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The long-term prognosis of hippocampal neurogenesis and behavioral changes of offspring from rats exposed to valproic acid during pregnancy

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Abstract

Aim: In pregnant women with epilepsy, it is essential to balance maternal safety and the potential teratogenicity of anticonvulsants. Recently, growing evidence has indicated that valproic acid (VPA) can produce postnatal congenital malformations and impair cognitive function. However, the mechanisms underlying cognitive dysfunction in long-term prognoses remain unclear.

Methods: Pregnant Wistar rats received daily intraperitoneal injections of VPA (200 mg/kg/day) from embryonic day 12.5 until birth. On postnatal day (PD) 149, the rats received an injection of bromodeoxyuridine (BrdU). On PD 150, the rats were subjected to the open field (OF), elevated plus-maze (EPM), and Y-maze tests. After behavioral testing, perfusion fixation was performed and the brain was dissected for immunohistochemistry.

Results: A significant marked decrease was seen in the number of BrdU-positive cells in the dentate gyrus of offspring of VPA-treated dams compared to those of control. However, no significant differences in hyperactivity were found based on the results of the OF test among the offspring on PD 150 of 200 VPA-treated dams. In addition, no significant differences were seen in the EPM test.

Conclusion: The behavioral abnormality observed in young offspring of VPA-treated dams was not significantly different from that of controls in adult offspring on PD 150. However, compared with controls, the number of BrdU-positive cells in VPA-treated rats was halved. The findings suggest that the behavioral abnormality seems to improve as they grow, even if some structural abnormalities may remain in the central nervous system.

KEYWORDS

attention deficit hyperactivity disorder, long-term, neurogenesis, offspring, prenatal toxicity, valproic acid

1 | INTRODUCTION

Prevention is an important issue for patients with epilepsy or bipolar disorder, and to achieve this, antiepileptic drugs and mood stabilizers, such as valproic acid (VPA), are often used continuously. However, an increasing body of evidence suggests that the offspring of pregnant women exposed to VPA can also increase risk of developing neurodevelopmental disorders such as autism spectrum disorder (ASD)[1–3] and attention deficit hyperactivity disorder (ADHD).[4–6].

In a rodent model, prenatal VPA exposure was found to be related to neurodevelopmental disorders in offspring.[7,8] Choi et al (2014) have reported that prenatal exposure to VPA causes hyperactivity disorders, such as ADHD, and that atomoxetine was effective for treating these abnormal behaviors. We also identified an increased locomotor activity and an insusceptibility to anxiety, and a positive correlation between spontaneous activity and neurogenesis in the dentate gyrus (DG) in offspring of rats exposed to repeated VPA use.[9] These findings suggest that changes in neurogenesis in the DG may be involved in the pathogenesis of hyperactivity.

Interestingly, Moffitt et al[10] reported follow-back analyses of ADHD cases diagnosed in adulthood alongside follow-forward analyses of ADHD cases diagnosed in childhood of one cohort, where fifty-eight cases (95%) remitted in adulthood.

Therefore, based on the neurodevelopmental abnormalities of young offspring in our study, we investigated the long-term prognosis of rats exposed to VPA, assuming that the long-term prognosis of hyperactivity is associated with the degeneration of neurogenesis in the DG.

2 | METHODS

2.1 | Animals

In this study, pregnant female Wistar rats (Sankyo Lab Service, Inc, Tokyo, Japan) weighing 190-210 g were randomized into two groups: VPA 200 mg (n = 3 dams) and controls (n = 3 dams). VPA (catalog No.: P4543; Sigma-Aldrich, Tokyo, Japan) was administered daily from embryonic day (ED) 12.5 to ED 21.5, covering the last 9-12 days of pregnancy. The control group received a daily injection of saline. Intraperitoneal injection was selected as the administration method to ensure a consistent daily dosage. The rats were housed in cages under a constant light-dark cycle (12 hours) and temperature (22-26°C). All rats had ad libitum access to a standard laboratory diet and water. Behavior and neurogenesis were assessed in the male offspring (weight: 300-400 g on postnatal day [PD] 150; VPA 200 mg offspring: n = 7, control offspring: n = 9).

2.2 | Immunohistochemistry

To evaluate the effect of VPA on cell proliferation, all rats were administered bromodeoxyuridine (BrdU) on PD 149 and decapitated on PD 150. A detailed description of the methods used in our immunohistochemistry experiments were provided elsewhere.[11] On PD 150, the rats were deeply anesthetized with sodium pentobarbital and then perfused with phosphate buffer (PB) for 3 minutes, followed by 4% paraformaldehyde in PB after behavioral tests. Brains were dissected and preserved in fixative solution (4% paraformaldehyde in PB) overnight. The brains were then transferred to 20% sucrose in PBS until they sank. We then sliced the brains on a freezing microtome at a thickness of 30 μm. Every 10th section containing the hippocampus was selected for subsequent analysis. Sections were then immunostained to detect BrdU. Stained slices were analyzed using a confocal microscope (Leica TCS Sp5 confocal laser microscope; Leica, Wetzlar, Germany). We counted BrdU-positive cells in every slice (whole hippocampus).

2.3 | Behavioral tests

The time course of this study is shown in Figure 1. To investigate the effects of prenatal exposure to VPA on behavior in the offspring on PD 150, we evaluated spontaneous locomotor activity using the open field (OF) test, spontaneous alternation behavior using the Y-maze test, and anxiety behavior using the EPM test, as previously reported.[9] To avoid the effect of prior experiments, we performed these experiments at intervals of an hour or more.

2.4 | Statistical analysis

Statistical analysis was conducted using IBM SPSS Statistics version 22 (IBM, Tokyo, Japan). One-way analysis of variance (ANOVA) was used to compare behavioral metrics and BrdU-positive cell count between groups. Two-way ANOVA was used to compare spontaneous

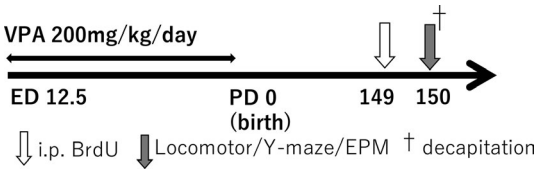

FIGURE 1 Outline of this study. This is an outline of the present study. VPA was administered daily from ED 12.5 to ED 21.5, covering the last 9-12 d of pregnancy. All animals were administered BrdU on PD 149. We evaluated spontaneous locomotor activity using the OF test, spontaneous alternation behavior using the Y-maze test, and anxiety behavior using the EPM test on PD 150. After that, all animals were decapitated

activities. When appropriate, post hoc comparisons were performed using a *t* test, with Bonferroni correction for multiple tests. Values of $P < .05$ were considered statistically significant.

3 | RESULTS

3.1 | Number of BrdU-positive cells in the dentate gyrus (DG)

BrdU-positive cells were typically observed in the sub-granular zone and granule cell layer of the DG in the offspring rats (Figure 2A). The number of BrdU-positive cells in the VPA 200 mg group was significantly decreased compared with the control group ($t[8] = 3.752$, $P = .00560$), especially in the anterior DG ($t[8] = 5.422$, $P = .000629$) (Figure 2B, C).

3.2 | Behavioral tests

We performed three kinds of behavioral tests. The time course of spontaneous activity is shown in Figure 3A. Spontaneous activity tended to decrease during the observation in both groups (Figure 3A). For every 10-minutes period, we compared the spontaneous activity between the VPA and control groups using two-way ANOVA. The main effect of treatment (VPA vs. control) was significant ($F[1,112] = 9.483$, $P = .00269$). Post hoc analysis (Bonferroni) showed that rats in the VPA 200 mg group had significantly higher activity levels than controls during the first 10-minutes period ($P = .00307$). We also compared total spontaneous activity between groups for the 60-minutes observation period (Figure 3B). No significant differences were found between the VPA 200 mg and control groups.

We performed the Y-maze test to evaluate memory function. No significant differences were observed between groups in terms of behavioral change in the Y-maze test ($t[14] = 0.326$, $P = .749$) (Figure 3C).

We also performed the EPM test to assess anxiety. No significant differences were found between groups in the time spent in the open arms, the time spent in the closed arms, or the ratio of the time spent in the open arms ($P = .779$, $P = .616$, and $P = .721$, respectively) (Figure 3D).

4 | DISCUSSION

We previously showed that VPA administration during pregnancy can lead to hyperactivity and related behavioral abnormality in offspring on PD 30.[9] However, the results of this study revealed that behaviors of offspring from VPA-treated pregnant rats were similar to those in controls on PD 150. The present results suggest that a part of these ADHD-like behavioral abnormalities improve with growth in rats. In humans, previous reports have found that ADHD symptoms also improve by 60%-90% with growth.[10,12–14]

However, in this study, the number of BrdU-positive cells in VPA-treated rats was halved compared with controls. As most of the progenitor cells in the DG differentiate into neurons,[9,15] VPA depletes the neuroprogenitor cells, which were SOX2-positive cells pool and reduces the number of proliferating neuroprogenitor cells.[15]

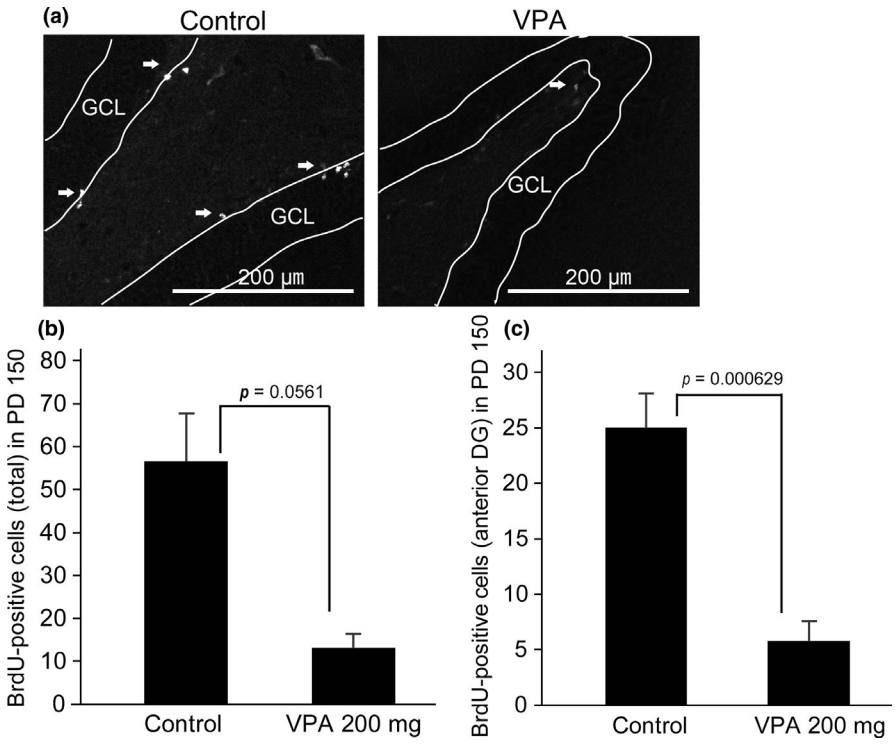

FIGURE 2 BrdU-positive cells in the dentate gyrus. Hippocampal dentate gyrus sections immunostained for BrdU. (A) 200× magnification; the photomicrograph on the left side is the control group and that on the right side is the VPA group. White spots are BrdU-positive cells. (B) The bar graph shows the number of BrdU-positive cells in whole DG. The number of BrdU-positive cells in the VPA group was significantly decreased compared with the control group ($P = .00561$). (C) The bar graph shows the number of BrdU-positive cells in the anterior DG. The number of BrdU-positive cells in the VPA group was significantly decreased compared with the control group ($P = .000629$). The decrease in BrdU-positive cells was remarkable in the anterior dentate gyrus. Error bars represent the standard error of the mean. GCL, granular cell layer

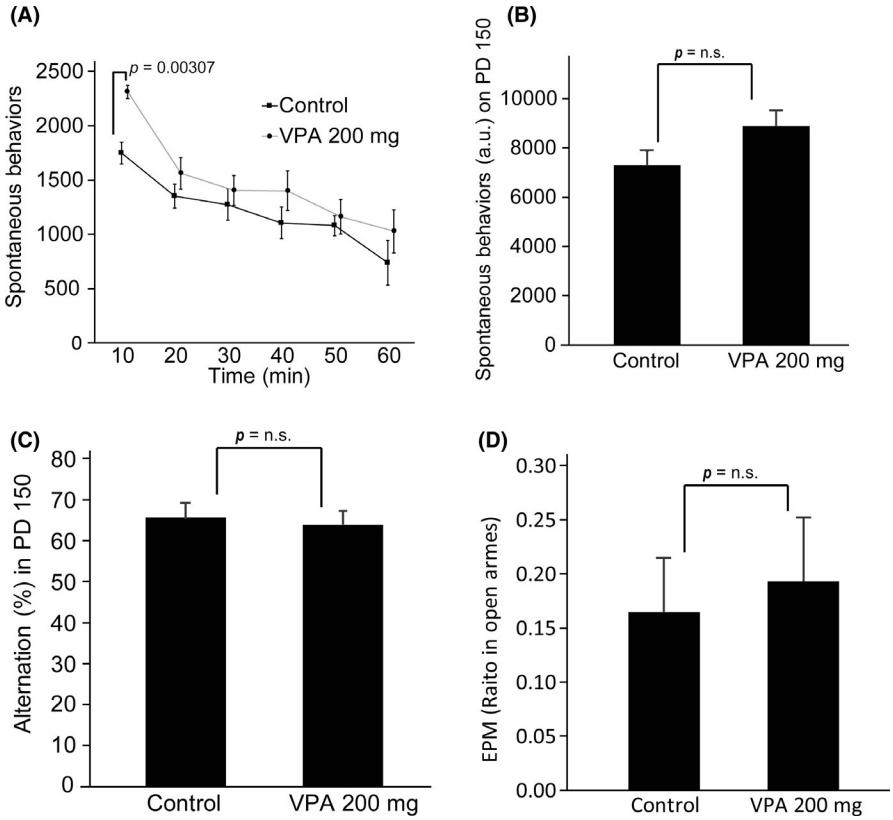

FIGURE 3 Behaviors. (A) Time course of spontaneous locomotor activity during the open field test. The magnitude of spontaneous activity tended to decrease in the first 30 min of observation, remaining stable thereafter, in all groups. Rats in the VPA 200 mg group showed higher levels of locomotor activity than those in the control group until 10 min ($P = .00307$). (B) No significant difference was seen in the amount of spontaneous locomotor activity. Error bars represent the standard error of the mean. (C) Y-maze test. No significant differences were seen between groups on alternation (%) metrics. Error bars represent the standard error of the mean. (D) EPM. The bar graph shows the results of the EPM test. No significant difference was observed between groups. Error bars represent the standard error of the mean

On the other hand, the number of BrdU-positive/Doublecortin-positive cells, which is immature neurons and cannot proliferate, was increased with prenatal treatment of VPA 200 mg/kg on PD 30.[9] It may indicate that VPA decreases the neuroprogenitor cells but promote neural differentiation. Therefore, some abnormalities may remain in the central nervous system of patients with ADHD, even if their symptoms seem to have improved. In a human study, Saad et al[16] reported that for inattentive ADHD, compared to both combined ADHD and controls, the nodal degree in MRI was higher in the hippocampus. This might be related to neurogenesis in the hippocampus.

The model of VPA exposure in one of the most frequently studied animal models of ASD.[17] This increase in PD 30 would be then followed by a decrease in PD 150, in a rebound phenomenon.[18] Hippocampus volume in human was relatively greater in ASD in analyses adjusting for hemisphere volume in a previous study.[19] This may be related to our result. Limitation of this study is that we found out only neurogenesis in hippocampus, and we could not perform a social interaction test of rats.

In conclusion, behaviors in VPA-treated offspring were similar to those in controls on PD 150. However, compared with controls, the number of BrdU-positive cells in VPA-treated rats was halved. Therefore, some abnormalities may remain in the central nervous system of patients with ADHD, even if their symptoms seem to have improved.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Tomoya Kinjo performed the behavioral tests and assessed neurogenesis using confocal microscopy. Masanobu Ito designed the experiments and performed the data analysis. Tatsunori Seki drafted the manuscript. Toshihito Suzuki summarized the study protocol, designed the experiments, and drafted the manuscript.

ETHICAL APPROVAL

All protocols in this study were approved by the Institutional Animal Care and Use Committee of Juntendo University Faculty of Medicine (approval No. 1148).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in Data S1.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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