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Original Article

Valproic acid promotes neuronal differentiation and GABAergic interneuron formation in primary hippocampal stem cells

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Abstract: Background: Neural stem cells (NSCs) are currently under investigation as a candidate treatment for neuronal damage and disrupted neuronal connections. Valproic acid (VPA), which is a widely prescribed drug for epilepsy in the clinic, has differential effects on growth, proliferation and differentiation in many types of cells. Whether VPA can induce the differentiation of NSCs into neurons and its possible molecular mechanism, however, remain largely unknown. Methods: The effects of VPA on the neuronal differentiation of NSCs were detected *in vitro*. The neuronal differentiation of NSCs from the embryonic hippocampus was examined after treatment with 0.75 mM VPA for three and seven days by immunochemical analysis. Western blot was employed to examine the levels of brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF) and neurotrophin-3 (NT-3). Results: The results indicated that there were more Dcx+ and GABA+ cells and fewer GFAP+ and O4+ cells in the VPA-treated group than in the control group. The expression of BDNF, GDNF and NT-3 in the VPA-treated group was significantly greater than that of the control group, suggesting that VPA enhances the fate determination of NSCs to produce more neurons and fewer gliocytes. Conclusion: Collectively, our findings indicated that VPA was able to induce the neuronal differentiation of NSCs and enhance the maturation of NSCs into GABAergic interneurons.

Keywords: Valproic acid, neural stem cells, neuronal differentiation, GABAergic interneurons, neurotrophic factors

Introduction

Neural stem cells (NSCs) are considered to be a feasible candidate for the treatment of neuropsychiatric disorders and rebuilding the disrupted neuronal circuitry because of their self-renewal and differentiation abilities in the mammalian central nervous system (CNS) [1]. Understanding the mechanism of the differentiation of NSCs in the CNS is of great importance in the field of regenerative medicine [2]. Replenishment of GABAergic interneurons through a transplantation strategy has proven to be a feasible and effective method to help reverse the symptoms in an epilepsy model [3]. However, NSCs exhibit a strong bias towards astrocytic differentiation due to the stimulation of inflammatory cytokines and other inhibitory growth components in some pathological states, which may substantially reduce and even impair functional restoration [4]. Therefore, further interventions are required to modulate this differentiation bias by improving neuronal differentiation and suppressing astrocytic differentiation, thereby reducing the side effects of NSC therapy.

The hippocampus plays a major role in memory formation and consolidation processes [5-7]. It is conceivable that specific hippocampal dysfunctions may be a possible causal link between seizures and memory impairment. The hippocampal subgranular zone contains cells that are capable of continuous division and differentiation throughout adulthood and can be enhanced by a number of extrinsic factors such as exercise, enriched environment, and learning [8]. As was reported by Scott et al, status epi-

lepticus (SE) can cause a marked increase in neurogenesis in adults. In addition, seizure-induced neurogenesis contributes to the deficits in hippocampal learning and memory that are associated with SE [9].

Valproic acid (VPA) has become the most prescribed antiepileptic drug, showing favorable antiepileptic effects and widely used in clinical practice. It has a broad antiepileptic spectrum with a simple structure of a short, branched fatty acid but with various mechanisms of action [10]. VPA has both neurotrophic and neuroprotective properties and can ameliorate impairments in neural plasticity of an animal model of epilepsy [11]. VPA may enhance GABAergic neurotransmission to modulate brain metabolism and decrease excitability by affecting intracellular signaling pathways [6]. Adequate evidence suggested that GABAergic interneurons play an important role in the process of seizures by regulating the excitatory/inhibitory balance in the hippocampus [12]. Some researchers are focused on other underlying mechanisms such as neurotrophic factors and cell neogenesis. Brain-derived neurotrophic factor (BDNF) and NT-3 promote neuronal differentiation and stimulate neurite out-growth in the development of the CNS [13, 14]. Cell neogenesis in the dentate gyrus of the hippocampus also plays crucial roles in epileptogenesis [13]. However, the effects of VPA on BDNF, neurotrophin-3 (NT-3) and cell neogenesis are not fully understood, and studies on these aspects are limited. Moreover, it is important to confirm whether there are possible mechanisms that account for the neuroprotective effect of VPA. In this study, we showed that VPA induced neural stem cells (NSCs) to differentiate predominantly into neurons, at least in part, by activating BDNF, GDNF and NT-3, coupled with promotion of GABAergic interneuron formation.

Materials and methods

Animals

Sprague-Dawley (SD) rats were obtained from the Shanghai Laboratory Animal Center. All animals were housed in the SPF animal room under controlled temperature (21°C ± 1°C), humidity (55%) and light conditions (12-h light/dark cycle), with ad libitum access to food and water. All experimental procedures used were in strict accordance with the animal care guidelines of the United States National Institute of Health [(NIH) publication no. 23-85, revised 1996]. All surgery was performed under sodium pentobarbital anesthesia, and every effort was made to minimize suffering.

Primary rat hippocampal stem cell culture and VPA treatment

Neural stem cells were isolated from the hippocampus of 12~14-day embryonic SD rats as previously described [15]. The tissue samples were then gently triturated and repeatedly pipetted in a neural basal medium containing 0.25% trypsin and deoxyribonuclease I (DNase I, 0.15 mg/ml). Then, the samples were dispersed for 20 min at 37°C (5% CO_2) and mechanically dissociated into a single-cell suspension. After centrifugation, the dissociated cells were resuspended at a density of 2×10^5 cells/ml in growth medium consisting of neural basal medium and 2% B27 supplemented with 20 ng/ml human recombinant basic fibroblast growth factor (bFGF, Gibico Invitrogen, USA), 20 ng/ml epidermal growth factor (EGF, Gibico Invitrogen, USA), 1 mM L-glutamine and 1% penicillin/streptomycin (Gibico Invitrogen, USA). The cells were incubated at 37°C in a humidified incubator containing 5% CO_2, and half of the medium was replaced every two days. The cell passage protocol was performed every 5-6 days to obtain neurospheres originating from a single primary cell. Secondary or tertiary neurospheres were used for subsequent experiments. The antibody against Nestin (1:500; Sigma-Aldrich, USA), a specific marker for NSCs, was applied to identify the prepared neurospheres before the differentiation assay.

The neurospheres from the 3rd passage were collected, dissociated, resuspended, and plated on coverslips coated with poly-L-lysine (Sigma) at a density of 1×10^5 cells/ml and then cultured with NB medium with 2% B27 supplement, fetal bovine serum (FBS, 1%), and VPA at a concentration of 0.75 mM [16]. The culture condition of the control group was the same as that of the treated group, except for the absence of VPA. The differentiation medium was half-exchanged every two days.

Dendritic number counting

After 6 days, the 3rd passage of neurospheres was observed as described in a previous study

[17]. Images were randomly taken in 3 different locations in each of the plates using an optical microscope (Olympus, Tokyo, Japan). The number of dendrites was counted in the images and divided by the number of cell bodies.

Immunocytochemistry

The immunocytochemistry (ICC) was carried out as described in a previous study [4]. Neurospheres from the 3rd passage were seeded on a cover-glass bottom dish with differentiation media in the presence or absence of VPA. After treatment, the cells were fixed in 4% paraformaldehyde in 0.2 M phosphate-buffered saline (PBS; pH 7.4) for 30 min at room temperature and rinsed with PBS. The cells were permeabilized for 10 min with PBS containing 0.25% Triton X-100 (PBST) and blocked for 1 h with 1% bull serum albumin (BSA) in PBST. Subsequently, primary antibodies (anti-DCX (1:250, ABIOB, Germany), anti-GABA (1:100, ABIOB, Germany), anti-GFAP (1:100, ABIOB, Germany), and anti-O4 (1:250, ABIOB, Germany)) were dissolved in blocking solution and added to the cells overnight at 4°C. The primary antibody solution was removed, and the cells were washed three times with PBST for 5 min. Cells were then incubated in fluorescein isothiocyanate (FITC)-conjugated goat anti rabbit secondary antibody (1:200, ABIOB, Germany) or TRITC-conjugated goat anti mouse secondary antibody (1:200, ABIOB, Germany) for 1 h. The nuclei were counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (1 μg/ml, Sigma, USA) for 5 min. Fluorescence signals were examined by a laser scanning confocal imaging microscope (Nikon C1Si; Nikon, Tokyo, Japan).

qRT-PCR analysis

The procedure was used as described by previous studies [18, 19]. Total RNAs were extracted from NSCs using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The two-step SYBR ExScript™ RT-PCR kit (Perfect Real Time, TaKaRa, Japan) for real-time RT-PCR was used to analyze the expression of DCX and GFAP. GAPDH served as the internal control. Quantitative RT-PCR was initiated using an activation step at 95°C for 15 min, followed by 40 amplification cycles of denaturation at 95°C for 10 s, annealing at 55°C for 30 s and extension at 72°C for 30 s. Quantification was achieved using standard curves derived from gene expression relative to the level of GAPDH gene expression. GenBank cDNA sequences or conserved regulatory sequences identified in the ECR browser were used to design gene-specific primers in the Universal Probe Library Assay Design Center (Roche Applied Science). The specificity of PCR primers was determined by BLAST run of the primer sequences. All primers were purchased from MWG Biotech. Primer sequences can be provided upon request.

Western blot analysis

The cells were lysed in RIPA buffer (Santa Cruz, Santa Cruz, USA) containing protease inhibitor cocktail (Sigma-Aldrich, USA), and total protein was harvested. Equal amounts of total protein (20 μg/lane), determined using the BCA Protein Assay Kit (Pierce, Rockford, USA), were separated using 8% SDS-polyacrylamide gels and electrophoretically transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, USA) via wet transfer. After blocking in PBS containing 0.05% Tween-20 (PBS-T) and 5% skim milk powder for at least 1 h to reduce non-specific antibody binding, the membranes were then incubated overnight at 4°C in primary antibodies targeting BDNF (1:5000, ABIOB, Germany), GDNF (1:3000, ABIOB, Germany), GFAP (1:3000, ABIOB, Germany), NT-3 (1:5000, ABIOB, Germany) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:3000, ZSGB-BIO, China). Then, the membranes were washed, followed by incubation with the corresponding horseradish peroxidase-conjugated secondary antibodies (1:5000; ZSGB-BIO) for 1 h at room temperature. The immunoblots were visualized with ECF in a VersaDoc 3000 imaging system (BioRad, Amadora, Portugal), following incubation of the membrane with the ECF reagent for 5 min. The protein levels were quantified via densitometry and normalized to GAPDH expression using Quantity One software (Bio-Rad, USA). Three independent experiments (n=3) were performed in triplicate.

Statistical analysis

The data obtained were expressed as the means ± standard deviation of at least three replicates. The software used for statistical analysis was GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). Differences between the groups were analyzed

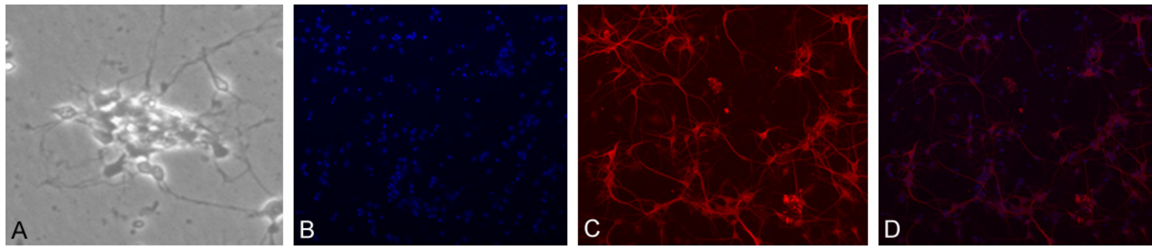

Figure 1. The growth and identification of embryonic NSCs. A. Representative photograph of neurospheres in culture. B. Representative photomicrographs of DAPI-stained nuclei (blue). C. Representative Nestin-positive NSCs dissociated from neurospheres (red). D. The merged image.

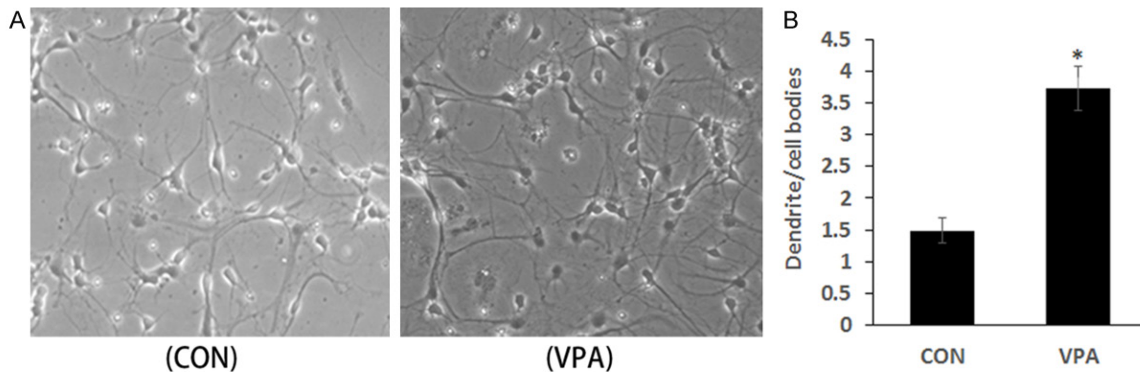

Figure 2. VPA induces dendrite formation. A. Representative photomicrographs of NSCs under optical microscopy. B. Representative quantitative analysis of the number of dendrites. The number of dendrites was counted in the images and divided by the numbers of cell bodies. The bar graph shows the mean dendrite number per NSC (n=3). Differences were statistically significant at $*P < 0.05$.

by two-tailed unpaired t-test or two-way ANOVA analysis. A two-tailed *P*-value less than 0.05 was considered statistically significant.

Results

Culture and seeding of rat NSCs

On the second day after primary culture, in the primary rat hippocampal cells cultured in neurobasal medium with B27 supplement, bFGF and EGF proliferated and formed neurospheres (**Figure 1A**). To validate that the cultured cells were NSCs, the 3rd passage of cells dissociated from the spheres were immunolabeled red for Nestin, a marker of neural precursors, and the nuclei were stained blue with DAPI. The ratio of Nestin-positive cells in the culture, calculated as the number of Nestin-positive cells divided by the total number of cells (stained with DAPI), was greater than 90% (**Figure 1B-D**).

Effects of VPA on dendrite extension of NSCs

To determine whether VPA affects NSC differentiation, NSCs were treated with differentiation media in the absence or presence of VPA for 6 days. The images taken by optical microscopy clearly showed that VPA-treated NSCs had a greater number of extended dendritic spines, a small membranous protrusion from the dendrite, than the control group (**Figure 2A**). Next, we counted the dendrite number per cell body. The VPA-treated NSCs extended 3.73 dendrites per cell (n=5), while the control NSCs had 1.49 dendrites per cell (**Figure 2B**). These data showed that VPA can promote the formation of dendrites and dendritic spines.

Effects of VPA on the neuronal differentiation of NSCs

To analyze the effects of VPA on neuronal differentiation, the 3rd passage of NSCs was cultured in 1% FBS-containing media in the absence or presence of 0.75 mM VPA. NSCs differentiated into at least three lineages: neurons (various subtypes), astrocytes and oligodendrocytes. Double-labeled immunocytochemistry was performed on the 3rd and 7th treatment day with the following markers: Dcx, a marker for neuroblast-like cells; GFAP, an

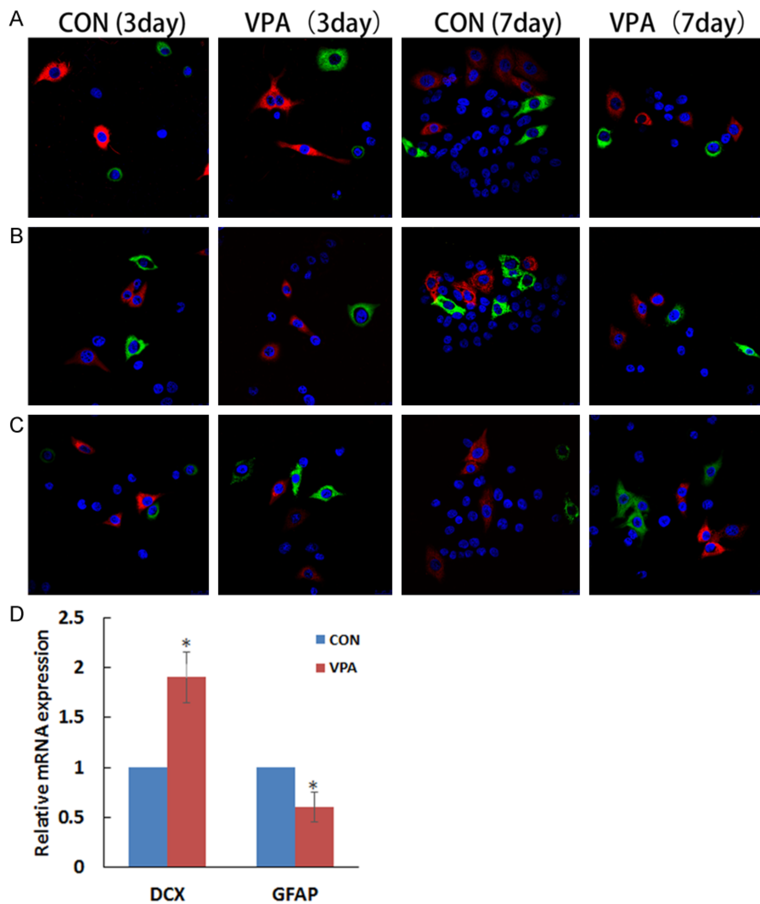

Figure 3. Identification and quantification of differentiated neural cells. A. Representative immunocytochemical staining of differentiated neural cells for DCX (red), GFAP (green) and DAPI (blue). B. Representative immunocytochemical staining of differentiated neural cells for DCX (red), O4 (green) and DAPI (blue). C. Representative immunocytochemical staining of differentiated neural cells for DCX (red), GABA (green) and DAPI (blue). D. RT-PCR analysis of DCX and GFAP expression in differentiated neural cells (n=3). $*P < 0.05$ indicates statistical significance compared with the control group.

astrocyte marker; and O4, a marker for oligodendrocytes. The percentage of differentiated cells was quantified by calculating the number of marker-positive cells relative to the number of DAPI-positive cells. Already 3 days after differentiation, cells positive for markers of both neuroblast-like cells and astrocytes were observed, with rare cells showing positive O4 staining. After 7 days, Dcx-positive cells were more abundant in the VPA group than the control group, with less expression of GFAP and O4 in the VPA-treated group than in the control group. Notably, VPA treatment increased the proportion of Dcx-positive cells to 50%, approximately 1.6-fold higher than the control group, suggesting that VPA additively improved the neuron and astrocyte differentiation of NSCs and suppressed oligodendrocyte differentiation. Moreover, GABA receptors, a marker for GABAergic neurons, could also be detected, indicating that there were more GABA-positive cells in the VPA-treated group than in the control group (**Figure 3A-C**).

To confirm the results of the ICC assays, we subsequently analyzed the mRNA levels of markers of neural and astrocytic precursors by qRT-PCR. After 48 h of treatment, there was a higher level of DCX mRNA in the VPA-treated group than in the control group (**Figure 3D**). In contrast, we also observed significantly lower mRNA levels of GFAP in the treated group than in the control group (**Figure 3D**). In summary, these results suggested that VPA markedly affected the differentiation process and lineage decision of NSCs.

VPA increases expression of neurotrophic factors

Neurotrophins play multiple roles in regulating differentiation, survival and plasticity of several different populations of neurons [19]. To determine if VPA could induce neurotrophic factor expression in the neuronal differentiation of NSCs, BDNF, GDNF and NT-3 were detected by western blot (**Figure 4**). The results indicated significant increases in BDNF, GDNF and NT-3 expression levels in NSCs after treatment with VPA for 7 days.

Discussion

NSCs, with their self-renewal and multiple differentiation ability, are a promising resource in cell therapies for various neurodegenerative diseases and neural tissue injuries [20]. Although the properties of NSCs have been extensively researched over the last decade, the problem for how to effectively promote NSC

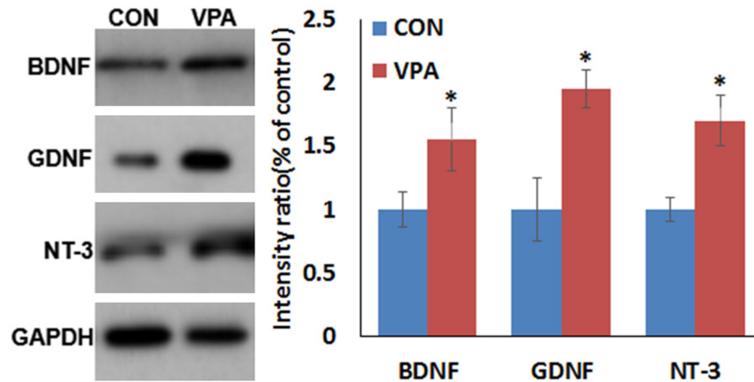

Figure 4. Effect of VPA on the expression of BDNF, GDNF and NT-3. Western blots analyses for BDNF, GDNF, NT-3 and GAPDH from differentiated NSCs. The expression levels of BDNF, GDNF, and NT-3 were normalized against GAPDH levels (n=3). *P < 0.05 indicates statistical significance compared with the control group.

proliferation and induce complete differentiation into neurons, which is critical for an accurate and safe cell therapy, has remained unsolved. In this study, we demonstrate that VPA promotes the fate determination and neurite outgrowth of NSCs, while suppressing gliocyte differentiation. In addition, VPA treatment significantly increased the expression levels of BDNF and other neurotrophic factors, which play essential roles in the development and survival of neurons. Moreover, our results demonstrated that VPA could promote the formation of GABAergic interneurons, which may partially describe the antiepileptic mechanism of VPA.

VPA is an effective and widely used antiepileptic and anticonvulsant drug that has been shown to protect cultured rat hippocampal neurons against amyloid and glutamate neurotoxicity and can protect cultured cerebral cortical neurons and neural progenitor cells from apoptosis [21, 22]. VPA has been shown to have neuroprotective effects in models of Alzheimer's disease (AD) by inhibiting β-amyloid production and neuritic plaque formation via inhibition of glycogen synthase kinase-3β (GSK-3β) signaling [23]. Conversely, some experiments have revealed that high-dose exposure to VPA could also induce the appearance of apoptotic markers in hippocampal cultures [24], indicating that this drug may have complex, dose-dependent effects. A previous study [16] showed that the effects of VPA on NSC differentiation concentration- and time-dependent, indicating that 0.75 mM is a desirable concentration for the differentiation of NSCs. Thus, in the present study, we chose 0.75 mM VPA to treat NSCs. Our results confirmed that VPA caused a significant neuronal induction accompanied by an inhibition of glial cell differentiation, as demonstrated consistently at both the mRNA and protein levels.

Neurotrophic factors are essential for the development and survival of neurons, and disruption of which may be implicated in several neurodegenerative diseases [25, 26]. Previous reports have shown that VPA upregulates the expression of diverse neurotrophic factors in the rat brain [27]. BDNF is a neurotrophin that plays multiple roles in regulating differentiation, survival and plasticity of several different populations of neurons. In our study, the expression levels of BDNF were upregulated by VPA, which may result in improved neuronal differentiation and neurite outgrowth, consistent with previous reports [28, 29]. The expression levels of GDNF and NT-3 in this study were also been detected and significantly elevated in the VPA-treated group compared with that of the control, suggesting that the effects of VPA on the NSCs could be due to the combined actions of elevated BDNF, GDNF and NT4. These effects might be initiated by the VPA-induced release of neurotrophic factors from astrocytes, as previously reported [28].

Previous studies have demonstrated that VPA could enhance the maturation of NSCs into synapse-forming neurons, especially GABAergic interneurons [1, 30]. GABAergic interneurons help modulate the firing patterns of projection neurons through forming inhibitory synapses to maintain the balance of inhibition and excitation in neuronal circuitry [3]. Dysfunction of GABAergic interneurons may be involved in a panel of neurological disorders including epilepsy, schizophrenia and autism [31, 32]. VPA is one of the most commonly used antiepileptic drugs with efficacy for the treatment of both generalized and partial epilepsies in adults and children [33]. Our results showed that VPA

treatment can exert powerful effects resulting in neuronal differentiation to GABAergic neurons as confirmed by ICC. In line with our results, a previous study [30] showed that VPA treatment could stimulate the differentiation of newly generated neurons into a GABAergic phenotype, indicating that VPA might produce an inductive effect on the genesis of GABAergic interneurons from endogenous stem cells or progenitors in the brain.

In conclusion, we have shown a significant neuronal promotion and gliocyte suppression induced by VPA treatment in NSCs from the embryonic hippocampus. Moreover, VPA-induced upregulation of neurotrophic factors, BDNF, GDBF and NT-3, may represent a novel therapeutic/neurogenic strategy to attenuate the dysfunction of GABAergic interneurons, which occur naturally or with the progression of epilepsy. More importantly, the effective promotion of GABAergic interneuron formation and neurotrophic factor upregulation induced by VPA may be partially related to the antiepileptic mechanism of VPA. However, the clear mechanism underlying the roles of BDNF, GDBF and NT-3 in the formation of GABAergic interneurons in primary hippocampal stem cells warrants further investigations.

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Disclosure of conflict of interest

None.

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