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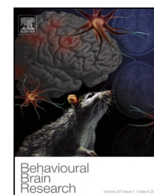

Short Communication

Downregulation of glutamatergic and GABAergic proteins in valproric acid associated social impairment during adolescence in mice

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HIGHLIGHTS

- NMDAR subunits 2A, 2B, 2C were downregulated in the cortices of VPA-induced autistic adolescent mice.
- $GABA_AR$ subunits GABRA1, GABRA5 and GABRB2 were downregulated in the cortices of VPA-induced autistic adolescent mice.
- Glutamic acid decarboxylase GAD65 and GAD67 were downregulated in the cortices of VPA-induced autistic adolescent mice.
- BDNF was downregulated in the cortices of VPA-induced autistic adolescent mice.

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ABSTRACT

The etiology of Autism Spectrum Disorder (ASD) remains controversial. Deficits in social communication are one of the key criteria for ASD diagnosis. Valproic acid (VPA), which is an anti-epileptic and anti-depressive drug, is one of the teratogens to cause ASD onset. Moreover, synaptic dysfunction is suggested as one of the major causative factor in VPA-induced ASD *in vitro* and *in vivo* studies. Herein, this study aimed to determine the excitatory/inhibitory synaptic mRNA and protein expression in VPA-induced autistic mice. Pregnant BALB/c mice were injected peritoneally with a single dose of 600 mg/kg VPA on embryonic day (E) 12.5. Social impairment was verified by three chamber sociability tests on postnatal days (PND) 28, 35, 42 and 49. Cortical synaptic mRNA and protein expressions were examined on PND 50. The excitatory synaptic proteins NR2A, NR2B, NR2C were significantly down-regulated by 80.0% ($p < 0.01$), 51.5% ($p < 0.05$) and 81.5% ($p < 0.05$) respectively. Furthermore, the NMDAR expression regulatory protein BDNF was also found to be significantly downregulated by 76.8% ($p < 0.05$). GAD65, GAD67, GABRA1, GABRA5, GABRB2 from the GABAergic inhibitory synaptic pathway were significantly downregulated by 21.3% ($p < 0.05$), 77.0% ($p < 0.05$), 53.9% ($p < 0.05$), 56.9% ($p < 0.05$) and 55.2% ($p < 0.01$) respectively in the cortex of VPA-induced mice. Taken together, our results suggested that synaptic dysfunction might be involved in the social impairments in VPA-induced ASD.

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1. Introduction

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder in which the pathophysiology remains elusive. Currently, there is neither clear prognosis nor cure for the disease. According to the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-V), ASD is diagnosed when a person has repetitive stereotypes and impairments in social communication [1]. Prenatal exposure to the anti-epileptic drug, valproic acid (VPA) during pregnancy is associated with a higher risk of ASD onset [2]. Autistic rodent models, which are induced by prenatal or postnatal VPA exposure, have been established in the past decades to understand the mechanisms involved in VPA-induced ASD [3]. Yet, its comprehensive etiology remains unknown.

Abbreviations: ASD, autism spectrum disorder; BDNF, brain derived neurotrophic factor; DSM-V, diagnostic and statistical manual for mental disorders; E, embryonic day; $GABA_AR$, γ-aminobutyric acid subtype A receptors; GAD65, glutamic acid decarboxylases 65 kDa; GAD67, glutamic acid decarboxylases 67 kDa; GABRA1, $GABA_AR$ subunit α1; GABRA5, $GABA_AR$ subunit α5; GABRB2, $GABA_AR$ subunit β2; NMDAR, *N*-methyl-D-aspartate receptor; NR2A, NMDAR subunit 2A; NR2B, NMDAR subunit 2B; NR2C, NMDAR subunit 2C; PND, post-natal day; VPA, valproic acid.

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Synaptic protein dysregulation plays a central role in the pathogenesis of ASD. *N*-methyl-D-aspartate receptors (NMDARs) and γ-aminobutyric acid subtype A receptors ($GABA_A$Rs) are the two major receptors in the pre- and post-synapses [4]. The decrease in protein expressions of NMDAR subunits 2A (NR2A) and 2B (NR2B) in the primary somatosensory cortices of VPA-treated adolescent rats was reported [5]. On the other hand, $GABA_A$Rs were reduced in the anterior cingulate cortex of ASD patients [6]. The GABA synthesizing enzymes, glutamic acid decarboxylases (GADs) 65 and 67, decreased in autistic parietal and cerebellar cortices in another post-mortem study [7].

In this study, we studied the effect of VPA towards cortical glutamatergic and GABAergic synaptic proteins expression functions using an autistic mouse model induced by prenatal administration of high dose VPA.

2. Materials and methods

2.1. Chemicals and antibodies

Valproic acid was obtained from Sigma-Aldrich (St. Louis, MO, USA). Rabbit anti-NR2A, rabbit anti-NR2B, rabbit anti-NR2C, rabbit anti-BDNF, mouse anti-GAD65, chicken anti-GAD67, mouse anti-GABRA1, mouse-GABRA5, rabbit anti-GABRB2, rabbit anti-GAPDH, goat anti-rabbit, goat anti- mouse and goat anti-chicken antibodies were purchased from Abcam (Cambridge, MA, USA).

2.2. Valproic acid animal model

BALB/c mice were housed in cages at 25 °C ± 2 °C with 12 h: 12 h light/dark cycle. Chow and water were available *ad libitum*. They were allowed to mate overnight. The presence of a vaginal plug indicated successful mating and the date was designated as embryonic day 0.5. Mice with vaginal plugs were isolated and kept in plastic cages. The pregnant mice were injected intraperitoneally with 600 mg/kg VPA on E12.5 that was the date of the closure of the neural tube [8]. Phosphate buffered saline (PBS) was used as the vehicle. The day when the pups were born was designated as postnatal day (PND) 0.5. The pups were housed with their mothers until they were weaned on PND 21.

2.3. Three chamber sociability test

To assess the deficits in social communication, male pups were subjected to the three chamber sociability test on PND 28, 35, 42 and 49 [9]. The test was performed in a Plexiglass box at a dimension of 60 × 40 × 30 cm, with three chambers of equal sizes. Two identical cylindrical cages were placed on the left and right chambers. The subjects were allowed to explore each chamber through the openings of the dividing walls between the chambers (Noldus, Wageningen, the Netherlands). The subject was first introduced and allowed to acclimate in the center chamber for 10 min; after 10 min, a stranger mouse was placed one of the cylindrical cages. The subject mouse was allowed to explore freely around for 10 min. Afterward, a novel mouse was placed in the empty cage and the subject mouse was allowed explore the chambers for another 10 min. The stranger and novel mice did not have any contact with the subject mice prior the test. The time of the subject spent in each chamber the stranger and novel mice were recorded and analyzed by Ethovision XT 11 (Noldus).

2.4. Real-time qPCR

Mice were euthanized on PND 50. The harvested cerebral cortices were frozen with liquid nitrogen and minced into small pieces using a mortar and pestle. Total mRNA was isolated using TRIzol (Thermo Fisher Scientific; MA, USA), following manufacturer's instructions. cDNA was synthesized using ImProm-II™ Reverse Transcriptase (Promega Corporation; Wisconsin, USA). Real-time qPCR was performed with StepOnePlus (Thermo Scientific; Carlsbad, MA, USA) using SYBR Premix Ex Taq (Tli RNase H Plus) (TAKARA; Shiga, Japan) with the following steps: 1 cycle 95 °C 30 s, 40 cycles (95 °C 5 s, 60 °C 30 s). β-actin was used as the housekeeping gene. The sequences of primers used are listed in Table 1.

2.5. Western blot

For total protein extraction, the cortices were lysed in N-PER Neuronal Protein Extraction Reagent (Thermo Fisher Scientific) containing EDTA-free Pierce™ Protease Inhibitor Tablets (Thermo Fisher Scientific). Protein samples of 30 μg were electrophoresed using 10–15% SDS-PAGE gels and transferred onto nitrocellulose membranes (Bio-Rad; Hercules, CA, USA). The membranes were blocked with 5% non-fat milk. The membranes were then incubated overnight with the designated primary antibodies, followed by incubating with HRP-conjugated secondary antibody for an hour on the next day. After washing the membranes with Tris-buffered saline containing Tween-20, the bands were visualized using ECL reagent kit (Thermo Fisher Scientific). The X-ray films (Fujifilm; Tokyo, Japan) were exposed for a period ranging from 30 s to 5 min. GAPDH was used as the housekeeping protein and the protein expression was quantified using Image J (National Institutes of Health; Bethesda, MD, USA).

2.6. Statistical method

Statistical analysis was carried out using Prism 6.0 (GraphPad Software; La Jolla, CA, USA). One-way ANOVA *post hoc* Tukey's test and Two-way ANOVA *post hoc* Bonferroni test were used for analyzing three chamber sociability test results. Unpaired *t*-test was used for analyzing mRNA and protein expression levels. $p < 0.05$ is regarded as statistically significant.

Table 1
Forward and Reverse primer sequences of the targeted genes.

Gene	Forward Primer (5′ → 3′)	Reverse Primer (5′ → 3′)
NR2A	ACCATTGGGAGCGGGTACAT	CCTGCCATGTTGTCGATGTC
NR2B	CCTCCTGAACCTCCTGTGTGAG	CGTGGTCATTCCCAAAGCGTC
NR2C	GTGGTTGCCATCACTGTCTT	GAACAGAGTTGTTGAAGACC
GABRA1	AAAAGCGTGGTTCCAGAAAA	GCTGGTTGCTGTAGGAGCAT
GABRA5	GATTGTGTTCCCCATCTTGTTTGGC	TTACTTTGGAGAGGTGGCCCCTTTT
GAD65	TCAACTAAGTCCCACCCTAAG	CCCTGTAGAGTCAATACCTGC
GAD67	CTCAGGCTGTATGTCAGATGTTC	AAGCGAGTCACAGAGATTGGTC
β-actin	GACAGGATGCAGAAGGAGATTACTG	CCACCGATCCACACAGTACTT

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