Valproic acid enhances neurosphere formation in cultured rat embryonic cortical cells through TGFβ1 signaling

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Abstract
This study aimed to investigate the effect and mechanism of valproic acid (VPA) on the neurosphere formation in rat embryonic cortical cells. We used free-floating neurosphere formation as a model system to evaluate the VPA on the proliferation of neural stem cells (NSCs). We found a time- and dose-dependent increase in neurosphere formation and NSC proliferation after VPA treatment. Further RNA-seq analysis demonstrated that the upregulated TGFβ1 signaling might attribute to the effect of VPA on the neurosphere formation and NSC proliferation. Consistently, the neurosphere formation and NSC proliferation were blocked by the treatment with SB431542, an inhibitor of TGFβ1 receptor. Moreover, in a coculture system, NSCs treated with VPA significantly reduced the oxygen-glucose deprivation-induced neuronal apoptosis. Taken together, our results showed that VPA could enhance neurosphere formation and NSC proliferation by activating TGFβ1, which might be a novel therapeutic strategy for neurological disorders.

Keywords: valproic acid, neural stem cells, neurosphere formation, proliferation, transforming growth factor β1

Introduction
Neural stem cells (NSCs), a class of stem cells with self-renewal ability, have the potential to differentiate into neurons, astrocytes, and oligodendrocytes. The NSCs are proposed to be used in the treatment of Huntington’s disease, stroke, Alzheimer’s disease, and other psychiatric and neurodegenerative diseases[1]. Though widely studied, there is still much work to do before the applications of NSCs in clinical practice. A stable and reliable source of NSCs has always been a challenge. Since differentiation and self-renewal can both be evaluated at the single cell level theoretically, the sphere forming assays are extensively used in stem cell biology. Thus, besides the quantitative readout of the stem cell number in vivo, the self-renewal and differentiation and functional properties of the stem cells can all be exhibited by the neurosphere assay[2].
It is believed that promoting neurosphere formation of NSCs in vitro is a relatively simple and efficient pathway to obtain more NSCs.

The differentiation and self-renewal of the NSCs have been shown to be closely associated with the epigenetic modulation according to the recent progress in the field[3]. In controlling the fate determination, self-renewal and pluripotency of stem cells, histone deacetylases (HDACs) have been reported to be coordinate with various signaling pathways and cell-intrinsic transcription factors[4]. In embryonic stem cells HDAC1 has been revealed by genetic studies to be a major deacetylase. Proliferation defects have been noticed in the HDAC1-null embryonic stem cells[5]. Moreover, increased NPC proliferation resulted in failed differentiation into mature neurons by deletion of HDAC1 and HDAC2 in the CNS[6]. Besides, both HDAC3 and HDAC5 coordinate with the transcription factor TLX to control NSC self-renewal and proliferation[7].

In the treatment of diverse neurodegenerative diseases, HDAC inhibitors could become novel therapeutic tools in view of the significant role of the HDACs in the control of NSC neurogenesis and proliferation[8]. Studied widely in various tissues and cell types along with the neural progenitor cells (NPCs), valproic acid (VPA) has been known as a HDAC inhibitor. The reduced adherent growth of NPC proliferation can be indicated by VPA[9]. In the promotion of the neuronal growth in cortical cells isolated from E18 embryos cultured in vitro, VPA can perform the same function as neurotrophic factors[10]. In the embryonic hindbrain, earlier studies have indicated a proliferative effect of VPA on NSCs. Contrarily, in the optic tectum of adult zebrafish, VPA decreases cell proliferation[11]. VPA can also inhibit neurosphere formation in the adult subventricular zone[12]. However, the effect of VPA on free-floating neurosphere formation in the embryonic cortical cells cultured in vitro is not fully understood.

In this study, we investigated the effect and mechanism of VPA on the neurosphere formation in cultured rat NSCs. By morphological observation and CCK8 assay, we observed that VPA treatment promoted the neurosphere formation of NSCs. Moreover, the results from RNA-seq and real-time quantitative PCR suggested that the TGFβ1 signaling pathway may be involved in this process. Furthermore, the co-culture of VPA-treated NSCs with neurons could significantly reduce the cell death induced by oxygen-glucose deprivation (OGD), which suggests a potential application of VPA in clinical practice.

Materials and methods

Cell culture

NSC culture

NSCs were isolated from the embryonic cortical tissues of Sprague-Dawley (SD) rats (Nanjing Medical University) at 12.5–14.5 days of pregnancy. Isolated fetal rat brain tissue was immersed in Hanks' balanced salt solution, and the tissue fragments were gently triturated using a 2 mL pasteur pipette to produce a suspension of single cells. The resulting cells were centrifuged for 3 minutes at 500 g; the supernatant was then aspirated and discarded. Then, NSCs were expanded as neurospheres in DMEM/F12 (Gibco, USA), which was supplemented with 2% B27 (Gibco), 20 ng/mL epidermal growth factor (EGF) (Gibco) and 20 ng/mL basic fibroblast growth factor (bFGF) (Gibco). Cell viability was assessed with trypan blue (Sigma, USA), and NSCs were plated in 10 cm Petri dishes at a density of 2×10⁵ cells/mL. NSCs were then incubated at 37 °C in a 5% CO₂ incubator (Thermo, USA). The medium was changed every other day by removing half of the medium and adding fresh growth medium. After 5 to 7 days, the NSCs were passaged by TrypLE Express (Gibco). The NSCs were used to study the neurosphere formation.

Hippocampus neuron culture

Based on the earlier reports the primary culture of rat hippocampal neurons was conducted. The SD rats were supplied by the Animal Centre of Nanjing Medical University. All the procedures adhered to the national standards. Subsequent to trypsinization of 4 mL of 0.25% trypsin (Gibco) for 2 minutes at 37 °C, the hippocampus from the fetal rats on embryonic 18 days was interrupted using a 0.5 mL fetal bovine serum. By centrifugation at 900 g for 10 minutes, the cells were collected. Using the minimum essential medium Neurobasal (Gibco), the pellet was resuspended. On a pretreated with 100 μg/mL Poly-D-Lysine (Sigma) 22 mm × 22 mm glass cover slip the 1×10⁶ cells were plated. Immediately before use, 2% B-27 supplement and the 2 mmol/L glutamine (Sigma) were added to the Neurobasal. With 50 μL culture medium, incubation of the 24 well plates was done with 5% CO₂/95% O₂ incubator at 37 °C. Every 3 days, half of the culture medium was changed. On day 10 all the in vitro experiments were conducted to set up the OGD-injured model.

The animal study protocol was reviewed and approved by the Ethics Committee of Nanjing Medical University.
Drug treatment

VPA (Sigma) was dissolved in water and stored in a stock solution of 1 mol/L or 10⁻³ mol/L at −20 °C. Then it was diluted to the final concentrations (1, 50, 100, 500, 1000, and 10 000 μmol/L) in the standard external solution before treatment.

SB431542 (Selleck, USA), the inhibitor of TGFβ1 receptor kinase activity, was dissolved in DMSO and stored in a stock solution of 10 mmol/L at −20 °C. SB431542 at the final concentrations of 1 μmol/L and 5 μmol/L was used to treat NSCs with or without 100 μmol/L VPA for 48 hours, respectively, before the effect of VPA on NSCs was observed.

Immunocytochemical staining

To identify NSCs, the immunocytochemical staining was performed. Using 4% paraformaldehyde, the NSCs were fixed at room temperature for 1 hour prior to three times of washing with phosphate buffered saline (PBS) for 5 minutes. Subsequently, for 10 minutes, the cells were permeabilized with 0.2% Triton X-100 through incubation. To block the nonspecific binding, the cells were treated at 37 °C for one hour with 5% bovine serum albumin. The cells were incubated with primary antibodies against SOX-2 or Nestin (Millipore, USA; dilution 1:400) overnight at 4 °C. To block the nonspecific binding, the cells were treated at 37 °C for one hour with 5% bovine serum albumin. The cells were incubated with primary antibodies against SOX-2 or Nestin (Millipore, USA; dilution 1:400) overnight at 4 °C, followed by incubation with secondary antibodies, i.e., Cy3-conjugated anti-rat IgG (Proteintech, China; dilution 1:500) against SOX-2 or FITC-conjugated goat anti-mouse IgG (Bioworld, China; dilution 1:500) against Nestin for 1 hour. The cells were then rinsed for 10 minutes and incubated with PBS thrice in the dark. Nuclei were stained with DAPI. An inverted fluorescence microscope was used to identify NSCs.

Cell viability and cell proliferation assay

Following the instructions of the manufacturer, the Cell Counting Kit-8 (Donjindo, Japan) was used to detect the cell viability. NSCs were seeded in 96-well plates at a density of 3×10⁴ cells/well in triplicate and subjected to different treatments. NSCs were treated with VPA at a concentration of 100 μmol/L and at different times (0, 24, 48, and 72 hours). Ten microliters of CCK8 solution were added to each well (containing 100 μL medium), and cells were cultured for an additional 30 minutes at 37 °C. The absorbance of each group at 450 nm was measured using an absorbance microplate reader (Tecan, Switzerland).

The lactate dehydrogenase (LDH) was identified as a stable enzyme expressed in all of the cell types. The LDH is rapidly released into the supernatant cell culture when the plasma membrane was damaged. Following the instructions of the manufacturer, the Cytotoxicity LDH Assay Kit-WST (Donjindo) was used to detect LDH release, while the supernatant from the NSCs was collected after treatment with 100 μmol/L VPA for 48 hours. After normalizing to the medium-only control, the data were expressed as the mean±SEM from the three independent experiments (3 wells per condition).

To obtain the single NSCs, the neurospheres were dissociated chemically using Accutase (Gibco). Using the appropriate concentration of VPA, the cells were incubated for 48 hours and dispensed into laminin and poly-L-ornithine coated 24-well chamber slides in triplicate for every treatment. The NSCs were fixed and treated by PCNA antibody (Proteintech; dilution 1:400) overnight at 4 °C.

The NSCs were treated with 20 μmol/L BrdU (Sigma, USA) for 2 hours, fixed and acid-treated, followed by rat anti-BrdU antibody (Abcam, USA; dilution 1:400) incubation overnight at 4 °C. Quantitative studies were based on three replicates.

Primary neurosphere and secondary neurosphere assay

The NSCs were cultured in a 60-mm dish at a density of 2×10⁵ cells/mL, and 100 μmol/L VPA was applied for 48 hours. Then, the diameter of the neurospheres was measured, and the number of neurospheres was counted to evaluate the proliferation activity of cells on day 2. The neurospheres in 3 random fields of view throughout the entire flask were recorded and photographed using an inverted light microscope (Nikon).

To generate secondary neurospheres, on the 7th day after initiating the primary culture, the primary neurospheres including control and VPA treatment group were dissociated with Accutase at 37 °C for 3 minutes. After centrifugation, cells were diluted to 2×10⁵ cells/mL using neurosphere media and plated onto 35 mm plates. The secondary neurospheres were counted with VPA for 48 hours. The numbers of neurospheres and their diameters were determined by Image-Pro Plus 6.0. (Media Cybernetics, USA). The total number of neurospheres and their diameters (as a percentage change relative to the control values) were compared among groups.

NSCs cocultured with VPA-treated OGD-injured cell groups

On the 10th day, the primary cultured hippocampus neurons were exposed to hypoxia in glucose-free PBS buffer. To establish the OGD injury model, the
cultured neurons were placed in an oxygen deprivation box and incubated with 95% N\textsubscript{2} and 5% CO\textsubscript{2} at 37 °C for half an hour. The non-OGD control neurons were kept under normal culture conditions for half an hour after being transferred to PBS with glucose. For the glucose and oxygen treatments, the cells were transferred for 48 hours with 5% CO\textsubscript{2} at 37 °C to the Neurobasal/B-27 cultured medium subsequent to OGD treatment.

The coculture system was set up in the 24-well plates hanging insert 0.4 μm PET (Millipore). In the lower chamber of the trans-well plates, the primary cultured hippocampus neurons were plated. In the upper insert chambers, the NSCs, VPA, and the NSCs cocultured with 100 μmol/L VPA were placed after the OGD or the control treatment. The cells were cocultured for 48 hours and then used in subsequent experiments.

Semi-quantitative reverse transcriptase PCR and real-time quantitative PCR

According to the instructions of the manufacturer, the total RNA was extracted from expanded neurospheres with Trizol (Life Technologies, USA) and later reverse transcribed with HiScript Q Select RT SuperMix (Vazyme, China). Following the instructions of the manufacturer, by using the Taq Polymerase (Vazyme), the semi-quantitative reverse transcriptase PCR (RT-PCR) analyses were conducted. The program was as follows: 35 cycles of denaturation for 30 seconds at 95 °C, annealing for 30 seconds at 55 °C, and elongation for 1 minute at 72 °C. Using the 1% agarose gel electrophoresis, the amplified PCR products were analyzed. On a 1% gel at 100 V, the products were electrophoresed. Primers used in this study were summed in Table 1.

Using a LightCycler 96 system (Roche, Switzerland), the measurement of the Tgf\textsubscript{b}1 mRNA levels was done using the real-time quantitative PCR. By measuring the binding of the fluorescence dye SYBR Green (Vazyme) to the double-stranded DNA, the amplification was monitored and analyzed. The primers used in this study were summed in Table 1. Gapdh was used as an internal control to normalize each primer group.

RNA-Seq and differential gene expression analysis

Total RNA was extracted from normal and paired VPA treated (100 μmol/L for 48 hours) NSCs. RNA sequencing was performed at Nanjing Vazyme BioTech Co., Ltd. Transcript abundance was quantified as fragments per kilo base of exon per million fragments mapped. Differential expression analysis was performed using paired t-tests, and genes with a nominal P-value <0.05 were determined to be differentially expressed. Enriched Gene Ontology (GO) terms were identified with ToppGene (https://toppgene.cchmc.org/).

Statistical analysis

The results are presented as the mean±SEM. All the data were statistically analysed using GraphPad Prism software. The normally distributed data were analyzed using the parametric unpaired two tailed t-test for between-group comparisons, and the nonparametric unpaired two-tailed Mann-Whitney test for between-group comparisons were used to analyze the data with non-normal distribution. For multiple comparisons amongst more than two groups, one-way ANOVA or two-way ANOVA followed by Bonferroni’s test were used to analyze the statistical differences.

Results

Bio-characteristics of NSCs and effect of VPA on NSCs

In the conventional NSC medium, the rat NSCs were isolated from the cerebral cortex of E12.5–14.5 embryos and cultured. On the 7th day after initiating the primary culture, the cells cultured in bFGF and

| Gene | Forward primer (5’-3’) | Reverse primer (5’-3’) |
|------|------------------------|-----------------------|
| Nes  | GGTAGGGCTAGGACCCCAA    | TGGGCAAATCAAGGATCCCC  |
| Sox2 | AACCAGTAGCACCCTAGCA    | TGCTGAGGAGACATGCCTG   |
| Tgf\textsubscript{b}1 | CACCATCCATGACATGAACC  | TCATGTTGAGCACACTGCTCC |
| Vit  | TGTAATGCTGCTGACATCAC   | TCCCATAAACGCTAATGCTTCC |
| Thbs1 | GCCAGATGACAGATCTCAGA  | CTGACACAGCTGCTGTCCT   |
| Cola1 | TGGTTTCCCTGGTGTCTGATG  | CTGCCATGAGACCCTTGG    |
| Gapdh | ATCAAGAAGGTGGTGAAGCA  | AAGGTG GAAGAATGGGAGTTG |

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EGF supplements had proliferated and formed neurospheres, exhibiting a regular round shape (Fig. 1A). The levels of cellular marker proteins and genes of the NSCs were observed to determine the properties of the NSCs under the present culture conditions. Co-immunostaining showed for Nestin and SOX-2 were highly expressed in our cultured NSCs (Fig. 1A). Strong expressions of Sox2 and Nes (the gene encoding Nestin) in the NSCs were showed in the semi-quantitative RT-PCR analysis (Fig. 1B). The gene expression patterns of the NSCs were found to be indistinguishable from those of the cultured NSCs.

To figure out whether VPA regulated the neurosphere viability of NSCs in vitro, the cultured NSCs were subjected to VPA exposure. The concentration effect curve of VPA was firstly explored by CCK8 assay. The concentration effect curve of VPA was firstly explored by CCK8 assay. As shown in Fig. 1C and D, the VPA treatment for 48 hours enhanced neurosphere viability in a concentration-dependent manner and 50% effective concentration (EC50) occurred between 10 and 100 μmol/L. Hence, the 100 μmol/L VPA treatment for 48 hours was selected for the subsequent experiments. Based on the LDH release, the cytotoxicity of the VPA was also assessed. The treatment of 100 μmol/L VPA for 48 hours did not increase LDH release compared with the control group (Fig. 1E).

**Effects of VPA on NSC viability and neurosphere formation**

The ERK pathway-dependent cortical neurogenesis and neuronal growth have been reported to be promoted by valproate in a time- and concentration-dependent manner[10]. After 100 μmol/L VPA treatment for 24, 48, and 72 hours, the images of neurospheres formation were captured (Fig. 2A). The results of CCK8 assay demonstrated that the viability of NSCs was increased in a time-dependent manner in the control group, while it was further enhanced by the 100 μmol/L VPA treatment for 48 hours or 72 hours (Fig. 2B). Moreover, the treatment with different concentrations of VPA enhanced the viability of NSCs in the same manner except that the cell viability was significantly suppressed by VPA at the concentration of 10 000 μmol/L (Fig. 2B).

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**Fig. 1** Identification of cultured NSCs and the effective concentration of VPA. Identification of NSCs by immunofluorescence and semiquantitative RT-PCR. A: Neurospheres were immunostained with Nestin (green) and SOX-2 (red), and nuclei were counterstained with DAPI (blue). Scale bar: 50 μm. B: Representative PCR products of Nes and Sox2; Gapdh was used as a loading control. C: Representative images of NSCs with the indicated concentrations of VPA (0, 1, 50, 100, 500, and 1000 μmol/L) treatment for 48 hours. Scale bar: 50 μm. D: The EC50 values indicating cell viability in NSCs. EC50 values were calculated using a mathematic function antilog of values provided by sigmoidal dose-response curves (N=3). E: Cytotoxicity assay of NSCs based on LDH release after the NSCs were treated with 100 μmol/L VPA for 48 hours (N=5, n=10). Data are presented as the mean±SEM; statistical analyses were performed using unpaired two-tailed t-test. EC50: 50% effective concentration; LDH: lactate dehydrogenase; N: experimental repetition times; n: total number of experimental samples.
Fig. 2  VPA treatment enhanced NSCs viability and neurosphere formation in culture. A: Representative images of neurospheres treated with 100 μmol/L VPA for 24, 48, and 72 hours. Scale bar: 50 μm. B: The effects of VPA on NSCs viability were evaluated using CCK8 assays (N=4, n=14). NSCs were treated with VPA (0, 50, 100, 500, 1000, and 10 000 μmol/L). Growth trend of NSCs was shown for each treatment at 24, 48, and 72 hours (N=4, n=14). Data are presented as the mean±SEM; statistical analyses were performed using one-way ANOVA; ***P<0.001, **P<0.01, *P<0.05 compared with untreated group. C: Representative images of NSCs with 100 μmol/L VPA treatment for 48 hours by the inverted fluorescence microscope. Scale bar: 50 μm. D: Quantitation of the number of neurospheres per field. The NSCs were treated with 100 μmol/L VPA for 48 hours (N=3, n=53). Data are presented as the mean±SEM; statistical analyses were performed using unpaired two-tailed t-test; ***P<0.001 compared with untreated group. E: The average diameter of neurospheres. The NSCs were treated with 100 μmol/L VPA for 48 hours (N=3, n=26–37). Data are presented as the mean±SEM; statistical analyses were performed using unpaired two-tailed Mann-Whitney test; ***P<0.001 compared with untreated group. N: experimental repetition times; n: total number of experimental samples.
We performed the neurosphere forming assays to identify the effects of VPA in NSCs. Neurospheres were cultured with or without 100 μmol/L VPA for 48 hours. More neurospheres per field were observed in the VPA group compared with the control group (Fig. 2C and D, 60.9±2.2 vs. 49.6±1.2). Moreover, the diameter of the neurospheres in the VPA group was significantly larger than that in the control group (Fig. 2C and E, [75.2±2.6] μm vs. [60.3±1.9] μm). These results indicated that VPA treatment increased the number and size of the formed neurospheres.

Role of VPA in NSC proliferation

Proliferating cell nuclear antigen (PCNA), a molecular marker, was used for proliferation assay. Immunofluorescent staining showed that compared with the control group, the VPA group had significantly increased number of PCNA+ cells (Fig. 3A and B, [37.4±2.5]% vs. [30.0±2.6]%).

Then after the 20 μmol/L BrdU treatment for 2 hours, the NSCs were double-stained against Nestin and BrdU. We observed that VPA administration in NSCs noticeably improved the number of BrdU+ cells compared with the control group (Fig. 3C and D, [77.7±5.1]% vs. [55.4±7.4]%). Collectively, these data indicated that VPA treatment promoted NSC proliferation.

VPA improved the secondary neurosphere formation

To study the effects of VPA treatment on the self-renewal ability of NSCs, a secondary neurosphere assay was developed. The neurospheres were digested by Accutase and cultured again in the complete medium, with a subset forming secondary neurospheres. Exogenous addition of VPA increased the size of secondary neurospheres (Fig. 4A and B, [56.4±1.8] μm vs. [43.2±1.6] μm).

VPA treatment of NSCs reduced the apoptosis of OGD-injured hippocampus neurons

To explore potential the beneficial effects of VPA-treated NSCs on the OGD-injured hippocampus neurons, the coculture system was set up. To assess the beneficial effect of VPA treatment on NSCs to the injured hippocampus neurons and the OGD-injured model, the apoptotic cells were stained by propidium iodide (PI) (Fig. 5A). As shown in Fig. 5B, the PI-
positive cells were increased to (58.5±6.1)% after OGD treatment, and the application of VPA alone exerted no effects on OGD-induced cell death ([51.3±6.4]%). However, the coculture of NSCs with the hippocampus neurons showed protective effects on OGD-induced cell death ([46.1±1.5]%, \(P<0.01\) compared with OGD group). Moreover, the protective effects of NSCs was further improved by the application of VPA ([30.3±3.2]%\(, P<0.01\) compared with OGD+NSCs group). Together, the cocultured data showed that VPA-treated NSCs could further attenuate the damage of neurons induced by OGD.

**Transcriptomic changes of NSCs in response to VPA and verification of candidate genes**

To systematically investigate the effect of VPA on NSCs, we performed RNA-seq of matched control and VPA-treated NSCs. We identified 454 significantly differentially expressed genes: 180 were increased in VPA-treated groups, and 274 were decreased. The differentially expressed genes that were upregulated by VPA (e.g., Tgfb1 and Vit) were enriched in GO terms such as cell proliferation and growth, as illustrated in the expression heatmap (Fig. 6A), suggesting that VPA treatment is responsible for facilitating the proliferation and the development of NSCs. Among the identified candidate genes, Tgfb1 particularly aroused our interest, as it is expressed in the brain and has been demonstrated to directly influence the production of neural crest cell fates\(^{[13]}\). To evaluate the RNA-Seq results and investigate the expression of Tgfb1 in NSCs with or without VPA treatment, Tgfb1 and the reference genes showing identical expression were selected for RT-PCR analysis. Consistent with the microarray data, our RT-PCR analyses confirmed an increased expression of Tgfb1 mRNA, and the mRNA levels of Vit were also dramatically increased in VPA-treated NSCs compared with the control, while the irrelevant mRNA levels of Thbs1 and Cola1 were not changed before and after VPA treatment (Fig. 6B and C).

**TGFβ1 regulated positive effects of VPA on neurosphere formation and proliferation of NSC**

To verify the contribution of TGFβ1 to the modulation of VPA treatment on the NSCs proliferation, the TGFβ1 receptor kinase activity inhibitor of SB431542 was added to NSCs, then each group was detected by a CCK8 assay. As shown in
Fig. 5 Repairing effects of the cocultured NSCs treated with VPA on OGD-injured neurons. A: DAPI/PI double staining of neurons. The experimental groups included the control, OGD-injured neurons, 100 μmol/L VPA-treated OGD-injured neurons for 48 hours, NSCs-treated OGD-injured neurons for 48 hours, and NSCs coupled with 100 μmol/L VPA-treated OGD-injured neurons for 48 hours. a, d, g, j, and m: DAPI-positive cells in neurons. b, e, h, k, and n: PI-positive cells in neurons. c, f, i, l, and o: DAPI-positive cells and PI-positive cells merged channels. Scale bar: 50 μm. B: Quantitative analyses of PI-positive cells in control, OGD-injured neurons, 100 μmol/L VPA-treated OGD-injured neurons for 48 hours, NSCs-treated OGD-injured neurons for 48 hours, and NSCs coupled with 100 μmol/L VPA-treated OGD-injured neurons for 48 hours. PI-positive cells/DAPI are shown (N=3, n=9). Data are presented as the mean±SEM; statistical analyses were performed using one-way ANOVA; ***P<0.001 compared with the control group; ^^^P<0.001 compared with the OGD-injured group; ##P<0.01 compared with the NSCs-treated OGD-injured group; ~P<0.01 compared with the OGD-injured group; mP<0.01 compared with the NSCs-treated OGD-injured group. N: experimental repetition times; n: total number of experimental samples.
Fig. 7A, the enhanced viability of NSCs by VPA was blocked by the treatment of 1 μmol/L or 5 μmol/L of SB431542. And the diameter of the neuropheres in the VPA+SB431542 group was remarkably smaller than that in the VPA group (<28.3±0.7 μm vs. 35.3±1.2 μm), but it had no difference with the control group ([28.3±0.7] μm vs. control group: [27.7±0.7] μm).

Furthermore, we observed the effect of SB431542 on VPA-treated NSCs by BrdU application for 2
hours. Then, the NSCs were double-stained against Nestin and BrdU. The administration of SB431542 with VPA in NSCs markedly reduced the number of BrdU+ cells compared with the group using VPA alone (Fig. 8A and B, [72.4±5.4]% vs. VPA group: [92.8±2.1]%), but had no difference with the control group ([72.4±5.4]% vs. [77.1±3.0]%). Collectively, these data indicated that the positive effects of VPA on neuropheres formation and proliferation of NSCs might be attributed to the activation of TGFβ1 signaling.

Discussion

VPA concentration and exposure period were significantly corrected with the cell survival rate. A previous study reported that VPA (1 mmol/L) simultaneously induced differentiation and decreased proliferation of embryonic day 14 (E14) rat cerebral cortex NPCs under adherent conditions in culture[9]. VPA (0.2 to 0.5 mmol/L) increases the proliferation of NPCs under adherent conditions in culture from rat E14 brain[14]. Additionally, it has been reported that valproate promotes E18 cortical neuronal growth and neurogenesis in a time- and concentration-dependent manner[10]. A proliferative effect of VPA (1 to 2 mmol/L) on the NSCs in the embryonic hindbrain was observed in the earlier studies. Contrarily, in the adult zebrafish optic tectum, VPA (500 μmol/L) reduced cell proliferation[11]. When separated from its niche in

Fig. 7  SB431542 reduced the VPA mediated increase of the size of secondary neurospheres. A: The indicated concentration of SB431542 (1 μmol/L or 5 μmol/L) was added to the medium, and a CCK8 assay was conducted after 48 hours. Data are presented as the mean±SEM (N=3); statistical analyses were performed using two-way ANOVA; **P<0.01 compared with untreated group. B: Representative images of neurospheres from the control group, VPA (100 μmol/L) treatment group or VPA (100 μmol/L) + SB431542 (1 μmol/L) group. Scale bar: 50 μm. C: Quantitation of the average diameter of secondary neurospheres per field after treatment with VPA (100 μmol/L) or VPA (100 μmol/L) + SB431542 (1 μmol/L) for 48 hours (N=3, n=37–41). Data are presented as the means±SEM; statistical analyses were performed using one-way ANOVA; *P<0.05 compared with the control group; ^^P<0.01 compared with the VPA group. N: experimental repetition times; n: total number of experimental samples.
vivo, the sphere-forming assays evaluate the potential of a cell as a stem cell[2]. The neurosphere formation by adult subventricular cells was inhibition of the VPA (1 mmol/L)[12]. Our research revealed that VPA increased neurosphere formation and NSC proliferation in a time- and dose-dependent. While the cell viability was significantly suppressed by VPA at the concentration of 10 mmol/L, the CCK8 assay also revealed that 100 μmol/L VPA increased neurosphere formation. The dose-responses make proper connection with the emerging principles of hormesis. Hormesis is a biphasic dose-response relationship characterized by low dose stimulation and high dose inhibition[15]. Hormesis can be used as a new approach in the investigation and translational therapy of amyotrophic lateral sclerosis in experimental and clinical research, thus showing a neuroprotective effect[16].

For replacing lost neurons and rescuing injured neurons subsequent to the ischemic cascade, the stem cell transplantation is considered as a potential treatment based on the current research on stem cells[17]. Moreover, cotreatment strategies were developed for neural regeneration by using NSCs coupled with selective drug therapy. According to our results, VPA promoted the proliferation of NSCs and might be beneficial for controlling the damage caused by OGD treatment. Increased differentiation of the transplanted NSCs was found to be induced by the stimulation of the increased growth factor production by the OGD lesion, as indicated by several studies. Both glia and neurons differentiated from the NSCs seem to have the potential to replace injured nerve cells[18]. Nonetheless, to identify the role of VPA in the differentiation of NSCs, further studies are needed.

Preconditioning ischemia is a typical representative of hormesis in which cells are exposed to moderate transient stress to protect themselves from more severe stress. Vitagenes are defined as cell survival stress responsive genes which give play to free radicals' detoxification and rescue the mitochondrial function under oxidative stress in the brain. Sirtuin-1 (Class Ⅲ HDACs), also known as a vitagene, helps to maintain protein homeostasis and cell redox under some pathological conditions[19]. The hormesis and vitagenes can be used to address various neurodegenerative diseases. Sirtuin-1 and related hormetic signaling pathways have been reported to cooperate with transcriptional regulators to promote cell survival[20]. The hormetic-preconditioning patterns and outcomes were supported to address the aging-associated neurodegenerative diseases including the Alzheimer's disease, Parkinson's disease, and Huntington's disease[21]. Based on the above results, preconditioning of VPA on OGD-injured model is valuable for our further research.

RNA-Seq analysis was performed to examine comprehensive changes in gene expression following VPA stimulation in NSCs. Our results revealed that the VPA-dependent genes were highly enriched for cell proliferation and growth, VIT has relatively high homology to Akhirin (AKH). AKH was expressed specifically in the ependymal cells of the central canal spinal cord during development. AKH regulated the

Fig. 8 SB431542 inhibited the VPA mediated proliferation of NSCs. Cultured adherent NSCs were treated with VPA (100 μmol/L) or VPA (100 μmol/L) + SB431542 (1 μmol/L) for 48 hours. A: Representative images from BrdU (red) and Nestin (green) immunofluorescent staining. Scale bar: 50 μm. B: Quantification of the number of proliferating NSCs (BrdU+) (N=3, n=8). Data are presented as the mean±SEM; statistical analyses were performed using one-way ANOVA; *P<0.05 compared with the control group; ^^P<0.01 compared with the VPA group. N: experimental repetition times; n: total number of experimental samples.
VPA regulates neurosphere formation of the neural stem cells by TGFβ1

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