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Valproic Acid Decreases Cell Proliferation and Color Preference in the Zebrafish Larvae

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Abstract

Valproic acid (VPA) is widely used as an antiepileptic drug or mood stabilizer. In this study, we evaluated the effects of treatment with 2 mM VPA for 3 h on cell proliferation in the telencephalic area of zebrafish larvae using bromodeoxyuridine (BRDU) to label dividing cells. It was demonstrated that 2 mM VPA exposure for 3 h at 2 and 3 days post-fertilization (dpf) larvae decreased cell proliferation in the telencephalic area of 5 dpf larvae. The reduced cell proliferation was not restored at 10 dpf larvae. The quantitative real-time PCR (qRT-PCR) data indicated that mRNA expression levels of WNT signaling pathway-related factors such as β-catenin, LEF1, and gsk3β were altered in the zebrafish larvae treated with 2 mM VPA at 2 and 3 pdf. It was also demonstrated that 2 mM VPA exposure affected color preference of the zebrafish larvae, reducing blue color preference at 5 dpf larvae. The altered color preference was restored at 10 dpf larvae. These results suggest that VPA exposure may cause molecular, cellular, and behavioral alterations in early developmental stage of the zebrafish.

Keywords

BRDU, Valproic acid, Color preference, β-catenin, Zebrafish

1. Introduction

Exposure to valproic acid (VPA) during the prenatal and postnatal period is known to have some deleterious effects on the brain development and behavior. Recently, it has been reported that VPA exposure to pregnant mice alters BNDF mRNA and protein levels in the fetal mouse brain, and may cause abnormal brain development [1]. Various behavioral deficit such as decreased raising and hole-poking along with increased locomotor activity in novel environments and increased anxiety were also found postnatally in rats prenatally exposed to VPA

[2] [3] [4].

Accumulating studies have shown that extensive reduction of neurogenesis in the developing brain may be involved in a variety of neurological functions in adult mice and humans [5] [6]. For example, severe cell proliferation defects are present in the developing neocortex, dentate gyrus, and cerebellum of mouse of Down Syndrome (DS) models [7], and the hippocampal and cerebellar regions of human fetuses with DS [8]. In the brain of zebrafish larvae cell proliferation and neurogenesis actively occur in diverse areas and are affected by external factors such as treatment with pentylenetetrazol, a GABA receptor blocker [9]. Therefore, the zebrafish is an appropriate model for researching behavioral consequences in adulthood that may be caused by alterations of cell proliferation in the brain during early development stages.

WNT signaling factors such as β-catenin and LEF1 are involved in regulation of cell proliferation in the mammalian brain [10]. A nuclear mediator of wnt signaling, LEF1 accelerates cell proliferation in the developing forebrain of the mouse [11]. These findings indicate the association between alterations in the WNT signaling gene expression and changes in cell proliferation following VPA exposure. In this study, the effects of VPA on the brain development and cell proliferation were studied in the telencephalic area of the zebrafish larvae. Furthermore, modification of color preference was also examined in the VPA-treated larvae.

2. Materials and Methods

2.1. Animals

Adult zebrafish (around 2.5 cm in length) were purchased from a local fish shop (Incheon, South Korea) and maintained at 28.5˚C ± 1.0˚C with a 14-h light/10-h dark cycle in aquariums as described previously [12].

2.2. Immunostaining

VPA (Sigma-Aldrich) was dissolved in distilled water at a concentration of 1 M, and then diluted to final concentrations of 2 mM in containers of aquarium water. Zebrafish larvae (2 and 3 days postfertilization [dpf]) were placed for 3 h in aquarium water containing 2 mM VPA. For the control experiment, age-matched larvae were placed in normal aquarium water during the corresponding period. After terminating the VPA treatment, the larvae were maintained for 12 h in aquarium water containing 10 mM 5-bromo-2'-deoxyuridine (BRDU; Sigma-Aldrich).

All larvae were fixed with 4% PFA in 0.01 M PBS immediately after termination of the BRDU treatment, and cryoprotected overnight in 30% sucrose at 4˚C. The larvae were then sectioned with a cryostat (CM1800; Leica, Wetzlar, Germany) in the coronal plane at a thickness of 10 μm. The sections were mounted onto coated slides (Matsunami Glass Ind., Ltd., Osaka, Japan), and stored at −20˚C until further use.

Double-labeling was conducted to detect colocalization of BRDU and proliferating cell nuclear antigens (PCNA, a cell proliferation marker). Sections were rinsed in 0.1% Triton X-100 in PBS (PBS-T) and blocked with 10% normal goat serum (Jackson Immuno Research, West Grove, PA, USA) in 0.1% PBS-T. After washing, the sections were incubated overnight at 4˚C in a cocktail containing antibodies specific for anti-rat monoclonal BRDU (1:1000; Abcam, Cambridge, UK) and PCNA (1:300; DAKO). The sections were incubated for 1 h at room temperature in a mixture of secondary antibodies including goat anti-rat IgG conjugated to Alexa Flour 594 (1:1000; Molecular Probes) to detect BRDU and goat anti-mouse IgG conjugated to Alexa Flour 488 (1:1000; Molecular Probes) to visualize PCNA. The slides were analyzed and photographed with ZEN Light Edition software and a Zeiss LSM 510 META (Carl Zeiss, Jena, Germany).

For the quantitative analyses, all cells stained for BRDU and PCNA in the pallium and dorsal subpallium of the telencephalic area were counted under 200X magnification using an Olympus IX71 inverted microscope. Data are expressed as the mean ± S.E.M and were analyzed with an ANOVA followed by post hoc Tukey's tests unless otherwise stated. P-values < 0.05 were considered significant.

2.3. Color Preference Tests

For measurements of color preference in the zebrafish larvae, a color maze kit (8.2 cm long × 12.4 cm wide × 2.1 cm high) was subdivided into eight compartments, each compartment with divided into two colors (blue and yellow) by color sleeves. The color preference of the zebrafish larvae was monitored using a digital camera; fish's position (blue or yellow sleeve) was monitored every minute for 1 h. The data were expressed as the means ± S.E.M and subjected to ANOVA, followed by post hoc Tukey's tests.

For the experiment, ten zebrafish larvae (5 and 10 dpf) were placed in a compartment of color maze filled with normal aquarium water (4 ml) on an illumination box.

2.3. Whole-Mount Skeletal Staining

Cartilage of the zebrafish larvae was stained as described previously [13]. The larvae were fixed at 10 dpf with 4% PFA in 0.01 M PBS (pH 7.4) for 4 h at room temperature, washed in 0.1% PBS-T, and stained overnight with 0.1% Alcian blue 8GX (Sigma-Aldrich) in 80% ethanol/20% acetic acid. For clarification, the larvae were treated with 1% KOH/3% H_2O_2 for 6 h, followed by digestion for 15 min with 0.02% trypsin (Sigma-Aldrich) dissolved in PBS. The larvae were then rehydrated in 70%, 50%, and 30% EtOH/0.01 M PBS, cleared in 30% glycerol/0.01 M PBS, and stored in 100% glycerol. Larvae stained with Alcian blue were dissected and flat-mounted. The cartilage was examined with a Leica MZ75 microscope at 50X magnification and images were captured by a Nikon D200 camera (Nikon, Tokyo, Japan).

2.4. Quantitative Real-Time PCR (qRT-PCR)

The heads of 50 larvae were flash-frozen on dry ice at 12 h, 2 d, 3 d, 7 d, and 8 d of recovery in normal water after 2 mM VPA treatment. Zebrafish larvae heads were lysed in TRIzol reagent (Invitrogen), and total RNA was isolated following the manufacturer's protocols. RNA was then treated with DNase I (Promega) to eliminate genomic DNA contamination and further purified using the RNeasy mini kit (Qiagen) following the manufacturer's protocols.

qRT-PCR was performed with a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with SYBR Green I and Taq polymerase. The following primers were used: β-actin, forward (5'-ATG GAT GAG GAA ATC GCT GCC-3') and reverse (5'-CTC CCT GAT GTC TGG GTC GTC-3'); hsp70, forward (5'-GCA GGC CGC CAT CCT CAT-3') and reverse (5'-GTA CTC CTC TTT ATC TGC CAG-3'); β-catenin, forward (5'-GAC ATC AAC GTG GTG ACG TGT-3') and reverse (5'-CAT CCT GGT GTC TGG ATG TGA-3'); LEF1, forward (5'-GAG GGA AAA GAT CCA GGA AC-3') and reverse (5'-AGG TTG AGA AGT CTA GCA GG-3'); and gsk3β, forward (5'-TGG CCT ACA TCC ATT CCT TTG-3') and reverse (5'-GTG GCT CCA AAG ATG AGT TCG-3').

3. Results

3.1. The Number of BRDU- and PCNA-Positive Cells Was Reduced by 2 mM VPA

The number of BRDU-positive cells in the telencephalic area of zebrafish treated with 2 mM VPA at 2 and 3 dpf was reduced significantly to 43.2% and 43.5% of the control at 12 h after BRDU injection ($p < 0.01$, $n = 8$) (**Figure 1(a)** and **Figure 1(b)**). The number of PCNA-positive cells was also significantly reduced to 34.0% and 32.6% of that found in the control ($p < 0.01$, $n = 8$) (**Figure 1(c)**). To find out whether the suppressive effect of VPA on cell proliferation is lasting, BRDU- or PNCA-positive cells in the telencephalic area of larvae that had been treated with 2 mM VPA for 3 h at 2 and 3 dpf were measured at 5 and 10 dpf. The numbers of BRDU- or PCNA-positive cells measured 12 h following BRDU treatment were not significantly different at 2, 3, 5 and 10 dpf in the control. However, the numbers of BRDU- or PCNA-positive cells were significantly lower in the 2 mM VPA-treated larvae of 2, 3, 5, and 10 dpf compared to the age-matched controls (**Figure 1**).

3.2. Expression Levels of Hsp70, β-Catenin, LEF1, and Gsk3β mRNA Were Altered by 2 mM VPA

Expression of hsp70, β-catenin, LEF1, and gsk3β mRNA was measured 2, 3, 5, and 10 dpf in the zebrafish larvae that had been treated with 2 mM VPA for 3 h at 2 and 3 dpf. qRT-PCR results showed that the expression of β-actin, a control gene, was not different between the control and VPA-treated zebrafish (data not shown). The expression levels of hsp70 increased 3- and 3.5-fold relative to the control 12 h after the VPA treatment at 2 and 3 dpf, respectively ($p < 0.01$, $n =$

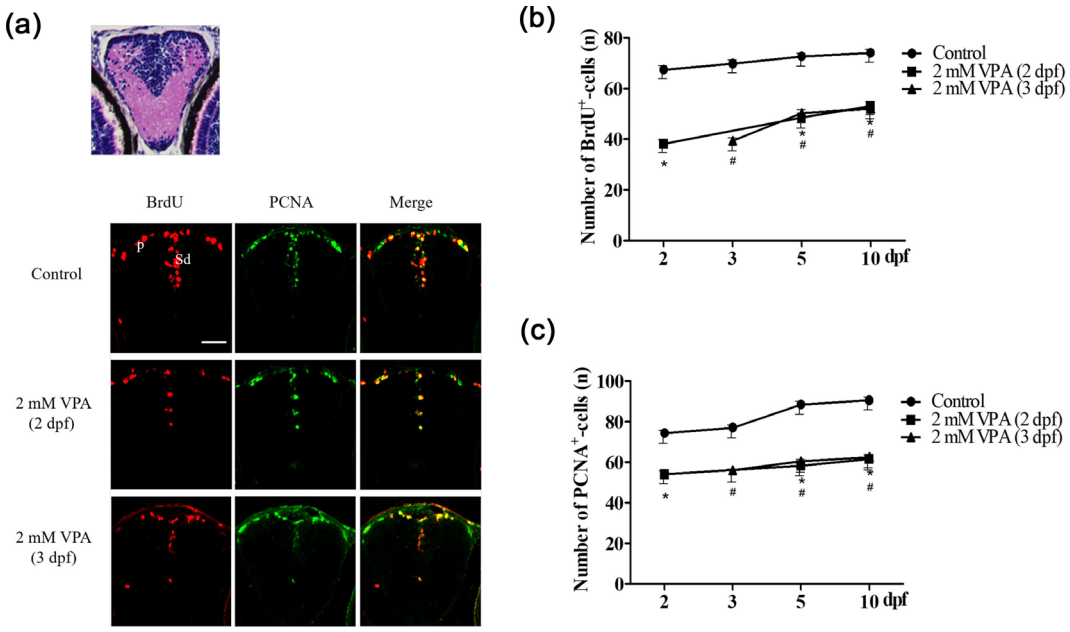

Figure 1. Reduced number of BRDU- and PCNA-positive cells in the zebrafish larvae treated with 2 mM VPA: (a) A representative hematoxylin and eosin stained section of the telencephalic area of zebrafish larvae (5 dpf) is shown at the top. Fluorescence microscope images show the immunostaining patterns corresponding to BRDU (red) and PCNA (green); (b) Quantification of BRDU-positive cells in the pallium (P) and dorsal subpallium (Sd); (c) Quantification of PCNA-positive cells in the pallium (P) and dorsal subpallium (Sd). Data were expressed as the means ± S.E.M and were analyzed via post hoc Tukey's multiple comparison tests. $^{*}P < 0.01$ (VPA-treated at 2 dpf); $^{\#}P < 0.01$ (VPA-treated at 3 dpf) compared to the control; Scale bar = 0.5 mm.

3). The levels of hsp70 mRNA decreased by 80.6% and 53.7% of the 2 dpf larvae treated with VPA in the 5 and 10 dpf larvae, respectively ($p < 0.01$, $n = 3$) (Figure 2(a)). Similarly, the levels of hsp70 mRNA decreased by 47.8% and 37.5% of the 3 dpf larvae treated with VPA in the 5 and 10 dpf larvae, respectively ($p < 0.01$, $n = 3$) (Figure 2(b)). β-catenin mRNA expression was not significantly different from the control 12 h after VPA treatment, but gradually decreased by 31.6% and 53.1% of the control in the larvae treated with VPA at 2 dpf, and by 45.7% and 56.2% of the control in the larvae treated with VPA at 3 dpf when measured at 5 and 10 dpf, respectively ($p < 0.01$) (Figure 2(c) and Figure 2(d)). LEF1 mRNA levels increased by 18.4% and 35.6% of the control 12 h after VPA treatment at 2 and 3 dpf. LEF1 mRNA levels gradually decreased by 40.6% and 49.7% of the control in the larvae treated with VPA at 2 dpf, and by 20.4% and 60.8% of the control in the larvae treated with VPA at 3 dpf when measured at 5 and 10 dpf, respectively ($p < 0.01$) (Figure 2(e) and Figure 2(f)). The expression levels of gsk3β were not significantly different from the control 12h after the VPA treatment at 2 dpf, but gsk3β mRNA expression increased by 16.4% in the VPA-treated zebrafish of 10 dpf relative to the control ($p < 0.01$, $n = 3$) (Figure 2(g)). In the VPA-treated larvae at 3 dpf gsk3β mRNA expression significantly increased by 19.5% of the control when measured at 5 dpf, respectively ($p < 0.01$, $n = 3$) (Figure 2(h)).

3.3. Cranial Malformation Was Not Observed in the VPA-Treated Larvae

Evaluation of the cartilage structures showed that the size and shape of the vis-

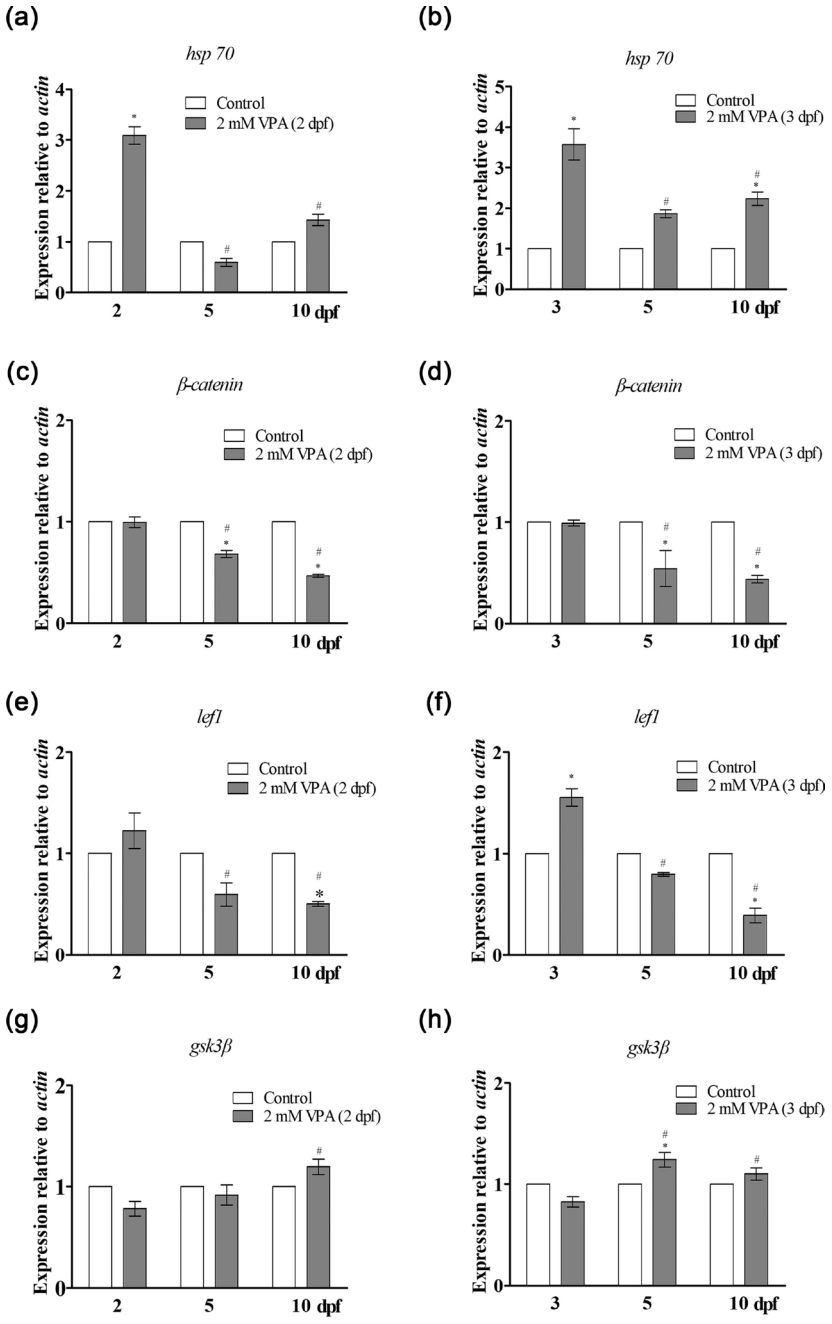

Figure 2. mRNA expression of hsp70, β-catenin, LEF1, and gsk3β in the zebrafish larvae treated 2 mM VPA at 2 and 3 dpf: ((a), (c), (e), (g)) Expression levels of hsp70, β-catenin, LEF1, and gsk3β mRNA zebrafish larvae treated with 2 mM VPA at 2 dpf compared to the control; ((b), (d), (f), (h)) Expression levels of hsp70, β-catenin, LEF1, and gsk3β mRNA zebrafish larvae treated with 2 mM VPA at 3 dpf compared to the control. The data were expressed as the means ± S.E.M and were analyzed with Student's t-tests along with post hoc Tukey's multiple comparison tests. $^{*}P < 0.01$ versus the control at each time point (Student's t-tests); $^{\#}P < 0.01$ compared to the 2 or 3d group (post hoc Tukey's tests).

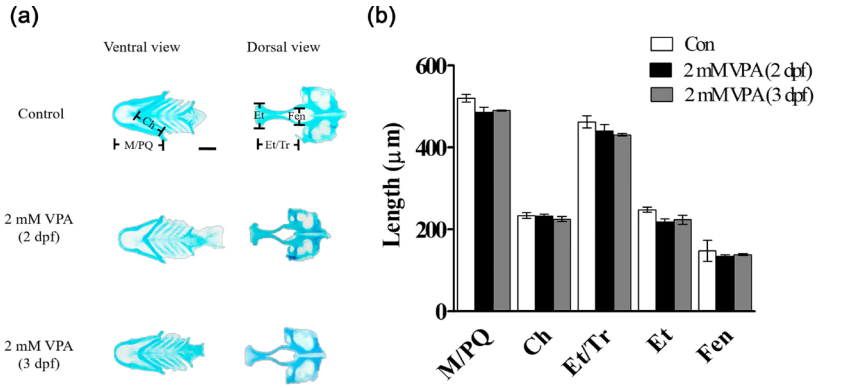

Figure 3. Effects of 2 mM VPA on craniofacial development in the zebrafish larvae: (a) Image of Alcian blue staining of the control and VPA-treated larvae of 10 dpf. Meckel's and palatoquadrate cartilage (M/PQ), ceratohyals (Ch) cartilage were clearly identified in the neurocranium (left); ethmoid plate and trabeculae (Et/Tr), ethmoid plate (Et), and fenestra (Fen) in viscerocranium (right) of the control and VPA-treated larvae; (b) Quantitative analysis of the M/PQ length, Ch length, Et/Tr length, Et width, and Fen width. Scale bar = 200 μm, n = 5.

cerocranium as well as neurocranium of the VPA-treated larvae were unaffected when measured at 10 dpf (Figure 3(a)). The Meckel's and palatoquadrate cartilage (M/PQ), ceratohyals (Ch), ethmoid plate and trabeculae (Et/Tr), ethmoid plate (Et), and fenestra (Fen) were 520.3 ± 2.8, 234.4 ± 3.4, 462.8 ± 4.4, 248.4 ± 3.1, and 148.0 ± 4.8 μm, respectively, in the control (n = 5); 485.7 ± 6.4, 232.5 ± 2.7, 440.1 ± 5.9, 218.7 ± 3.6, and 135.1 ± 1.5 μm in the larvae treated with VPA at 3 dpf (n = 5); 490.6 ± 0.6, 225.2 ± 3.1, 431.2 ± 1.8, 224.0 ± 5.2, and 138.8 ± 1.5 μm in the larval treated with VPA at 2 dpf (n = 5). No significant differences in these elements were observed the three groups (Figure 3(b)).

3.4. Color Preference Was Transiently Altered by 2mM VPA

In the color preference test at 5 dpf, blue color preference for the control were measured 9.01 ± 0.06 and yellow color preference were 0.98 ± 0.06 ($p < 0.01$, n = 7) (Figure 4(a)). In contrast, blue color preference in the larval treated with VPA at 2 and 3 dpf significantly decreased to 5.17 ± 0.24 and 6.11 ± 0.21 and yellow color preference significantly increased to 4.82 ± 0.24 and 3.88 ± 0.21 for the 5 dpf larvae, respectively ($p < 0.01$, n = 7) (Figure 4(a)). However, altered color preference was recovered at 10 dpf. Blue color preference in the control was measured to 7.11 ± 0.03 and yellow color preference was 2.92 ± 0.02 at 10 dpf (n = 8) (Figure 4(b)). Similarly, blue color preference in the larval treated with VPA at 2 and 3 dpf were 6.56 ± 0.05 and 6.81 ± 0.15 and yellow color preference were to 3.43 ± 0.05 and 3.18 ± 0.15, respectively ($p < 0.01$, n = 8) (Figure 4(b)). Locomotion activity increased as zebrafish larvae grew with no significant difference between groups (data not shown).

4. Discussion

This study showed that 2 mM VPA reduced cell proliferation in the telencephalic

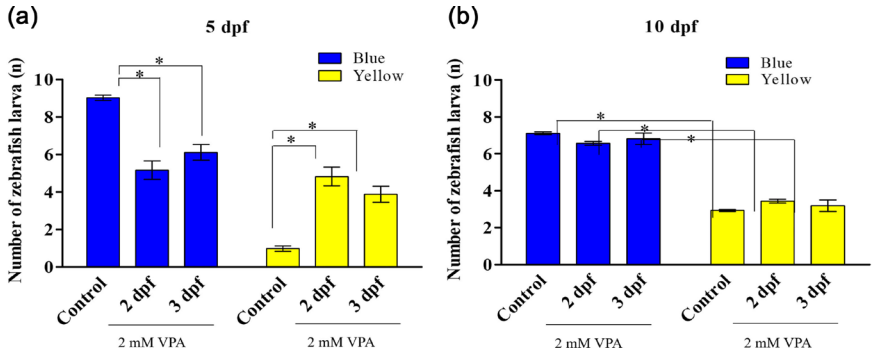

Figure 4. Altered color preference by 2 mM VPA: (a) Color preference test measured at 5 dpf. Yellow color preference was increased in the zebrafish larvae treated with 2 mM VPA at 2 and 3 dpf compared to the control; (b) Color preference test measured at 10 dpf. Color preference was recovered in the zebrafish larvae treated with 2 mM VPA. The data were expressed as the means ± S.E.M and were analyzed via post hoc Tukey's multiple comparison tests. $^{*}P < 0.01$ compared to the control.

area of zebrafish larvae. Expression levels of LEF1 and β-catenin mRNA, nuclear mediators of wnt signaling, were also decreased following treatment with 2 mM VPA. LEF1 and β-catenin have been shown to increase cell proliferation in the developing brain of the mouse and neural stem cell [10] [11] [14]. In addition, a previous research has shown that VPA increases hsp70 protein levels in the rat cortical neurons [15]. Similarly, hsp70 mRNA levels were increased in the zebrafish brain treated with 2 mM VPA for 3 h in this study.

It has been reported that young epilepsy patients suffered hyperammonemic encephalopathy following treatment with VPA for 1 ~ 2 weeks [16]. The serum valproic acid level ranged 50 ~ 269.9 mg/L, which roughly corresponds to 0.5 ~ 2.5 mM [16] [17]. Our previous study has also shown that treatment with 2 mM VPA induced learning impairments in the adult zebrafish [18]. In this study, therefore, 2 mM VPA was applied for 3 h in aquarium water in order to evaluate effects of 2 mM VPA on cell proliferation and behavior of zebrafish larvae.

The decreased cell proliferation in the larvae of 5 and 10 dpf treated with 2 mM VPA for 3 h at 2 and 3 dpf is consistent with a decreased cell proliferation in the larvae treated with the same dose of VPA for 3 h at 5 dpf [11]. An interesting difference is that the decreased cell proliferation was gradually recovered within 10 days following VPA treatment at 5 dpf, while it was not recovered following VPA treatment at 2 and 3 dpf. This discrepancy in the recovery of cell proliferation depending on the treatment time may be attributed to different expression patterns β-catenin and LEF1 mRNA in the two groups. Expression patterns of β-catenin and LEF1 mRNA were altered following VPA treatment at 2 and 3 dpf larvae in this study. When 2 mM VPA was treated at the 2 and 3 dpf larvae, β-catenin and LEF1 expression was lower in the VPA-treated larvae than the control at 2 ~ 8 d following VPA treatment. In contrary, gsk3β mRNA expression was higher in the VPA-treated larvae. Conversely, when the same does of VPA was treated at the 5 dpf larvae, β-catenin and LEF1 expression was higher in the VPA-treated larvae than the control 2 ~ 10 d following VPA treatment

[11]. On the contrary, gsk3β mRNA expression was lower in the VPA-treated larvae.

The reduced cell proliferation in the brain didn't cause either the cranial malformation or abnormal locomotive activity in this study. Locomotive activity increased as the larvae grew, but it was not significantly different between the control and VPA-treated larvae. However, color preference was altered in the 5 dpf larvae treated with 2 mM VPA at 2 and 3 dpf. VPA-treated larvae showed a decreased blue color preference compared to the control. Interestingly, the color sense disorder has been reported from adolescent patient who underwent seizure treatment by carbamazepine (CBZ) and VPA [19].

5. Conclusion

VPA is known to detrimentally affect the embryonic and fetal development when a pregnant woman is exposed to it in pregnancy. Taken our studies together, 2 mM VPA had molecular and cellular effects on the brain development of zebrafish larvae. Especially, VPA treatment at 2 and 3 dpf reduced cell proliferation in the telencephalic area of zebrafish larvae of 5 and 10 dpf. In addition, it was also demonstrated that VPA exposure might cause visual deficits. Furthermore, VPA altered expression patterns of LEF1, β-catenin, and gsk3β RNA, which might underlie the reduced cell proliferation. Thus, our study suggests the possible cellular and molecular factors underlying detrimental effects of VPA on the brain development.

Acknowledgements

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