

Original Article

Effects of valproic acid on the differentiation of endogenous neural stem cells of the hippocampus in rats

Xiaopu Cui^{1,5}, Siqi Hong¹, Daochao Huang², Yue Hu¹, Peng Wu¹, Li Jiang^{1,2,3,4}

¹Department of Neurology, Children's Hospital of Chongqing Medical University, Chongqing, China; ²Ministry of Education Key Laboratory of Child Development and Disorders, China; ³Key Laboratory of Pediatrics in Chongqing, China; ⁴Chongqing International Science and Technology Cooperation Center for Child Development and Disorders, China; ⁵Department of Neurology, Children's Hospital of Hebei Province, China

Received November 29, 2016; Accepted January 1, 2017; Epub March 15, 2017; Published March 30, 2017

Abstract: Objective: To observe the effects of valproic acid (VPA) on the differentiation of endogenous neural stem cells of the hippocampus in rats. Methods: Hippocampus primary neural stem cells (NSCs) of newborn rats were prepared according to the methods of trypsin digestion combined with mechanical dissociation. The expressions of Nestin and Brdu proteins were identified by immunofluorescence. The neurospheres were exposed to a differentiation media and added into 250 μ M (Group B), 500 μ M (Group C), 1 mM (Group D) VPA respectively for 7-day induction. The positive rate of neuron specific enzyme (NSE) and glial fibrillary acidic protein (GFAP) positive cells were detected by immunofluorescence, and the amount of NSE and GFAP proteins were detected by Western blot. Results: NSCs were able to express Nestin, and had the ability to differentiate into neurons and astrocytes. Compared with the control group, the number of NSE positive cells in the other 3 groups significantly increased ($P < 0.05$), and the number of GFAP positive cells significantly decreased ($P < 0.05$). With the increase of the concentration of VPA, the number of positive NSE cells gradually increased ($P < 0.05$), and the number of GFAP positive cells decreased gradually ($P < 0.05$). Compared with the control group, the relative expression of NSE protein significantly increased in the other 3 groups ($P < 0.05$), and that of GFAP protein significantly decreased ($P < 0.05$). With the increase of the concentration of VPA, the relative expression of NSE gradually increased ($P < 0.05$), and that of GFAP gradually decreased ($P < 0.05$). Conclusion: NSCs which derived and cultivated from newborn rats' hippocampus, by mechanical digestion, have perfect proliferated effects. VPA can apparently accelerate the differentiation of neural progenitor cells, interfere with the orientation of differentiation, significantly promote neuronal differentiation and inhibit differentiation of astrocytes.

Keywords: Neural stem cells, valproic acid, differentiation

Introduction

Neural stem cells (NSCs) are common in central nervous systems of mammals, which mainly distribute in the hippocampus, striatum, sub-ventricle zone, olfactory bulb, as well as cerebral cortex and spinal cord during development [1, 2]. NSCs are a kind of cell population with the ability of multidirectional differentiation and proliferation. The nerve cells formed by the differentiation from NSCs in the hippocampus have the functions of learning and memorizing. Studies have indicated that many factors such as teratogen, cytokines, growth factors, etc., can lead to the change of differentiation and

proliferation of NSCs and affect the survival, division and cell cycle of NSCs [1-4].

Clinically, Valproic acid (VPA) is commonly used as one of the broad-spectrum anti-epileptic drugs at present, with certain neurotoxicity [5, 6] and if used in the embryonic stage, there exist possibilities of complications such as development delay and cognitive disorder of children. However, some scholars suggest that epileptic pregnant women should be cautious but not be prohibited from using VPA, due to the good effects of VPA and greater adverse effects of other anti-epileptic drugs. But, whether VPA can truly influence the differentiation of NSCs in

the embryonic stage, how strong its neurotoxicity is and its mechanism still have controversies.

By cultivating NSCs of the rats' hippocampus isolated *in vitro* and then inducing its differentiation, this study researched the effects of VPA on the differentiation of NSCs during the process of differentiation, providing the experimental basis *in vitro* for the VPA's promotion of differentiation of nerve cells.

Materials

Laboratory animals

Sprague-Dawley (SD) neonatal rats of 3 d old were provided by the Department of Laboratory Animal Science of Shanghai Jiao Tong University School of Medicine. Animals were put to death in accordance with the ethical and moral standards of animals.

Main equipment and materials

DMEM/F12 culture medium (Gibco, USA), Fetal bovine serum (Sigma, USA), basic fibroblast growth factor (bFGF) (Sigma, USA), B-27 SUPPLEMENT W/O VIT A (Invitrogen, USA), mouse anti-rat BrdU monoclonal antibody (Chemicon, USA) and GFAP monoclonal antibodies (Gibco, USA), rabbit anti rat Nestin antibody (Sigma, USA) and NSE monoclonal antibodies (Gibco, USA), goat anti-rabbit red fluorescent secondary antibody TRITC, goat anti-mouse green fluorescently labeled secondary antibody FITC (Abcam, USA) and 4,6-diamidino-2-phenylindole (DAPI) (Chemicon, USA), Valproic acid (Sigma, USA).

Super Clean bench (Shanghai Instrument & Equipment Factory, China); cell culture incubator (Invergon, USA); Automatic balance micro centrifuge (Shanghai Instrument & Equipment Factory, China); inverted phase microscope (Nikon, Japan); fluorescence microscopy (Olympus).

Methods

Separation and cultivation of neural stem cells

After being sterilized with 75% alcohol, the rats of 3 d old were killed to collect their hippocampi were removed under sterile conditions and cut by ophthalmic scissors in the medium. After the above, the samples were filtered by 200 mesh

strainer and then centrifuged of 3000 r/min for 15 min (centrifugal radius of 6 cm). Then, supernatant was discarded. In the culture medium, re-suspended cells in the medium were cultured under the condition at 37°C and 5% CO₂. Lastly, the medium was changed once every 2 d and passaged once every 5 d.

Identification of neural stem cells

Neurospheres kept in primary culture for 7 d, were inoculated on glass slide with polylysine. After that, keep incubating neurospheres under the condition at 37°C and 5% CO₂ for 2 h. Then the neurospheres were washed twice by PBS and fixed by 4% paraformaldehyde for 20-30 min. Perform rupture of membrane with 0.2% Triton-100 and then block by goat serum. Neurospheres were washed, added into Nestin first antibody (1:100) and BrdU first antibody (1:100) in order, and then incubated at 4°C overnight. On the second day, rewarm for 20 min at room temperature and wash four times by PBS for 5 min. Then the slides were incubated at 37°C for 40 min after the red fluorescent secondary antibody TRITC (1:200) was added. After that, the samples were washed four times by PBS for 5 min again and added into green fluorescently labeled secondary antibody FITC (1:200), then they were incubated at 37°C for 40 min. After that, wash the slides four times by PBS for 5 min for the third time. Eventually, use Olympus inverted phase contrast fluorescence microscope to observe and photograph.

The induced differentiation and drug intervention of neural stem cells

Extract neural stem cells which have passaged three times to prepare single cell suspension after five-minute centrifuge at a speed of 1500 r/min. And inoculate cells into 24-well culture plates with density of 1×10^5 /ml. Each well contained 100 μ l. And divide single cell suspension into 4 groups based on different inducers. There were 4 groups including Group A, which was considered as the control group (neural stem cells medium and 10% fetal bovine serum), Group B (neural stem cells medium and 250 μ M VPA), Group C (neural stem cells medium and 500 μ M VPA); Group D (neural stem cells medium and 1 mM VPA). Culture at 37°C, 5% CO₂ and replace medium every two days. Observe the morphological changes of cells every day and detect the index of differentiation after 7 d and 14 d.

Effects of valproic acid

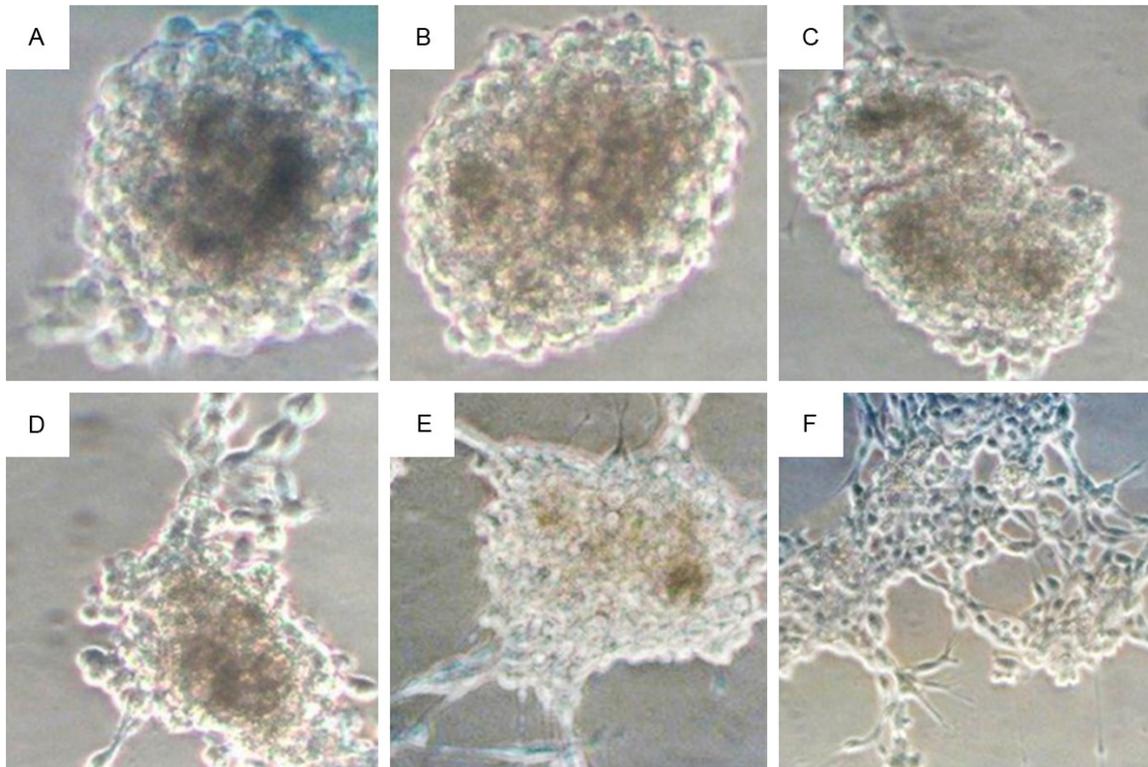


Figure 1. Morphological observation of NSCs (400 ×). A: Primary culture of neurospheres for 5 d; B: Primary culture of neurospheres for 7 d; C: Adherent culture of neurospheres for 24 h; D: Adherent culture of neurospheres for 5 d; E: Adherent culture of neurospheres for 7 d; F: Induced differentiation of neurospheres for 14 d.

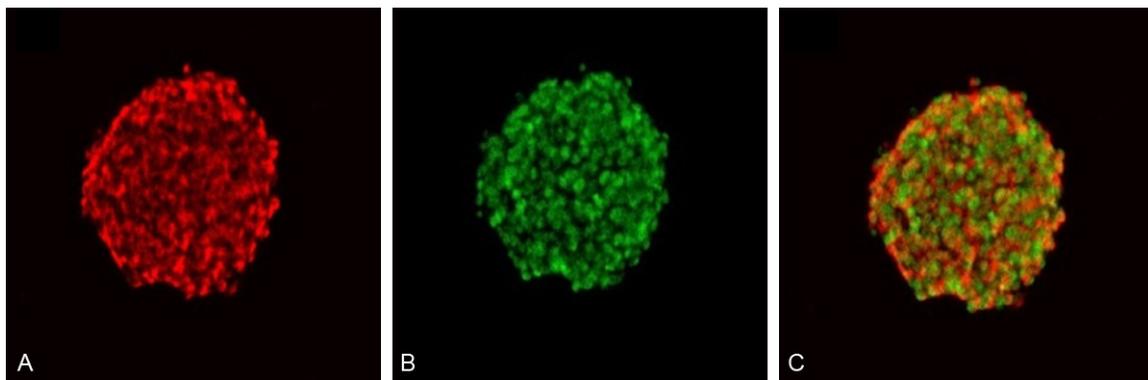
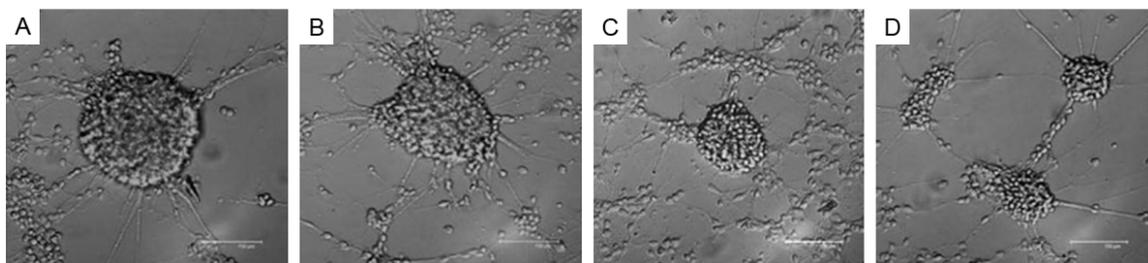


Figure 2. Expression of Nestin and Brdu proteins in the neurospheres (400 ×). A: Nestin protein is shown in red fluorescence; B: Brdu protein is shown in green fluorescence; C: Combined picture A with picture B.



Effects of valproic acid

Figure 3. Effects of different concentrations of VPA on the differentiation of NSCs (200 ×). A: Control group; B: 250 μM Valproate acid; C: 500 μM Valproate acid; D: 1 mM Valproate acid.

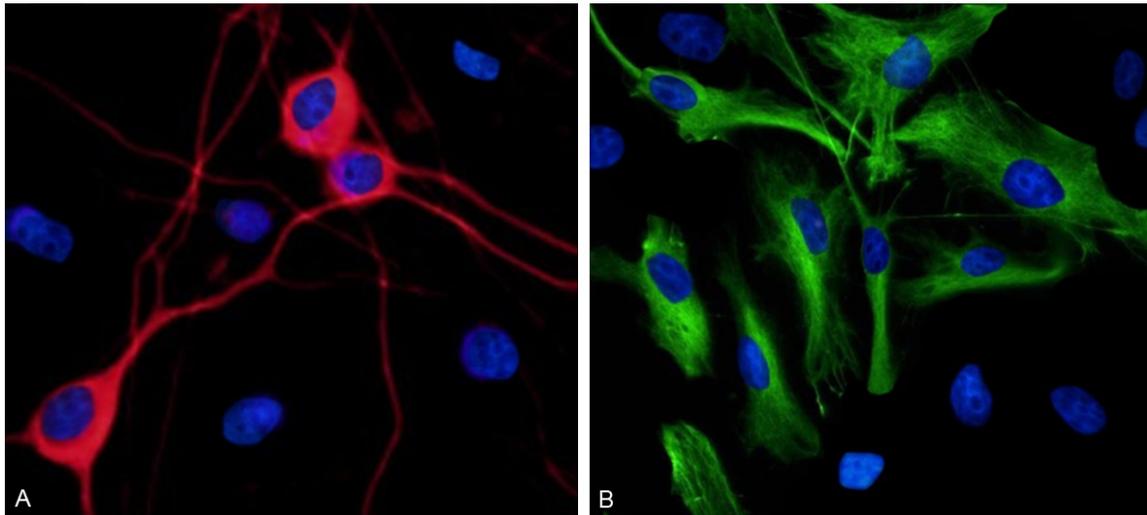


Figure 4. Expressions of NSE and GFAP in NSCs cells (400 ×). A: NSE protein is shown in red fluorescence, and the nucleus is shown in blue fluorescence; B: GFAP protein is shown in green fluorescence, and the nucleus is shown in blue fluorescence.

Detection of expressions of differentiated cells NSE and GFAP by immunofluorescence technique

Differentiated cells which have incubated for 7 d and 14 d, were washed twice by PBS and fixed by 4% paraformaldehyde for 20-30 min. Then perform rupture of membrane by 0.2% Triton-100 and block by goat serum. After washing the cells, add NSE and GFAP first antibody, red fluorescent secondary antibody TRITC (1:200) and green fluorescently labeled secondary antibody FITC (1:200) respectively. And incubate for 1 hour at 37°C and 5% CO₂. After being washed three times by PBS, cells were incubated with 50 μg/ml DAPI for 20 min at room temperature. Wash three times by PBS. Adopt Olympus inverted phase contrast fluorescence microscope to observe and photograph. Count the amount of NSE and GFAP positive cells with blind stereoscopic counting. Choose 4 non-overlapping visual fields randomly at 400-fold magnification to count the amount of positive cells and then take the average.

Analysis of the expressions of NSE and GFAP proteins by western blot

Extract total proteins of cells from 4 groups, 100 μg of which were performed electrophoresis. The transfer-membrane current of NES was

150 mA for 90 min and that of GFP was 200 mA for 110 min. Then block at room temperature for 2 hours with 5% skimmed milk. After that, incubate at 4°C overnight with first antibody NSE (1:150) and GFAP (1:250), respectively. Wash the membranes three times by scrubbing solution and incubate for 2 h on shaker with horseradish peroxidase labeled secondary antibody. Color exposure appeared after washing with scrubbing solution. Use Gene Genius Bio Imaging system to collect image and use BandScan to analyze optical density signal of the bands.

Statistical methods

Use SPSS16.0 statistical software for statistical analysis. All of the data were in normal distribution. Adopt $\bar{x} \pm s$ to express data which consistent with normal distribution. F test was used in 4 groups and χ^2 test was used for comparison of the positive ratio of cells. $\alpha=0.05$ was considered as test level. $P<0.05$ was considered statistically significant.

Results

Culture and identification of NSCs

The border of the neurospheres cultured in serum-free medium was clear with strong refrac-

Effects of valproic acid

Table 1. Positive expression rates of NSE and GFAP in each group were higher than those in the control group ($\bar{x} \pm s$, %)

Group	NSE	GFAP
Group A	36.9±8.4	56.2±11.6
Group B	44.6±7.9*	40.8±10.5*
Group C	59.7±10.2*.#	34.1±9.4*.#
Group D	60.1±10.7*.#	32.8±9.7*.#
F	38.28	40.17
P	<0.05	<0.05

*Compared with the control group, $P < 0.05$; #compared with other dose groups, $P < 0.05$.

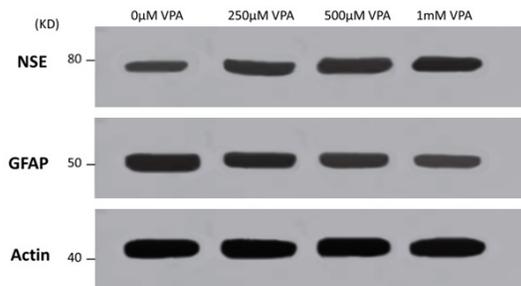


Figure 5. Analysis of the protein expressions of NSE and GFAP by western blot.

Table 2. Relative expressions of NSE and GFAP proteins in each group ($\bar{x} \pm s$, %)

Group	NSE	GFAP
Group A	0.308±0.012	0.409±0.021
Group B	0.359±0.015*	0.385±0.017*
Group C	0.437±0.022*.#	0.351±0.025*.#
Group D	0.446±0.019*.#	0.311±0.018*.#
F	25.09	29.64
P	<0.05	<0.05

*Compared with the control group, $P < 0.05$; #compared with other dose groups, $P < 0.05$.

tion and the expressions of Nestin and Brdu proteins were positive. When the neurospheres were added to fetal bovine serum to induce differentiation, they adhered rapidly and the marginal cells migrated outward. After 5-6 d, the cells were further pleomorphic differentiated. After 14 d of induced differentiation, the cells in the neurospheres gradually moved out and they spread and formed extensive connections with each other. The above results confirmed that the cells we cultured were hippocampus neural stem cells, as shown in **Figures 1 and 2.**

Effects of VPA on the differentiation of NSCs

The differentiation rate of NSCs was significantly increased after adding VPA to the induction medium. After 24 h, the marginal cells of Neurospheres were observed to differentiate into peripheral cells. With the increase of concentration of VPA, the number of cells moving out gradually increased while there was little cell in control group moving out. With the increase of culture time, move-out cells increased and with the increase of VPA concentration, the neuritis of nerve cells were longer, and the number of neuritis branches increased significantly, as shown in **Figure 3.**

The proportions of neurons after the differentiation of NSCs for 7 days

Immunofluorescence staining technique showed that NSE (red fluorescence) was observed in the cytoplasm of NSCs and had elongated neuritis, indicating these were neuronal cells (**Figure 4A**). Some cytoplasm expressed GFAP (green fluorescence) with long and straight neuritis that had less branches, suggesting these were astrocytes (**Figure 4B**). The blue-fluorescent ovals were nuclei (DAPI staining). Compared with the control group, the rates of NSE positive cells in the other 3 groups were significantly increased ($P < 0.05$), while the rates of GFAP positive cells were significantly decreased ($P < 0.05$). With the increase of VPA concentration, the number of NSE positive cells gradually increased ($P < 0.05$), while the number of GFAP positive cells gradually decreased ($P < 0.05$), as shown in **Table 1.**

Expressions levels of GFAP, NSE proteins

Compared with the control group, the relative expression of NSE protein in the other three groups significantly increased ($P < 0.05$), while that of GFAP protein significantly decreased ($P < 0.05$). With the increase of VPA concentration, the relative expression of NSE protein gradually increased ($P < 0.05$), and that of GFAP protein decreased ($P < 0.05$), as shown in **Figure 5; Table 2.**

Discussion

VPA, as a kind of clinical first-line drugs for the treatment of convulsion, epilepsy and unstable

emotions, has been widely used for 40 years. It is a sort of short-chain fatty acids with very low molecular weight and can cross the placental barrier promptly. Recently, it has been proved that VPA can affect fetuses' brain development by interfering with the hyperplasia, differentiation and migration of NSCs [7].

In this study, the tissues in rats' hippocampi were chosen to be isolated and cultured for the reason that isolated and cultured in-vitro NSCs can be induced to differentiate into nerve cells, such as neurons, glial cells, etc. Cells with good abilities of division and proliferation, obtained from the hippocampi of 24-hour-old new-born rats by trypsin chemical digestion combined with mechanical isolation, were observed that they can rapidly proliferate to form some neurospheres in serum free medium. Especially, the brain tissues in hippocampi are accumulation parts of NSCs. Nestin, as a member of intermediate silk protein family, is mainly expressed in cytoplasm, which is also one of the mark proteins in NSCs [8] Nestin won't express until the neural plate is formed, and its expression quantity declines gradually as the neuronal migration, differentiation and mature of neuroepithelium. In addition, 5-Bromodeoxyuridine (BrdU), as a kind of thymidine analogue, can be mixed into the synthesis of DNA of cells just like thymidine. And this BrdU is in the DNA of cell nucleus as long as the cell exists. BrdU tagging technology is routinely employed to keep track of division, survival, differentiation and functional status of cells [9]. Recently, it has been also used in the studies on stem cells [10]. In our study, we found that the cultured cells were proved to be nestin-positive NSCs through nestin immunofluorescence staining technique. What's more, through BrdU immunofluorescence technique, it was discovered that these cells had amplification ability to produce progeny cells. Besides, these cells also can differentiate into neuron and glial nerve cells, which demonstrates that they possess potential of multidirectional differentiation. All the results showed that NSCs were obtained successfully, which sets a solid foundation for the study of toxicity of nerve cells.

NSCs are a group of precursor cells in nervous system. The activities of NSCs, such as proliferation and multidirectional differentiation, are under regulation of surrounding microenvironment (cytokines, neurotransmitter, hormone,

etc.). Through various intracellular signal pathways, these regulatory factors can activate the corresponding transcription factors and promote NSCs to differentiate into different nerve cells in the specific time and space [11, 12]. In this process, the change of microenvironment can interfere with the growth and development of brain nerve cells. VPA may increase the concentration of GABA and affect the differentiation and development of NSCs by inhibiting the degradation of inhibitory neurotransmitter gamma amino butyric acid (GABA). Some studies showed that, after being given GABA enhancer, young rodents could lead to a wide range of neurocyte apoptosis in their brains [13]. In addition, some animal experiments demonstrated that, after the intraperitoneal injection of VPA to rats, the immunohistochemical detection found that the quantities of neurons had increased significantly in the part of the outer surface of the brain while they had decreased apparently in the subventricular zone and the middle cerebral zone, suggesting that VPA can affect fetal rats' proliferation and differentiation of NSCs [14]. Meanwhile, VPA has the pharmacological effects of histone deacetylase inhibitors (HDACIs). Some studies showed that it was necessary to regulate the process of stem cells' proliferation and differentiation as well as HDACIs could promote the differentiation of neural stem cells to neurons [15]. Through the research, Hsieh found that HDACIs could promote the differentiation of pluripotent neural progenitor cells to neurons [16]. Costas' study also confirmed that the inhibition of HDAC activity could promote the transformation of oligodendrocyte to neural stem cells which can be differentiated to astrocytes and neurons [17]. Doughty ML found that VPA could inhibit the formation of neurospheres' clone in neural stem cells by the lithium-sensitive mechanism [18].

GFAP is the marker of astrocytes and NSE is the marker of the early neuron cells [19, 20]. In this study, we applied VPA to NSCs, and observed the expressions of GFAP and NSE in the differentiation of NSCs after 7 d. The result showed that, after using VPA, the ratios of positive cells and the protein expressions of NSE in all three groups were significantly higher than that in the control group, while the ratios of positive cells and the protein expressions of GFAP in all three groups were lower than that in the control group. These indicated that VPA could obvious-

ly expedite the differentiation of NSCs and promoted it to the differentiation of neurons, and inhibited the differentiation of astrocytes. VPA can interfere the differentiation direction of human hippocampus NSCs obviously and at the same time, it may have the same developmental neurotoxicity. Also, effects of VPA on the differentiation process are obviously dose dependent. Effects of 250 μ M VPA are not obvious while 500 μ M and 1 mM VPA can easily interfere the differentiation of NSCs. However, the exact mechanism for how VPA promotes the differentiation of NSCs needs further studies.

In conclusion, NSCs of rat's hippocampi region can differentiate to neurons or neural glial cells, and VPA can promote the differentiation of hippocampi NSCs to neurons. As for how the VPA promotes the proliferation and differentiation of NSCs in hippocampi region, it still needs further study.

Acknowledgements

The authors thank valuable guidance of Prof. Li Jiang and acknowledge financial support from Project 81201507 supported by National Natural Science Foundation of China.

Disclosure of conflict of interest

None.

Address correspondence to: Peng Wu, Department of Neurology, Children's Hospital of Chongqing Medical University, No. 136. Zhongshan Rd. (II), Yuzhong District, Chongqing 400014, China. Tel: +86-187-23471115; E-mail: dr_wupeng@126.com

References

- [1] Kim DY, Rhee I and Paik J. Metabolic circuits in neural stem cells. *Cell Mol Life Sci* 2014; 71: 4221-4241.
- [2] Zhang Z and Chopp M. Neural stem cells and ischemic brain. *J Stroke* 2016; 18: 267-272.
- [3] Rodriguez-Martinez G and Velasco I. Activin and TGF-beta effects on brain development and neural stem cells. *CNS Neurol Disord Drug Targets* 2012; 11: 844-855.
- [4] Radice PD, Mathieu P, Leal MC, Farias MI, Ferrari C, Puntel M, Salibe M, Chernomoretz A and Pitossi FJ. Fibulin-2 is a key mediator of the pro-neurogenic effect of TGF-beta1 on adult neural stem cells. *Mol Cell Neurosci* 2015; 67: 75-83.
- [5] Wang L, Liu Y, Li S, Long ZY and Wu YM. Wnt signaling pathway participates in valproic acid-induced neuronal differentiation of neural stem cells. *Int J Clin Exp Pathol* 2015; 8: 578-585.
- [6] Zhou Q, Dalgard CL, Wynder C and Doughty ML. Valproic acid inhibits neurosphere formation by adult subventricular cells by a lithium-sensitive mechanism. *Neurosci Lett* 2011; 500: 202-206.
- [7] Satar M, Ortakoylu K, Batun I, Yildizdas HY, Ozlu F, Demir H and Topaloglu AK. Withdrawal syndrome and hypomagnesaemia and in a newborn exposed to valproic acid and carbamazepine during pregnancy. *Turk Pediatri Ars* 2016; 51: 114-116.
- [8] Johnson K, Barragan J, Bashiruddin S, Smith CJ, Tyrrell C, Parsons MJ, Doris R, Kucenas S, Downes GB, Velez CM, Schneider C, Sakai C, Pathak N, Anderson K, Stein R, Devoto SH, Mumm JS and Barresi MJ. Gfap-positive radial glial cells are an essential progenitor population for later-born neurons and glia in the zebrafish spinal cord. *Glia* 2016; 64: 1170-1189.
- [9] Dozawa M, Kono H, Sato Y, Ito Y, Tanaka H and Ohshima T. Valproic acid, a histone deacetylase inhibitor, regulates cell proliferation in the adult zebrafish optic tectum. *Dev Dyn* 2014; 243: 1401-1415.
- [10] Zhao Y, Zuo Y, Jiang J, Yan H, Wang X, Huo H and Xiao Y. Neural stem cell transplantation combined with erythropoietin for the treatment of spinal cord injury in rats. *Exp Ther Med* 2016; 12: 2688-2694.
- [11] Maurange C. Temporal specification of neural stem cells: insights from Drosophila neuroblasts. *Curr Top Dev Biol* 2012; 98: 199-228.
- [12] Imayoshi I, Ishidate F and Kageyama R. Real-time imaging of bHLH transcription factors reveals their dynamic control in the multipotency and fate choice of neural stem cells. *Front Cell Neurosci* 2015; 9: 288.
- [13] Giachino C, Barz M, Tchorz JS, Tome M, Gassmann M, Bischofberger J, Bettler B and Taylor V. GABA suppresses neurogenesis in the adult hippocampus through GABAB receptors. *Development* 2014; 141: 83-90.
- [14] Sabers A, Bertelsen FC, Scheel-Kruger J, Nyengaard JR and Moller A. Corrigendum to "Long-term valproic acid exposure increases the number of neocortical neurons in the developing rat brain" [*Neurosci.Lett.* 580 (2014) 12-16] A possible new animal model of autism. *Neurosci Lett* 2015; 588: 203-207.
- [15] Yu IT, Park JY, Kim SH, Lee JS, Kim YS and Son H. Valproic acid promotes neuronal differentiation by induction of proneural factors in association with H4 acetylation. *Neuropharmacology* 2009; 56: 473-480.

Effects of valproic acid

- [16] Hsieh J, Nakashima K, Kuwabara T, Mejia E and Gage FH. Histone deacetylase inhibition-mediated neuronal differentiation of multipotent adult neural progenitor cells. *Proc Natl Acad Sci U S A* 2004; 101: 16659-16664.
- [17] Lyssiotis CA, Walker J, Wu C, Kondo T, Schultz PG and Wu X. Inhibition of histone deacetylase activity induces developmental plasticity in oligodendrocyte precursor cells. *Proc Natl Acad Sci U S A* 2007; 104: 14982-14987.
- [18] Clarke DL, Johansson CB, Wilbertz J, Veress B, Nilsson E, Karlstrom H, Lendahl U and Frisen J. Generalized potential of adult neural stem cells. *Science* 2000; 288: 1660-1663.
- [19] Yoshikawa T, Samata B, Ogura A, Miyamoto S and Takahashi J. Systemic administration of valproic acid and zonisamide promotes differentiation of induced pluripotent stem cell-derived dopaminergic neurons. *Front Cell Neurosci* 2013; 7: 11.
- [20] Lee IS, Jung K, Kim IS, Lee H, Kim M, Yun S, Hwang K, Shin JE and Park KI. Human neural stem cells alleviate Alzheimer-like pathology in a mouse model. *Mol Neurodegener* 2015; 10: 38.