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Valproic acid selectively suppresses the formation of inhibitory synapses in cultured cortical neurons

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HIGHLIGHTS

- Exposure of valproic acid to cultured cortical neurons decreased VGAT expression.
- The reduction of VGAT by valproic acid was through its HDAC inhibition activity.
- Valproic acid suppressed the formation of inhibitory synapses.
- Valproic acid may disturb the brain development.

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ABSTRACT

Valproic acid (VPA) has been used to treat epileptic patients because of its ability to potentiate GABA signaling in the brain. Despite its clinical significance, VPA administration during pregnancy increases the risk of congenital abnormalities, such as neural tube defects and neurodevelopmental disorders including autism. Furthermore, recent studies revealed that early postnatal administration of VPA also leads to neurodevelopmental deficits in rodents. Here, using cultured cortical neurons derived from postnatal day 1 rats, we examined whether exposure to VPA would affect synapse formation. When neurons were exposed to 1 mM VPA during early development, expression of the vesicular GABA transporter (VGAT) was selectively reduced, whereas other synaptic markers, including the vesicular glutamate transporters 1 and 2 (VGLUT1 and 2), were not affected. This VPA effect was mediated through inhibition of histone deacetylases (HDACs), since the effects were mostly recapitulated by an HDAC inhibitor, trichostatin A, but not by a VPA derivative, valpromide, which lacks HDAC inhibitor activity. Immunocytochemical analysis demonstrated that VPA exposure resulted in a retardation of axonal growth specific to GABAergic neurons and a decrease in VGAT-positive synapses. Since disturbance of the excitatory and inhibitory (*E–I*) balance has been implicated as a potential cause of multiple psychiatric disorders, our results may account for one of the cellular mechanisms underlying the pathogenesis of VPA-induced neurodevelopmental impairments.

1. Introduction

A precise balance between excitatory and inhibitory neurotransmission is crucial for brain function. It has been demonstrated that disturbance of the *E–I* balance is frequently associated with a number of neurological and neurodevelopmental disorders, such as epilepsy, schizophrenia, and autism [1–5]. Although some molecules regulating the *E–I* balance have recently emerged [6,7], the underlying mechanisms responsible for the pathophysiology of these neurodevelopmental disorders have yet to be elucidated.

Valproic acid (VPA) is an established anti-convulsant drug. It is suggested that VPA inhibits GABA transaminase, an enzyme that degrades GABA, and probably stimulates GABA synthesis, thereby facilitating the inhibitory drive in the brain, which exerts an anti-epileptic effect [8,9]. Despite its clinical usefulness, VPA has contra-indications, especially during pregnancy, as it causes congenital anomalies in the fetal brain and autism-like behavior [10–13]. Administration of VPA to rats around gestational day 11–12.5 causes abnormal neurological symptoms in their offspring, which are similarly observed in autistic patients, such as

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increased repetitive behavior and impaired social interaction [14]. Notably, electrophysiological measurements revealed that prenatal administration of VPA in rodents increased the connectivity of local cortical pyramidal cell networks and diminished the intrinsic excitability of these pyramidal cells [15]. Although many studies have focused on the effects of VPA during mid-pregnancy as a possible animal model of autism, it has also been demonstrated that administration of VPA at postnatal day 14 induces neuronal apoptosis and social, motor, and cognitive deficits [16,17], indicating that early postnatal exposure to VPA may also lead to neurodevelopmental deficits.

In addition to its effect on GABA metabolism, VPA affects numerous enzymes involved in multiple cellular pathways such as histone deacetylases (HDAC) and multiple kinases [18,19]. Of these, activation of the ERK pathway by VPA was proposed to facilitate neuronal growth in cultured cortical neurons and neurogenesis in the hippocampus [20]. Moreover, VPA induced the differentiation of adult neural progenitor cells into neurons via inhibition of HDAC activity [21]. Inhibition of HDAC causes epigenetic modification of various kinds of genes. Because of the effect VPA has on HDAC activity, it was also reported that a short exposure of VPA (12 h) to cultured cortical neurons caused alterations in mRNA expression of multiple genes, including genes that are essential for GABAergic synapses, such as GAD65, GAD67, and $GABA_A$ receptor subunits. In addition, BDNF mRNA was also increased [22], and BDNF is associated with the maturation of both excitatory and inhibitory neurons [3,23]. In these studies, however, it was unclear whether VPA exposure directly affects the formation of synapses. In the present study, we utilized cultured rat cortical neurons derived from postnatal day 1 (P1) animals to examine whether postnatal VPA exposure would affect excitatory and inhibitory synapse formation.

2. Materials and methods

2.1. Cell culture

Cultured cortical neurons were prepared from P1 rats, as reported previously with slight modifications [24] (see also Supplemental information). On the next day of the culture, day in vitro 1 (DIV1), VPA (0.3–5.0 mM), VPM (0.3–5.0 mM) or TSA (100 nM) (all obtained from Sigma–Aldrich) were added to the cultures. In the control experiments, the same volume of the solvents, of either water or DMSO, was added to the medium. The usual clinical dose of VPA is 0.2–0.6 mM in serum, and it sometimes reaches up to several mM in a fetus [8,10]. Therefore, we set experimental concentrations of VPA between 0.3 and 5.0 mM. In a typical experiment, the medium was exchanged at DIV4 for fresh medium without any additives. Neurons were lysed or fixed at the various time points indicated in the figure legends.

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All animals were purchased from Shimizu Laboratory Supplies Co., Ltd. (Japan) and treated according to our institutional guidelines for the care and use of animals.

2.2. Western blotting

SDS-PAGE followed by western blotting was performed as previously described [24]. The protein concentrations in cell lysates were determined using a BCA Protein Assay kit (Pierce). Equivalent amounts of total protein were assayed for each experiment. As primary antibodies, anti-VGAT [25], anti-VGLUT1 [26], anti-VGLUT2 [26], anti-GluN1 (Synaptic Systems), anti-Homer1 (Synaptic Systems), and anti-TUJ1 (Berkeley Antibody) antibodies were used. Monoclonal antibodies against anti-synaptotagmin1, anti-synaptophysin, anti-synaptobrevin, anti-synapsin1, and anti-SNAP25 antibodies were kind gifts from Dr. R. Jahn (Max-Planck-Institute for Biophysical Chemistry). The signals were detected and analyzed as described in Supplemental information

2.3. Immunocytochemistry

Cells were fixed and permeabilized before incubation with primary antibodies as described in Supplemental information. Immunofluorescence was observed with a confocal microscope (LSM710; Zeiss, Japan), and the images were analyzed with Zen2009 software. The methods for quantifying axonal growth and counting cell numbers are indicated in Supplemental information.

2.4. Statistics

Quantitative data from western blotting and immunocytochemistry are expressed as the mean ± standard deviation (SD). Student's *t*-tests were performed to compare two experimental groups. To evaluate the statistical significance of the data among multiple groups, ANOVA and Bonferroni tests were performed. All analyses were conducted using the Statistical Package for Social Science (SPSS) version 11.0 (SPSS, Japan). *P* values <5% were considered statistically significant.

3. Results

3.1. Immature cortical neurons exposed to VPA have reduced VGAT expression

To explore the effect of VPA on neuronal development, cultured neurons derived from P1 rat cerebral cortex were exposed to 1.0 mM VPA from DIV1 to DIV4, and were maintained until DIV14. Protein composition in the whole cell lysate was evaluated by semi-quantitative western blotting using a set of antibodies against synaptic marker proteins (Fig. 1A). Intriguingly, the expression level of the vesicular GABA transporter (VGAT), which accumulates GABA into the synaptic vesicles of inhibitory synapses, was dramatically reduced by the VPA treatment (Fig. 1A). In contrast, the levels of many major synaptic proteins were not affected. VGAT expression was reduced by VPA in a dose dependent manner (Fig. 1B and C). In these conditions, expression of VGLUT1, synaptophysin, and TUJ1 were not affected (only quantification of VGLUT1 is shown in Fig. 1D). Since high VPA (5 mM) concentrations apparently induced cell death, we performed all our remaining experiments with 1.0 mM VPA. These data suggest that the VPA exposure selectively affected GABAergic synapses.

3.2. Timing of VPA exposure is critical for the suppression of VGAT expression

To survey the time-course of this VPA effect, lysates were subjected to analysis at DIV4, 7, 12, and 14 (Fig. 1E). As previously reported, expression of both VGLUT1 and VGAT were developmentally upregulated during this period in non-treated cells [27]. Immediately after the three-day treatment with VPA, VGAT expression was slightly upregulated at DIV4, but started to decrease at DIV7 compared to the control (Fig. 1E and F). In contrast, VGLUT1 expression was not affected at any of the time points examined (Fig. 1E and G). To further investigate if the timing of VPA exposure was critical for this effect, cells were exposed to VPA at different time points. When cells were incubated with VPA from DIV4 to DIV7, VGAT expression was also