice daily until the autonomous bladder voidance reflex was exhibited.

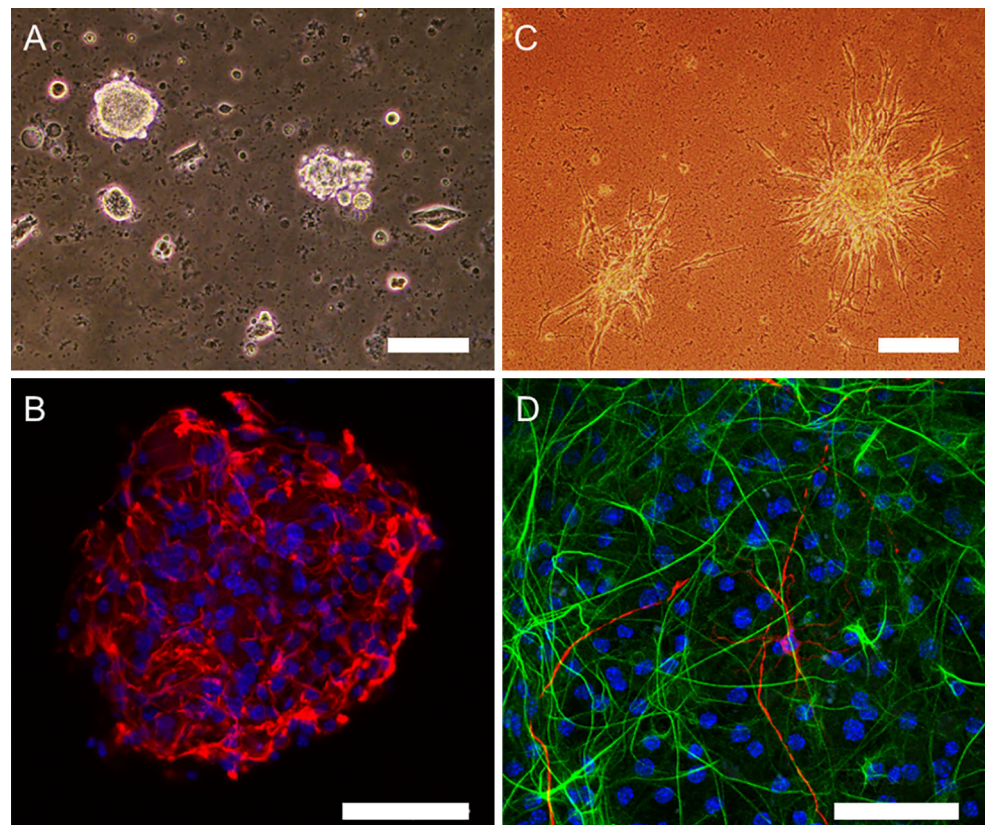
Primary Culture and Identification of Adult Spinal NSPCs from SCI Rats

Primary culture of adult rat spinal cord-derived NSPCs was performed as described previously, with slight modifications [24, 25]. At day 15 after compressive SCI, the rats were anesthetized and perfused with artificial cerebrospinal fluid. A 20-mm injury site-centered segment was harvested immediately. Microdissection of the spinal cord into 1–2 mm³ pieces was performed in PBS. The dissected tissue pieces were washed three times with D-hank's

solution and resuspended in 0.125 % trypsin/0.02 % EDTA solution. Then, the tissue was incubated for 5 min at 37 °C and triturated using a 5-mL pipette until the cell suspension was free of large tissue pieces. The remaining tissue pieces were removed via filtration using a nylon mesh. The filtered cell suspension was centrifuged at $1000\times g$ for 3 min, and the trypsin–EDTA solution was gently removed. The cells were suspended in DMEM/Ham's F-12 medium containing 10 % fetal bovine serum (FBS) (Invitrogen) and washed three times via centrifugation. After aspirating the medium, the cells were resuspended in N2 medium containing 10 % FBS (Invitrogen) and seeded at 1×10^4 cells/cm² on 10-cm uncoated tissue culture plates. The next day, the medium was changed to serum-free N2 medium (Invitrogen) containing 20 ng/mL basic fibroblast growth factor (bFGF) and 20 ng/mL epidermal growth factor (EGF) (PeproTech, USA). For clonal culture, the suspended cells were seeded at 5×10^4 cells/cm² on poly-L-ornithine hydrobromide (Sigma-Aldrich)/laminin (Invitrogen)-coated dishes with serum-free N2 medium containing 20 ng/mL bFGF and 20 ng/mL EGF. To feed the cultures, half of the medium in the cultures was replaced with fresh medium every 2–3 days. After 10–14 days, the clones reached a target size (diameter $>50 \mu\text{m}$, Fig. 1a).

To examine the self-renewal and differentiation potential of these cells into a neural lineage, primary neurospheres were dissociated and subcultured in serum-free proliferation medium or differentiation medium (containing 5 % FBS without growth factors). The much more abundant neurospheres were passaged and stained with a rabbit anti-Nestin (Santa Cruz, USA) antibody (Fig. 1b). In differentiation medium, the neurospheres grew adherently and sprouted differentiated cells (Fig. 1c, d), which were characterized using specific antibodies, including rabbit anti-GFAP (Sigma-Aldrich, USA), a specific astrocyte marker, and mouse anti- β -tubulin III (Abcam, USA), a specific neuronal marker. Standard immunofluorescent procedures were used for the identification of differentiated cells [26]. Briefly, cell culture slides were fixed with 2 % paraformaldehyde in PBS, permeabilized with 0.1 % Triton X-100 and blocked with 5 % normal goat serum. Then treated with primary antibodies in PBS containing 0.5 % horse serum overnight at 4 °C. After removal of the primary antibodies by washing with PBS, the slides were incubated in secondary antibodies for 1 h at 37 °C and then mounted using 90 % glycerol. Fluorescence staining was examined and photographed using a confocal laser scanning microscope (LSM 710, Carl Zeiss, Germany). The primary antibodies used in this study including rabbit anti-

Fig. 1 Culture and identification of adult spinal NSPCs from SCI rats. **a** Primary cultured neurospheres; **b** Nestin-positive cells (red) in the neurospheres; **c** neurospheres grew adherently and sprouted cells in differential medium; **d** GFAP⁺ (green) and β -tubulin III⁺ (red) cells in neurospheres cultured under differential condition. DAPI: blue. Bar **a**, **c** 100 μm ; **b**, **d** 50 μm (Color figure online)



Nestin (Santa Cruz, USA, 1:100), rabbit anti-gial fibrillary acidic protein (GFAP, 1:300), mouse anti- β -tubulin III (1:200) (Abcam, USA). The secondary antibodies included fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit, Cy3-conjugated goat anti-mouse and Cy3-conjugated goat anti-rabbit (Santa Cruz, USA).

NSPCs Proliferation Assays

The neurospheres were separated using 0.125 % trypsin/0.02 % EDTA for 1 min and were gently triturated with a glass pipette. Then, individual NSPCs were seeded at 1×10^4 /well on 12-well plates and were cultured in 1 mL of serum-free N2 medium containing 20 ng/mL bFGF and 20 ng/mL EGF. Five final concentrations (0.001, 0.01, 0.1, 1 and 10 mmol/L) of VPA were applied to six wells of the subcultured cells for each concentration. The number and diameter of the neurospheres were measured under six random fields (CKX31-A12PHP, Olympus, Japan) at day 7. For the cells viability assays, cell counting kit-8 (CCK-8, Dojindo, Japan) was used according to the manufacturer's instructions. Briefly, after culturing for 0, 24, 48 or 72 h, 10 μ L of CCK-8 buffer was added to each well, followed by incubation for another 2 h. The optical density (OD) values at 490 nm were measured using a microplate reader (Thermo MK3, Thermo, Finland). Each assay was performed in triplicate.

Lactate Dehydrogenase (LDH) Release Assay

LDH is a stable enzyme that is expressed in all cell types and is rapidly released into the extracellular environment upon damage to the plasma membrane. Therefore, LDH is the most widely used marker for cytotoxicity studies.

After treatment with VPA for 48 h, the supernatant from the NSPCs was collected, and LDH release was detected using a CytoTox-ONE™ Homogeneous Membrane Integrity Assay kit (Promega, USA) according to the manufacturer's instructions. The data from three independent experiments (5 wells per condition) were normalized to the medium-only control and were expressed as the mean \pm SEM.

Cell Cycle Assay

The cell cycle assay was performed via flow cytometry (FCM) as reported previously [27]. Adult SCI rat spinal cord-derived NSPCs were treated with 0.01 or 1 mmol/L VPA for 48 h. Then, the cells were harvested and washed three times with PBS, fixed in 70 % ethanol, stained with propidium iodide (PI, 25 μ g/mL) (Sigma-Aldrich) and incubated for 30 min at 37 °C in RNase A (20 μ g/mL)

(Roche Applied Science, USA). Then, the cellular DNA content was evaluated via flow cytometry using a fluorescence activated cell sorting (FACS) scanner (BD Biosciences, USA).

Differentiation Assay

The neurospheres were dissociated using 0.125 % trypsin/0.02 % EDTA for 1 min. Then, the suspended NSPCs were seeded at 1×10^4 cells/well on 12-well plates with differential medium. The next day, differential media containing 0.01 or 1 mmol/L VPA was applied to the subcultured cells and was replaced every 2 days. On day 12 of VPA administration, the cells were harvested for immunofluorescence, FCM and Western blot. For FCM analysis, the cells were fixed using 4 % paraformaldehyde for 30 min at 4 °C, permeabilized with 0.1 % Triton-100 for 10 min at room temperature, and then stained with either Alexa Fluor® 488 Mouse anti- β -tubulin III or Alexa Fluor® 488 Mouse IgG2a, κ Isotype control (BD Pharmingen™, USA). Then, the cells were washed and resuspended in cold PBS and analyzed using a FACS scanner (BD Bioscience) [28]. Western blot was performed as described above.

VPA Administration, Grouping and Tissue Preparation

A dose of 150 mg/kg VPA (Sigma-Aldrich, USA) or saline vehicle control was intraperitoneally injected every 12 h from day 15 to day 22 after SCI. The sham group did not receive any of these injections. Ninety-eight (98) animals were randomized to the four groups (Sham, Sham + VPA, SCI and SCI + VPA) and sacrificed at day 15, 22, 29 and 36 after SCI for tissue examination. Other ten (10) animals of the group SCI and SCI + VPA were injected biotinylated dextran amines (BDA) for tracking of the nerve fibers in corticospinal track. The rest 27 rats were randomized to the three groups (Sham, SCI and SCI + VPA) and maintained for behavioral test.

For tissue preparation, rats were deeply anesthetized using chloral hydrate (500 mg/kg) and were intracardially perfused with 0.1 M PBS followed by 4 % paraformaldehyde. A 25-mm section of the spinal cord centered at the compression site was immediately harvested, post-fixed in 10 % formalin overnight and cryoprotected in 30 % (w/v) sucrose in PBS for 2 days. This section of the spinal cord was cut into three parts (epicenter, adjacent segment, distant segment) at 5-mm intervals from the lesion epicenter (Fig. 4a) and then embedded in paraffin according the standard procedures [29]. Ten (10) μ m-thick horizontal sections were sliced and stained with specific antibodies.

Reverse Transcription (RT) and Real-Time Quantitative PCR (qPCR)

Total RNA was isolated from spinal cord tissues or cultured cells using Trizol reagent (Invitrogen, USA). Then, 0.5 µg of total RNA was used for RT using MMLV-reverse transcriptase according to the manufacturer's instructions, and qPCR was performed using a CFX96™ Real-Time PCR Detection System (Bio-Rad, USA) and iTaq universal SYBR Green supermix (Bio-Rad). PCR amplification was performed for 5 min at 94 °C, followed by 35 cycles of 45 s at 94 °C, annealing for 30 s at 47–57 °C and extension for 30 s at 72 °C. The results were normalized to the expression of GAPDH and were expressed as a ratio of the internal reference. The specific primer sequences were as follows (Table 1).

Western Blot

The three portions of the harvested spinal segments (epi-center, adjacent segment and distant segment) were stored in liquid nitrogen and homogenized using a disposable grinding stick. Proteins were extracted from cultured cells using CellLytic MT Cell Lysis Reagent (Sigma-Aldrich, USA). Western blot was performed as described previously [30]. The protein samples were probed using the following antibodies: rabbit anti-DCX (1:800), rabbit anti-NeuN (1:1000) (EMD Millipore, USA), mouse anti-β-actin (1:5000) (Santa Cruz, USA), mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:5000) (Chemicon, USA), rabbit anti-GFAP (1:1000), Olig2 (1:800), β-tubulin III (1:800) (Abcam, USA), rabbit anti-P21 (1:800) (Boster, China). The secondary antibodies included IR Dye 700-conjugated anti-rabbit (1:5000, Rockland, USA) and IR Dye 800-conjugated

anti-mouse (1:5000, LI-COR Biosciences, USA). The membranes were scanned using an Odyssey imager (LI-COR Biosciences, USA), and the band intensity was determined using Quantity One 4.40 software.

Immunofluorescence

The tissue sections were incubated in PBS containing 0.1 % Triton X-100 for 20 min and then treated with primary antibodies in PBS containing 5 % horse serum overnight at 4 °C. After removal of the primary antibodies by washing with PBS, the sections were incubated in secondary antibodies for 1 h at room temperature and then mounted using 90 % glycerol. Fluorescence staining was examined and photographed using a confocal laser scanning microscope (LSM 710, Carl Zeiss, Germany). The primary antibodies used in this study included rabbit anti-doublecortin (DCX, 1:300), rabbit anti-neuron-specific nuclear protein (NeuN, 1:200) (Santa Cruz, USA). The secondary antibodies included fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit and Cy3-conjugated goat anti-rabbit (Santa Cruz, USA). All of the antibodies were used according to the manufacturer's instructions.

BDA Tracking of Corticospinal Tract

Five rats in SCI and SCI + VPA groups were anesthetized by intraperitoneal injection of 5 % chloral hydrate in the day 23 of modeling and fixed in the prone position. A scalp incision was made in the center to expose the primary motor cortex and 4 injection points was selected in each hemisphere. The bregma plane indicated the horizontal axis, and the sagittal suture indicated the vertical axis. The coordinates for the four points on the right side were (2.5,

Table 1 Primers used for real-time PCR experiment

Gene	Forward	Reverse	Annealing T (°C)
P21	CGACTTTGTCACCGAGACAC C	GGCAGAAGATGTAGAGCGGG	57
Sox2	AACCGATGCACCGCTACGA	TGCTGCGAGTAGGACATGCTG	57
Pax6	TTACGAGACTGGCTCCATCA	CCGCTTATACTGGGCTATTT	55
Mash1	CCAGTTGTA CTTCAGCACC	TGCCACTTTGAGTTTGAC	55
Ngn2	CCTGGAAACCATCTCACTCA	TACCCAAAGCCAAGAAATGC	55
NeuroD1	CCACGGATCAATCTTCTCAG	CATGATGTGAATGGCTATCG	55
NeuroD2	CAGTAAGGACTTTTAGAA	TGATTATTGGTAGTAGTG	47
Math1	GAGAGAGCATCCCGTCTA	GAATGTAGCAAATACTGG	50
Math2	TGAATCCTTCTATGAAAG	GAAAATATCCCATATAGTTA	47
Tbr1	GAATCAGTCAGATACAGA	AAGACAGGAGAGAGTTTA	50
Tbr2	CAAGGTTCTGTATTTATTT	TTTAACTCATCTGATAGC	50
DCX	GGCCAGGTGAGTTGCAAAAGGT	GCCGCACACGTCAAAGAATG	57
NeuN	GGCAATGGTGGGACTCAAAA	GGGACCCGCTCCTTCAAC	57
GAPDH	AAGGGTCATCATCTCCGCCCA	TGCCCCATGTTTGTGATGG	55

2.7 mm), (1, 2.5 mm), (0.4, –1.5 mm), and (1.8, –1.2 mm), and 4 symmetrical points were selected on the left for a total of eight injection sites. At each point, 2 μ L of 5 % BDA was injected using a microinjection pump. For injection sites rostral to the bregmatic suture, the needle depth was 3.5 mm, and for injection sites caudal to the bregmatic suture, the needle depth was 3.2 mm. The injection was completed within 5 min, and the needle remained in the injection position for 5 min to prevent drug overflow. After the microsyringe was removed, the skull defect was filled with gelatin, and the scalp was sutured. The spinal cords were dissected 2 weeks after the injection of BDA (i.e., 37 days after injury), followed by sectioning and staining according to the manufacturer's instructions. BDA positive nerve fibers were counted in five randomly selected high power field in every cross section of T12.

Behavioral Assessment

The functional deficits after injury was assessed by two blinded observers using the BBB locomotor rating scale [31]. Briefly, the rats were placed on a plastic platform for 4 min. Then, the scores were recorded according to the 21-point locomotor rating scale. A score of 0 corresponded to no spontaneous movement, and a score of 21 indicated normal locomotion. When an animal exhibited plantar stepping with full weight support and complete forelimb–hindlimb coordination, it attained a score of 14 points. This assessment was performed once a week for 8 weeks post-SCI.

Statistical Analysis

The data are presented as the mean \pm standard error of the mean (SEM). Two-way repeated measures ANOVA was used for comparison of the weekly BBB scores. The differences between the two groups were compared using Student's *t* test. Multiple comparisons between the different groups were performed via one-way ANOVA. All statistical analyses were performed using SPSS 17.0 software (SPSS, Chicago, IL, USA). The differences were considered to be statistically significant when **P* < 0.05 or ***P* < 0.01.

Results

VPA Inhibits NSPCs Proliferation in Proliferative Medium

We cultured primary spinal cord NSPCs from adult SCI rats, and confirmed that these cells possessed the abilities of self-renewal in proliferative medium and differentiation into neural and glial lineages in differential medium (Fig. 1a–d).

The subcultured cells were treated with VPA at five concentrations (0.001, 0.01, 0.1, 1 or 10 mmol/L), then number and diameter of the neurospheres were measured under six random fields (CKX31-A12PHP, Olympus, Japan) at day 7. 1 mmol/L VPA-treated adult spinal cord NSPCs revealed an 5.2-fold-decrease in the number of neurospheres compared to PBS-vehicle control group (diameter \geq 50 μ m, *n* = 4). The number of small clusters (diameter <50 μ m) increases 4.1-fold in VPA-treated group (*n* = 4) consistent with the continued survival of non-neurosphere forming cells. The average diameter of the neurospheres was 2.7-fold-decrease in VPA treated cultures (*n* = 4) (Fig. 2a–c).

The viability of the NSPCs was assayed using a CCK-8 kit and the growth curves were delineated. VPA treatment at 0.001 mmol/L did not affect NSPCs viability, but at 0.01, 0.1, 1 or 10 mmol/L, VPA significantly suppressed NSPCs proliferation, and the higher concentrations exerted stronger inhibitory effects (Fig. 2d).

The cytotoxicity of VPA was also assessed based on LDH release. VPA treatment at 0.001, 0.01, 0.1 or 1 mmol/L did not increase LDH release; only VPA treatment at the highest concentration (10 mmol/L) caused significant damage to the NSPCs (Fig. 2e).

VPA Arrests NSPCs in the G0/G1 Phase and Increased p21^{Cip/WAF1} Expression Under Proliferative Condition

The cell cycle of NSPCs was detected by FCM (Fig. 3a) at 72 h after subculture. Compared to the vehicle-treated control group, administration of VPA remarkably arrested the NSPCs in the G0/G1 phase (72.12 \pm 3.33 % at 0.01 mmol/L VPA and 80.99 \pm 3.34 % at 1 mmol/L VPA vs 45.3 \pm 3.24 % vehicle-treated control) (Fig. 3b). The proliferation index (PI) [PI = (S + G2/M)/(G0/G1 + S + G2/M) \times 100 %] of NSPCs also significantly decreased when treated with VPA (Fig. 3c).

The p21^{Cip/WAF1} protein binds to and inhibits the activity of the cyclin-CDK2, -CDK1, and -CDK4/6 complexes, thus acting as a regulator of cell cycle progression at the G1 and S phases [32]. In this study, we found that p21^{Cip/WAF1} expression was increased at both the mRNA and protein levels after administration of VPA (0.01, 1 mmol/L) compared to vehicle-treated control (Fig. 3d, e).

VPA Increases the Number of β -Tubulin III⁺ Cells and Proneural Genes Expression in Differential Medium

To define the effect of VPA in neural differentiation, NSPCs were induced to differentiation in medium that containing 5 % FBS without growth factors. When NSPCs

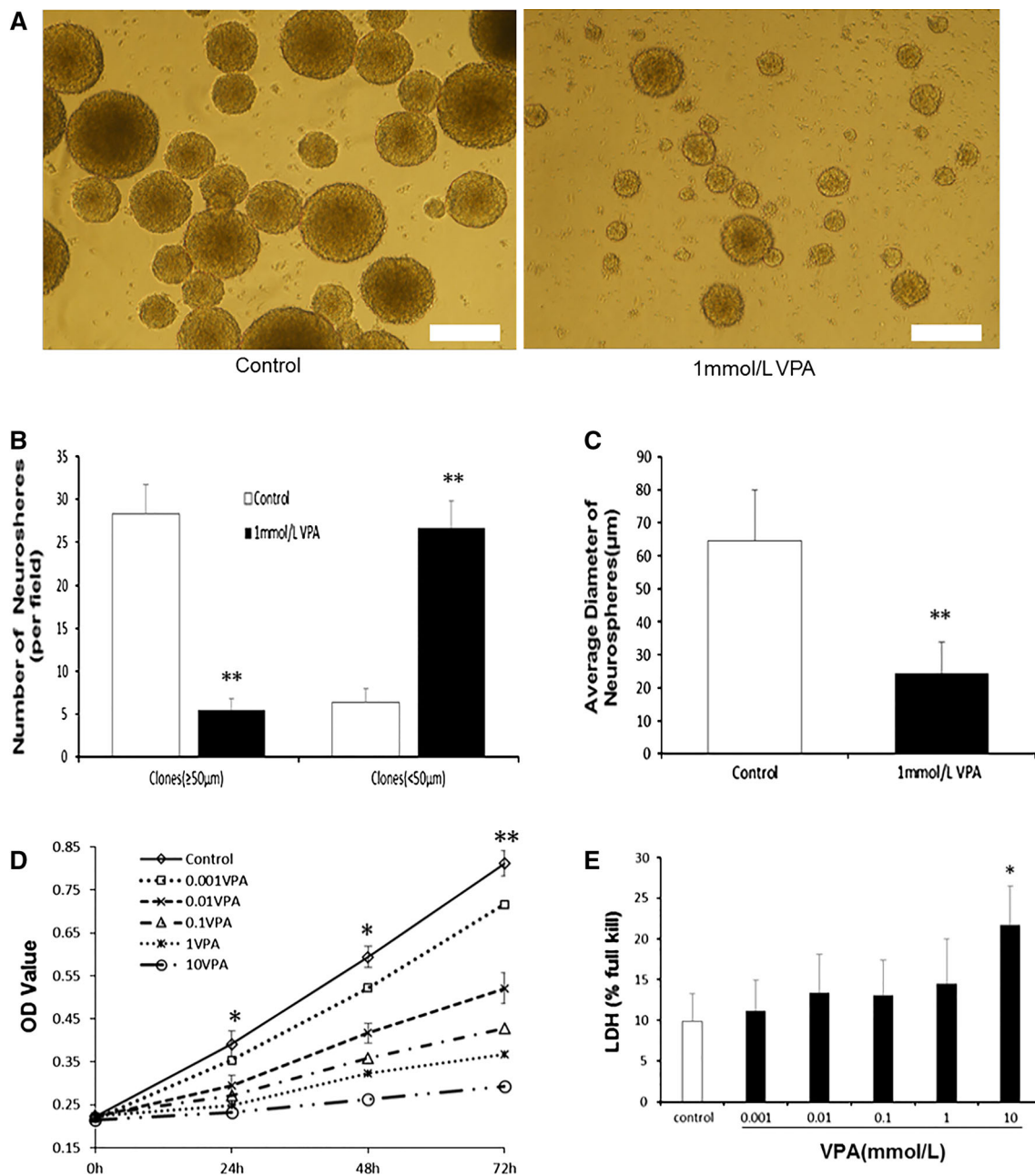


Fig. 2 VPA treatment inhibited proliferation of adult spinal NSPCs. **a** The representative photos of neurospheres with VPA treatment. **b**, **c** Quantitative analysis of number and size of neurospheres. One (1) mmol/L VPA-treated adult spinal NSPCs revealed an 5.2-fold-decrease (diameter $\geq 50 \mu\text{m}$) and 4.1-fold-increase (diameter $< 50 \mu\text{m}$) in the number of neurospheres (**b**), the average diameter of the neurospheres was 2.7-fold-decrease in VPA treated-cultures (**c**), compared to PBS-vehicle control group. $**P < 0.01$, compared to control group, $n = 3$. **d** Growth curves of the NSPCs were generated

using a CCK-8 kit. VPA dose-dependently inhibited the proliferation of NSPCs in vitro. $*P < 0.05$, $**P < 0.01$ compared to the 0.01 mmol/L VPA-treated group. **e** Cytotoxicity assay of VPA treatment based on LDH release. VPA treatment at 0.001, 0.01, 0.1 or 1 mmol/L did not increase LDH release; only treatment with the highest concentration of VPA (10 mmol/L) that showed a significant damage to the NSPCs. $*P < 0.05$ compared to the PBS-vehicle control group, $n = 3$

were differentiated for 10 days, the VPA-treated cells exhibited about 2.3-fold (0.01 mmol/L) and 5.9-fold (1 mmol/L) increase in the β -tubulin III-positive neuronal populations, meanwhile, 1.4-fold decrease GFAP-positive

astrocytic populations (Fig. 4a, b). In a parallel experiment, the neuronal differentiation of NSPCs was assessed by FCM using Alexa Fluor[®] 488 mouse anti- β -tubulin III. After VPA treatment at 0.01 or 1 mmol/L for 10 days, the

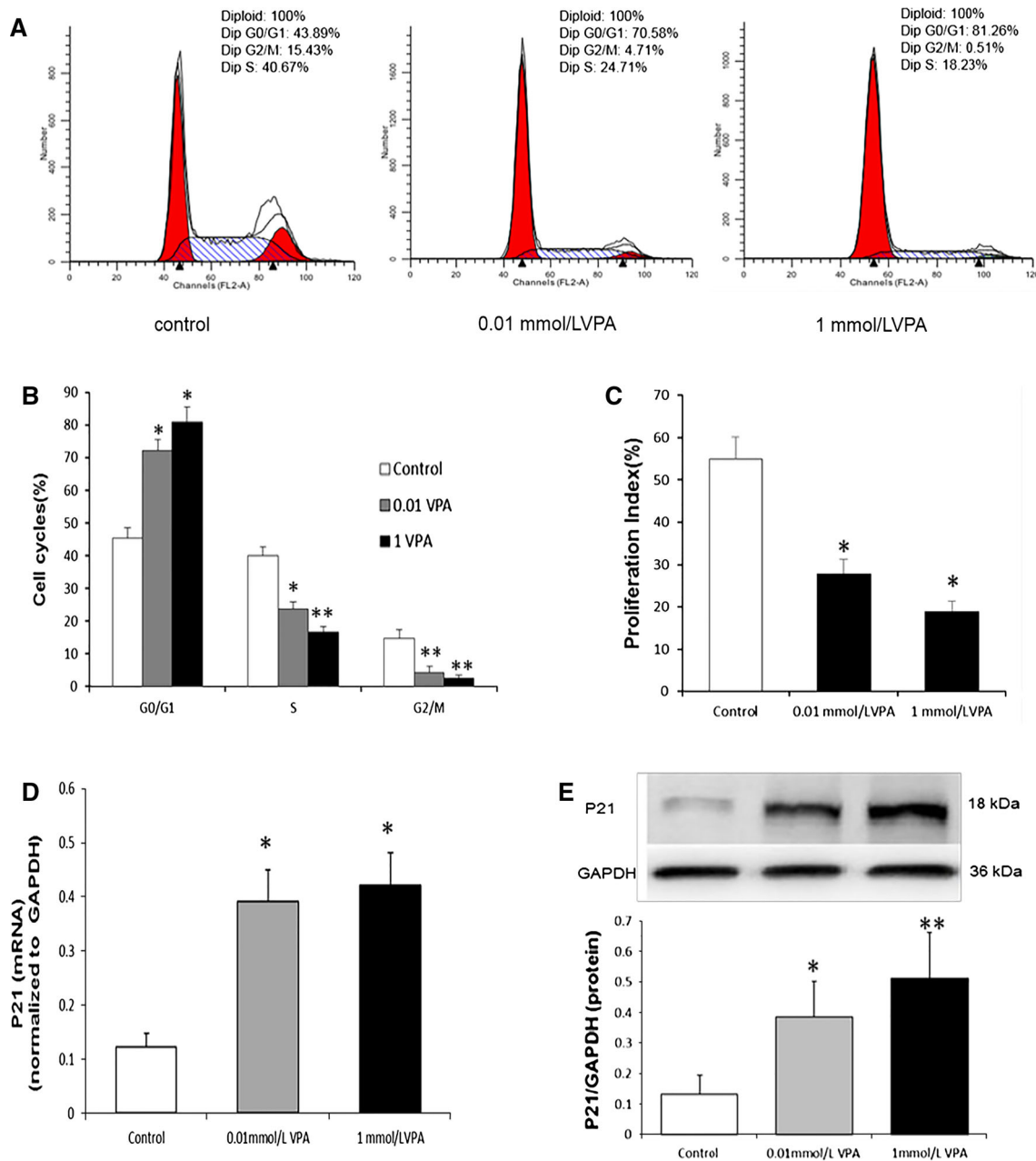


Fig. 3 Administration of VPA arrested NSPCs in the G0/G1 phase and enhanced P21 expression in proliferative medium. **a** Representative cell cycle analysis of NSPCs by FCM; **b, c** quantitative analysis of the cell cycles. **b** Administration of VPA remarkably arrested the NSPCs in the G0/G1 phase ($72.12 \pm 3.33\%$ at 0.01 mmol/L VPA and $80.99 \pm 3.34\%$ at 1 mmol/L VPA vs $45.3 \pm 3.24\%$ vehicle-

treated control). **c** The proliferation index (PI) [$PI = (S + G2/M) / (G0/G1 + S + G2/M) \times 100\%$] of NSPCs significantly decreased when treated with VPA. **d, e** VPA treatment (0.01 or 1 mmol/L) increased the expression of P21 at both mRNA (**d**) and protein (**e**) in NSPCs (normalized to GAPDH). * $P < 0.05$, ** $P < 0.01$, compared to the vehicle-treated control, $n = 3$

percentage of β -tubulin III⁺ cells was significantly increased to $13.98 \pm 2.76\%$ ($P < 0.05$) and $39.53 \pm 5.66\%$ ($P < 0.01$) respectively, compared to the vehicle-treated control ($6.75 \pm 1.85\%$) (Fig. 4c, d). Western blot also showed that β -tubulin III expression was markedly increased and that the expression of the astrocytic marker GFAP was decreased; the expression of the

oligodendrocyte marker Olig2⁺ was increased numerically, but hadn't statistical significance ($P = 0.11$, $n = 3$) (Fig. 4e, f).

To gain insight into the molecular mechanisms involved in neuronal differentiation of NSPCs after VPA treatment, we analyzed the proneural genes expression at day 5 after differential induction by real-time quantitative PCR.

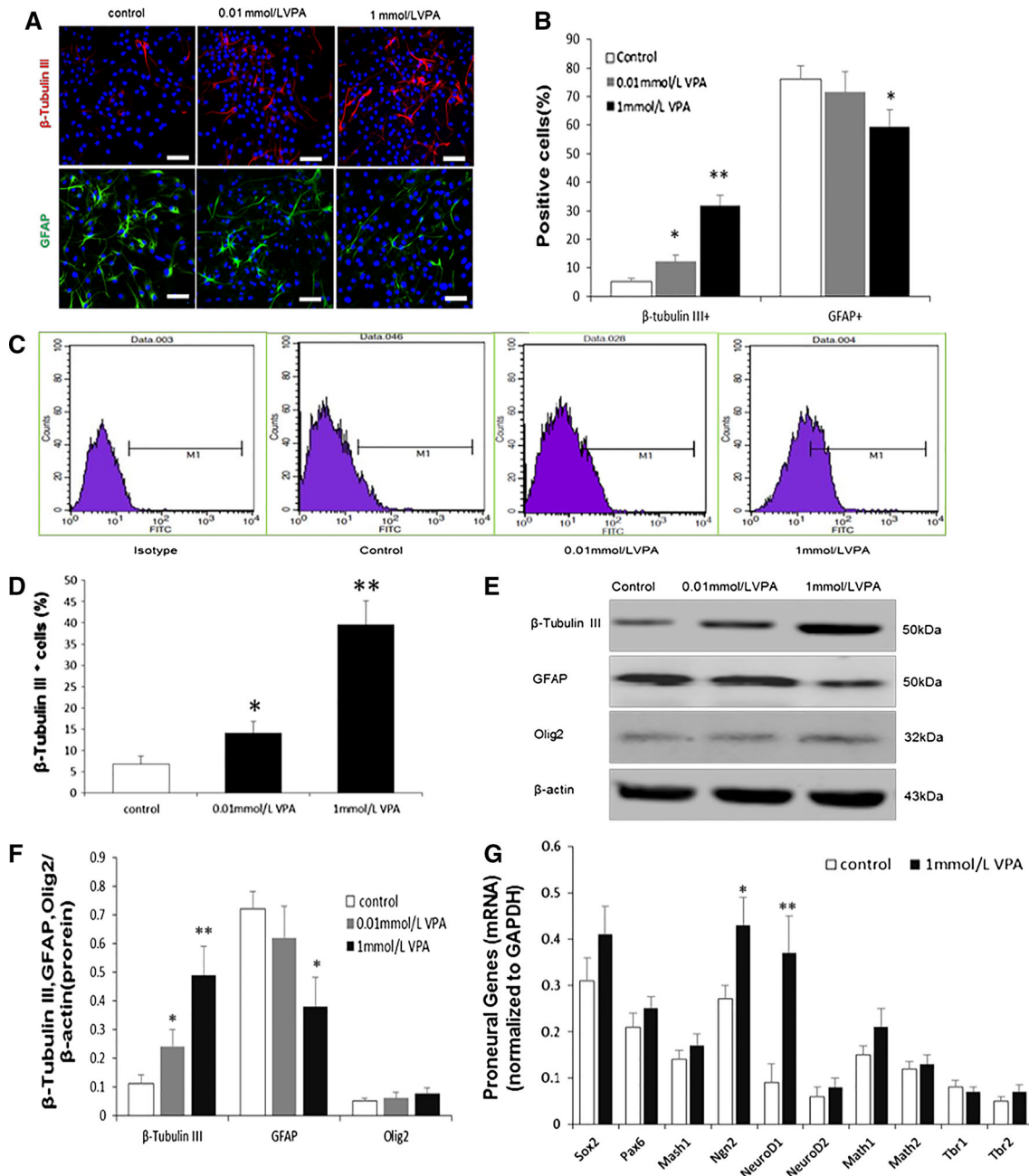


Fig. 4 Administration of VPA promoted neuronal differentiation and increased the expressions of Ngn2 and NeuroD1 in differential medium. **a** Representative immunofluorescent photos of β -tubulin III⁺ cells and GFAP⁺ cells in NSPCs under differential condition. Bar 50 μ m. **b** Quantitative analysis of the β -tubulin III⁺ and GFAP⁺ cells population among the NSPCs. VPA-treated cells exhibited about 2.3-fold (0.01 mmol/L) and 5.9-fold (1 mmol/L) increase in the β -tubulin III⁺ neuronal populations and 1.4-fold decrease GFAP⁺ astrocytic populations. **c, d** FCM analysis of β -tubulin III⁺ cells in NSPCs. After VPA treatment (0.01 and 1 mmol/L), the percentage of β -tubulin III⁺ cells was significantly increased to $13.98 \pm 2.76\%$ ($P < 0.05$) and $39.53 \pm 5.66\%$ ($P < 0.01$) respectively, compared to the vehicle-treated control ($6.75 \pm 1.85\%$); **e, f** Western blot also

showed that VPA treatment (0.01 and 1 mmol/L) increased β -tubulin III expression, decreased GFAP expression ($*P < 0.05$, $**P < 0.01$ compared to the control). The expression of the oligodendrocyte marker Olig2⁺ was increased numerically, but hadn't statistically significant ($P = 0.11$, $n = 3$); **g** expression of proneural bHLH genes (Pax6, Sox2, Ngn2, NeuroD1, NeuroD2, Mash1, Math2, Tbr1 and Tbr2) in NSPCs after differential medium. 1 mmol/L VPA treatment increased Ngn2 and NeuroD1 expression and a trend was observed for Sox2 ($P = 0.12$) and Mash1 ($P = 0.19$), while the expression of Pax6, NeuroD2, Tbr1 and Tbr2 wasn't changed (normalized to the expression of GAPDH; $*P < 0.05$, $**P < 0.01$, compared to the control, $n = 3$)

Proneural bHLH genes Pax6, Sox2, Ngn2, NeuroD1, NeuroD2, Mash1, Math2, Tbr1 and Tbr2 were expressed in NSPCs under differential medium. When treated with 1 mmol/L VPA, Ngn2 and NeuroD1 were increased significantly and a trend was observed for Sox2 ($P = 0.12$) and Mash1 ($P = 0.19$), while the expression of Pax6, NeuroD2, Tbr1 and Tbr2 wasn't changed (Fig. 4g).

VPA Delayed Treatment Enhances Expression of Neuronal Markers in SCI Rat

To minimize the possible adverse effects cause by the inhibitory effect of VPA to the proliferation of NSPCs as well as avoid other neuroprotections of VPA in acute phase of SCI [21, 33–35], we carried out a delayed intraperitoneal injection of VPA (150 mg/kg/12 h) to SCI rats from day 15 to day 22 after injury. Then, mRNA and protein expression levels of two neuronal markers, DCX and NeuN, in epicenter, adjacent segment and distant segment were analyzed (Fig. 5a). DCX is a microtubule-associated protein that is expressed by neuronal precursor cells and immature neurons. Due to the nearly exclusive expression of DCX in developing neurons, this protein has increasingly been used as a marker of neurogenesis [36]. NeuN is a neuronal nuclear protein that is commonly used as a marker of mature neurons [37].

Compared to the saline-treated SCI control group, qPCR analysis of the epicenter revealed mRNA expression of DCX and NeuN were both increased significantly in VPA-treated SCI group. DCX mRNA presented a maximum 2.6-fold increase at 22 days and NeuN mRNA reached a maximum 1.8-fold increase at 29 days after injury compared with control counterparts (Fig. 5b, c). The protein expression of DCX and NeuN in the epicenter, adjacent and distant segments were measured via western blot. The results revealed that both of DCX and NeuN were much more strongly expressed in adjacent segments than the epicenter. VPA treatment significantly increased the protein level of DCX at 22 days (1.8-fold in the epicenter and twofold in adjacent segments, Fig. 5d, e) and NeuN at 29 days (2.5-fold in the epicenter and 1.6-fold in adjacent segments, Fig. 5f, g) following SCI compared to the saline-treated control group. In the sagittal section of epicenter, immunofluorescence staining also showed that VPA treatment increased the DCX-positive cells at 22 days and NeuN-positive cells at 29 days after SCI respectively (Fig. 5h).

VPA Delayed Treatment Facilitates Motor Function Recovery After SCI

HE staining of sagittal section revealed that there were a lot of bleeding spots at the epicenter injured site at the first day

following SCI. Cyst and glial scar were distinct in saline-treated rats at the 36 day after SCI, but they were obviously reduced in the VPA-treated group (Fig. 6a). BDA tracking showed the nerve fibers of corticospinal tract had a numerical increase after VPA delayed treatment, but there was no statistically significant difference when compared to SCI group (Fig. 6b, c). Functional recovery was evaluated based on the BBB scale every week from the 0 day to 8 weeks after injury. Compared to the saline-treated SCI control group, the VPA-treated SCI group exhibited a significantly increased BBB score from 4 weeks to the end point ($P < 0.05$, $n = 9$) (Fig. 6d).

Discussion

The results of this study show that although dose-dependently inhibited NSPCs proliferation and arrested NSPCs in the G0/G1 phase in proliferative medium, VPA treatment markedly promoted neuronal differentiation (β -tubulin III⁺ cells) as well as decreased astrocytic differentiation (GFAP⁺ cells) under differential condition. Cell cycle regulator p21^{Cip/WAF1} and proneural genes Ngn2 and NeuroD1 were increased in these processes. In a chronic compressive rat SCI model, a delayed administration of VPA enhanced the expression of DCX and NeuN, the newborn neuron marker and mature neuron marker respectively, in the epicenter and adjacent segments of the injured spinal cord. In addition, delayed administration of VPA didn't distinctly increase the nerve fibers of impaired corticospinal track, but facilitated motor function recovery after SCI with BBB scale. Collectively, our data provide the first evidence that administration of VPA enhances the neurogenic potential of NSPCs after SCI.

Several lines of evidence demonstrate that endogenous NSPCs are present in the ependymal layer of the adult rodent [9, 38] and human spinal cord [39, 40], providing the potential for self-replacement of lost neurons after SCI. Indeed, increasing studies have showed that SCI results in the expansion of endogenous NSPCs in the ependymal region [9, 10, 12]. In this study, although it is very difficult to culture adult spinal NSPCs from normal rat (data not show), we successfully cultured considerable adult spinal NSPCs from SCI rat, which may be attributed to the expansion of endogenous NSPCs after injury [12, 41].

VPA was first introduced in 1964 and is currently prescribed worldwide as a well-tolerated, relatively safe and effective anticonvulsant and mood stabilizer [42]. Moreover, as a potent class-selective histone deacetylase (HDAC) inhibitor, VPA displays broad pharmacological effects on cellular biology including cell fate regulation. For example, VPA can arrests cancer cells in the G0/G1 phase, inhibits proliferation, induces the differentiation and

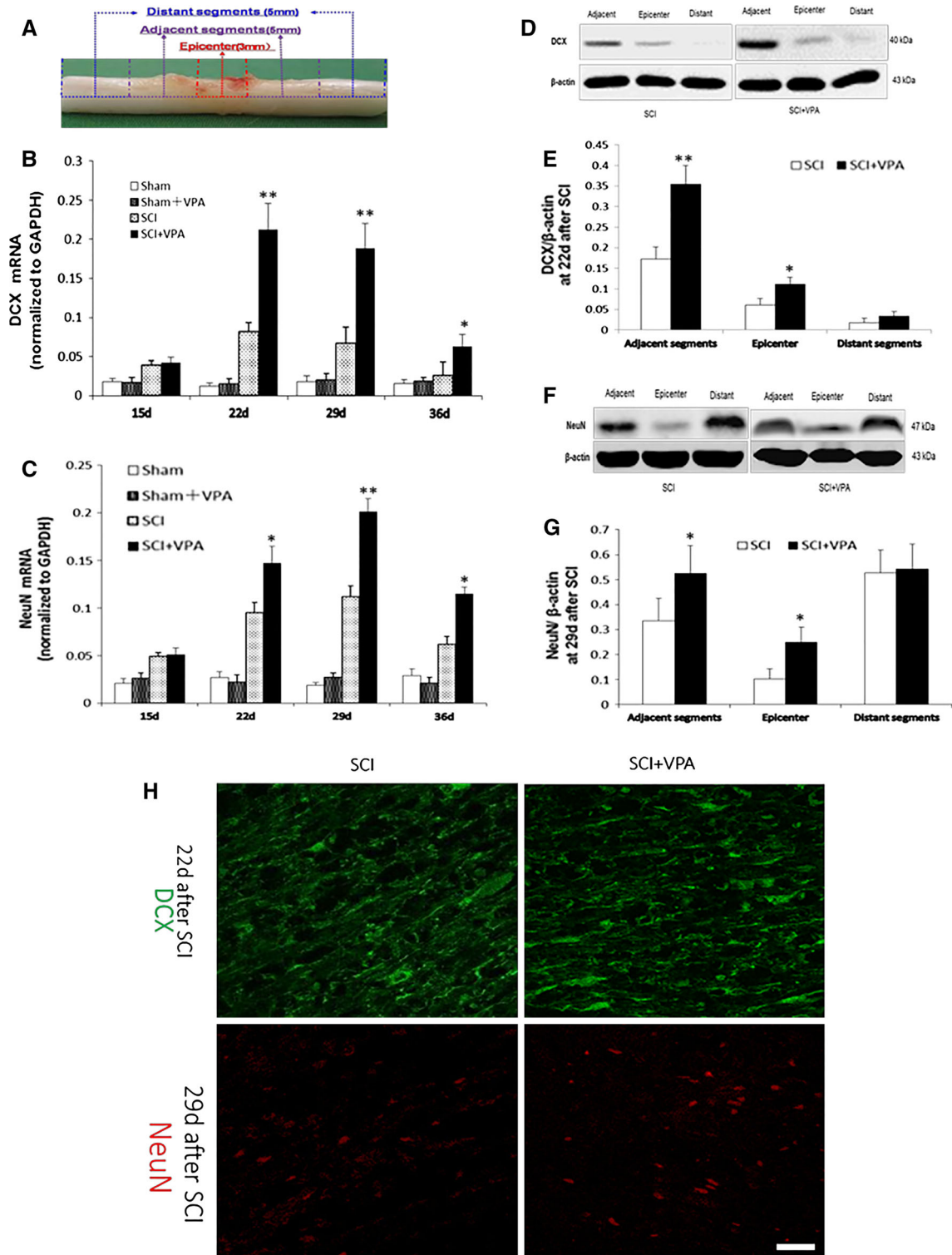
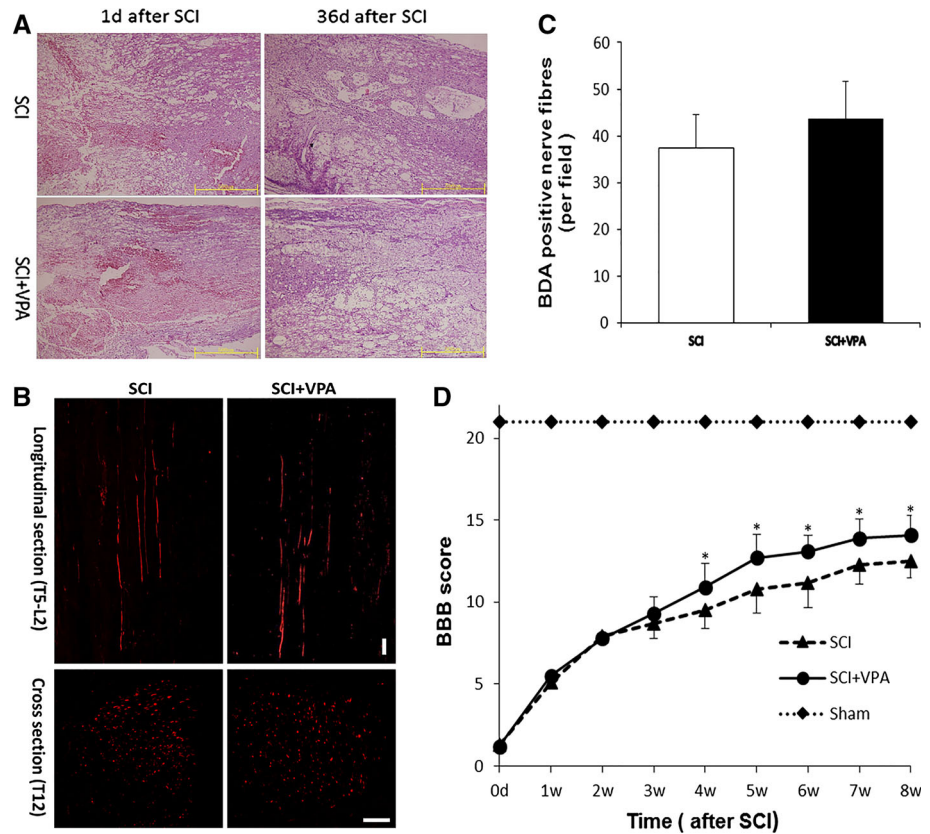


Fig. 5 VPA delayed treatment enhances expression of DCX and NeuN in SCI rat. **a** Schematic of the sampling protocol. The epicenter was the approximately 3 mm region surrounding the injured site. The adjacent segments were the 5 mm regions proximal to epicenter, and the distant segments were the 5 mm regions proximal to the adjacent segments. **b, c** VPA delayed treatment increased the mRNA level of DCX (**b**) and NeuN (**c**). DCX mRNA presented a maximum 2.6-fold increase at 22 days and NeuN mRNA reached a maximum 1.8-fold

increase at 29 days after injury compared with control counterparts (normalized to GAPDH; * $P < 0.05$, ** $P < 0.01$, $n = 5$). **d–g** VPA treatment increased the protein expression level of DCX at 22 days (**d, e**) and NeuN at 29 days (**f, g**) after SCI. The expression levels of DCX (**e**) and NeuN (**g**) were normalized to β -actin (* $P < 0.05$, ** $P < 0.01$, $n = 5$). **h** Immunofluorescence staining of DCX (green) and NeuN (red) in the epicenter at 22 and 29 days after SCI respectively. Bar 25 μ m (Color figure online)

Fig. 6 Administration of VPA facilitated motor function recovery in SCI rats. **a** HE staining of sagittal section showed bleeding spots in the injured epicenter 1 day following SCI. Cyst and glial scar were distinct at the 36 day after SCI, but they were obviously reduced after VAP delayed treatment. *Bar* 500 μ m. **b** Longitudinal (T5–L2) and cross (T12) sections of BDA tracking showed the nerve fibers of impaired corticospinal tract in SCI and SCI + VPA rats. *Bar* 20 μ m. **c** Counting of BDA positive nerve fibers. There was no statistically significant difference between SCI group and SCI + VPA group. **d** The VPA treatment rats exhibited higher BBB scale scores compared with the saline-treated SCI control group beginning at 4 weeks following injury ($*P < 0.05$, $n = 9$)



apoptosis of tumor cells, increases cancer cell immunogenicity and inhibits angiogenesis and metastasis [43]. An increasing body of study demonstrates that VPA facilitates stem cell differentiation, such as hematopoietic stem cells [44], bone marrow-derived MSCs [45] and ESCs [46]. Abematsu et al. [47] reported that VPA treatment promoted the differentiation of transplanted NSCs into neurons rather than glial cells in SCI. Hsieh and colleagues verified in adult hippocampal neural progenitor cells that VPA increases the neuronal differentiation but inhibits astrocyte and oligodendrocyte differentiation, even in conditions that favor lineage-specific differentiation [48]. Our data also show that VPA induces the neuronal differentiation of adult spinal NSPCs and inhibits astrocyte differentiation, but oligodendrocyte differentiation is not changed and even improved in trend. This difference may be due to regional heterogeneity of NSPCs.

The role of epigenetic regulation in stem cell fate decisions is crucial based on increasing evidence [49, 50]. As an HDAC inhibitor, VPA simultaneously regulates the expression of numerous genes by increasing the level of acetylated chromatin [20, 51]. It is believed that VPA-mediated proliferation inhibition and neuronal differentiation of spinal NSPCs also due to the change in genes expression by chromatin acetylation. Screening of whole

genomic is a very huge work, an alternative mechanism of the effect of VPA could be the direct activation of cell cycle regulator and neurogenic transcription factors. Indeed, Our results shows the expressions of cell cycle regulator p21^{Cip/WAF1} and transcription factors Ngn2, NeuroD1 were increased significantly after VPA treatment, implying these factors involved in the two processes. p21^{CIP1/WAF1} is a potent cyclin-dependent kinase inhibitor, binds to and inhibits the activity of cyclin-CDK2, -CDK1, and -CDK4/6 complexes, thus acting as a regulator of cell cycle progression at the G1 and S phases [32]. Both Ngn2 and NeuroD1 are the member of basic helix-loop-helix (bHLH) transcription factors, involved in neuronal differentiation, activates transcription by binding to the E box [52, 53]. Further studies are required to determine whether the up-regulation of p21^{Cip/WAF1}, Ngn2 and NeuroD1 after VPA treatment is merely correlative or is causal. Interestingly, Su et al. [54] recently converted endogenous spinal astrocytes to proliferative DCX-positive neuroblasts by a single transcription factor SOX2 in SCI and VPA can further enhanced the neuronal survival and maturation. In our study, an increased SOX2 was also observed in NSPCs under differentiation condition when treated with VPA although no statistically significant. It implies that the neuron-promoting effect of VPA maybe not only from the

regulation of NSPCs fate but also from the reprogramming of astrocytes.

Expansion of endogenous NSPCs after SCI is a definitely positive signal that reveals the potential of endogenous replace of lost neurons by mobilized NSPCs. But the progeny of these activated NSPCs is primarily restricted to astrocytes that contribute to glial scar and have a negative effect to neurogenesis and remyelination [55, 56]. Optimizing the activity of NSPCs to produce more neurons and fewer astrocytes would be a promising therapeutic strategy for SCI. Our previous work and many other studies show that damage-induced endogenous neural stem cell proliferation usually reach the peak at 2 week after injury [10, 14, 57]. Therefore, we treated with VPA in SCI rats at the 15th day after injury in order to utilize its neuron-promoting effect, meanwhile to minimize the possible adverse effect to the NSPCs proliferation. On the other hand, in consideration of the neuroprotections of VPA in acute phase of SCI, such as attenuates blood–spinal cord barrier disruption [35], inhibits apoptosis and autophagy [33, 58] and reduces of oxidative stress and endoplasmic reticulum (ER) stress [21], a delayed administration of VPA will avoid most of these actions and focus on its neuron-promoting effect. Our results show that a delayed intraperitoneal injection of VPA to SCI rats from day 15 to day 22 after injury remarkably increased the expression both of the newborn neuron marker DCX and the mature neuron marker NeuN. Although the contribution of neurogenesis to recovery from neural injury is currently uncertain, the formation of new neurons in the periphery of the injury site may represent an endogenous self-repair mechanism that attenuates neuronal loss and the functional impairments [59–61]. Thereby, the increased neuronal markers reveal the therapeutic value of delayed treatment of VPA to SCI. Actually, we observed a better motor function recovery in the VPA-delayed-administration SCI rats, although their impaired nerve bundles without a better reservation.

In conclusion, our study shows that VPA treatment arrests proliferation and promotes neuronal differentiation of adult spinal NSPCs from SCI rats; A delayed administration of VPA enhances neuronal markers expression and facilitates motor function recovery in a chronic compressive SCI model. These results suggest that VPA provides supplementary pharmacological stimulation by optimizing the activity of NSPCs after SCI and promoting neuronal regeneration following SCI, thereby providing a potential alternative or complementary approach to cell transplantation.

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