Social behavior, neuroimmune markers and glutamic acid decarboxylase levels in a rat model of valproic acid-induced autism

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ABSTRACT — Autism is a complex neurodevelopmental disorder characterized by impaired social communication and social interactions, and repetitive behaviors. The etiology of autism remains unknown and its molecular basis is not yet well understood. Pregnant Sprague-Dawley (SD) rats were administered 600 mg/kg of valproic acid (VPA) by intraperitoneal injection on day 12.5 of gestation. Both 11- to 13-week-old male and female rat models of VPA-induced autism showed impaired sociability and impaired preference for social novelty as compared to the corresponding control SD rats. Significantly reduced mRNA expressions of social behavior-related genes, such as those encoding the serotonin receptor, brain-derived neurotrophic factor and neuroligin3, and significantly increased expression levels of proinflammatory cytokines, such as interleukin-1 β and tumor necrosis factor-α, were noted in the hippocampi of both male and female rats exposed to VPA in utero. The hippocampal expression level of gamma amino butyric acid (GABA) enzyme glutamic acid decarboxylase (GAD) 67 protein was reduced in both male and female VPA-exposed rats as compared to the corresponding control animals. Our results indicate that developmental exposure to VPA affects the social behavior in rats by modulating the expression levels of social behavior-related genes and inflammatory mediators accompanied with changes in GABA enzyme in the hippocampus.

Key words: Developmental neurotoxicity, Valproic acid (VPA), Social behavior, Autism, Glutamic acid decarboxylase (GAD), Rat

INTRODUCTION

Autism is characterized by impaired social interaction, language/communication, range of interests and activities, and repetitive behaviors (Gadia et al., 2004; Rapin and Tuchman, 2008). Autism spectrum disorder (ASD) prevalence in the US and Canada is 1 in 68 individuals (CDC MMWR, 2014; Ouellette-Kuntz et al., 2012) and it represents a public health issue and a large burden for education, social service and economy. The precise etiology of autism remains unknown. Both genetic and environmental factors contribute to ADS (Goines and Ashwood, 2013). Chronic neuroinflammation, gamma aminobutyric acid (GABA) imbalance, immune dys-regulation, microglial activation and genetic factors are proposed to play important role in the pathogenesis of ASD (Vargas et al., 2005; Morgan et al., 2010; Goines and Ashwood, 2013). Exposure to teratogens, such as thalidomide, valproic acid (VPA) and ethanol, may be associated with an elevated risk of development of autism (Strömland et al., 1994; Christianson et al., 1994; Williams and Hersh, 1997).

VPA has been used for the treatment of epilepsy and psychiatric disorders, including bipolar disorder, based on its effect on the inhibitory neurotransmitter, GABA (Haddad et al., 2009). Epidemiological evidence has indicated that the association exists between prenatal VPA exposure and increased risk of autism. The mater-
nal use of VPA during pregnancy increased the risk of ASD 4.42% in the offspring of a Danish population (Christensen et al., 2013). Moreover, it has been reported that a 6-fold increase in the prevalence of neurodevelopmental disorders including ASD in children prenatally exposed to VPA (Bromley et al., 2013). A single administration of VPA in utero was shown to induce autism-like behaviors, such as impairment of social communication, anxiety-like behaviors and lifelong motor deficits in the offspring (Schneider and Przewlocki, 2005; Kolozsi et al., 2009). Rats exposed to VPA showed abnormalities similar to those observed on autopsy and brain imaging of autistic patients (Chomiak et al., 2013).

The mechanism by which exposure to VPA during pregnancy causes autism-like behaviors in both humans and rodents is not known clearly. GABA plays a critical role in maturation of neural networks in the developing brain (Baltz et al., 2010). It was reported that GAD65 and GAD67 protein levels were reduced in 20- to 25- year old autistic patients by postmortem examination (Fatemi et al., 2002). It is possible that changes in GABA levels due to exposure to VPA during early development could disrupt the developing neuronal circuitry and result in autistic behavioral phenotypes. Disruption to the GABA system may be common to VPA exposure and ASD, and an excitatory/inhibitory (E/I) imbalance may trigger to altered information processing in autism models (Rubenstein and Merzenich, 2003). The interaction between immune system and GABAergic system which contributes to altered behavior and communication in ASD is not known yet. It was suggested that neuroimmune crosstalk in response to exposure to environmental factors, altered genetic factors, changes in synaptic neurotrophic factors and dysregulated immune system may contribute to the development of ASD (Ohja et al., 2018).

It is of interest to examine the crosstalk between the immune and nervous systems while considering health and disease. Previously, our research group examined neurological and immunological markers to demonstrate the effects of low-level exposure to toluene, an environmental chemical, on neuroimmune crosstalk in a mouse model (Win-Shwe et al., 2007, 2010, 2012). We hypothesized that prenatal exposure to VPA induced ASD by modulation social behavior-related genes, inflammatory cytokines, GABAergic neurotransmission and oxidative stress accompanied with impaired social behavior. In the present study, we attempted to investigate the social behaviors (using a 3-chamber social behavioral test) and hippocampal expressions of social behavior-related genes, such as those encoding serotonin, brain-derived neurotrophic factor (BDNF), Nlgn3 and proinflammatory cytokines, in a rat model of VPA-induced autism. Furthermore, we also aimed to examine the glutamic acid decarboxylase (GAD), a rate limiting enzyme of GABA, in the hippocampus of VPA-exposed rats for understanding the mechanism of action of VPA.

MATERIALS AND METHODS

Animals

Pregnant Sprague-Dawley (SD) rats (n = 16) were purchased from Charles River Laboratories Japan, Inc., Kanagawa, Japan and were allocated to two different groups (n = 8 per group) as the control group and VPA group. The control group was administered by normal saline alone and VPA group was administrated 600 mg/kg of VPA (Wako Pure Chemical Ind., Ltd., Osaka, Japan) dissolved in saline by intraperitoneal injection on embryonic day (ED) 12.5 of gestation. Injection volume was 200 µL/300 g body weight. We selected the VPA injection date as ED 12.5 according to studies indicating that this approach generates VPA-induced autism model rats successfully (Schneider and Przewlocki, 2005; Kim et al., 2011; Kim et al., 2013). Food (a commercial CE-2 diet, CLEA Japan, Inc., Tokyo, Japan) and water were given ad libitum. The pups were housed in the individual plastic cages with their own dam till weaning (3 weeks old) and after that we picked up one male and one female pup from each mother and used 8 male and 8 female pups from each group for social behavioral test and gene expression assay. Male and female offspring were kept separately, 2 per cage, under controlled environmental conditions (temperature, 22 ± 0.5°C; humidity, 50 ± 5%; lights on 0700-1900 hr). Then, 11- to 13-week-old male and female offspring (n = 8) from the control and VPA-exposed dams were subjected to the 3-chamber social behavioral test with the Any-maze software video-assisted tracking system (Muromachi Kikai Co. Ltd., Tokyo, Japan). Twenty-four hours after completion of the social behavioral tests, the rats from each group (n = 8) were sacrificed under deep pentobarbital anesthesia. The hippocampi from six mice of each group were collected, and right hippocampi were used for the mRNA analyses and left hippocampi were used for protein analyses. The whole brains from two mice of each group were used for histopathological analyses. The experimental protocols were approved by the Ethics Committee of the Animal Care and Experimentation Council of the National Institute for Environmental Studies (NIES), Japan.

Behavioral tasks

All the behavioral test procedures were video-record-
Sociability and preference for social novelty

The apparatus used was a rectangular, three-chambered Plexiglas box (100 cm x 100 cm x 35 cm), with equal sizes of the three chambers. The dividing partitions were also made of clear Plexiglas, with small doorways on each (10 cm x 10 cm) that allowed free access of the animals among the chambers. Wired cups (diameter 15 cm; height, 30 cm) were placed in each of the side chambers to house unfamiliar animals. For habituation, the subject rats from four different groups (male and female VPA-exposed and control rats) were placed in the middle chamber and allowed to explore for 5 min. During the habituation phase, the wired cup in each of the side chambers was empty. Following habituation, for the sociability test, an unfamiliar rat (stranger 1, age-matched rat) was placed in the wired cup in one of the side chambers; the subject rats were allowed to explore for 10 min. The location of stranger 1 in the left or right-side chamber was systematically alternated between trials. The social novelty preference test was performed immediately after the sociability test. For this test, another unfamiliar rat (stranger 2, age-matched rat) was placed in the wired cup on the other side that had been empty during the first 10-minute session, and the subject rat was allowed to explore the two strangers for 10 min. The time spent in exploring the wired cups on either side was measured. The time that the subject rat spent exploring the wired cup was measured as the time spent with its head facing the cup from a distance of within 1 cm.

Quantification of the mRNA expression levels of relevant genes

The right hippocampi from six mice of each group were used for the mRNA analyses. The hippocampal tissue samples were frozen quickly in liquid nitrogen and then stored at −80°C until the total RNA was extracted. Briefly, total RNA extraction from the hippocampal tissue was performed using the BioRobot EZ-1 and EZ-1 RNA tissue mini kits (Qiagen GmbH, Hilden, Germany). The purity of the total RNA was examined, and the quantity was estimated using the ND-1000 NanoDrop RNA Assay protocol (NanoDrop, Wilmington, DE, USA), as described previously (Win Shwe et al., 2006, 2008a, 2008b). Next, we performed first-strand cDNA synthesis from the total RNA using SuperScript RNase H-Reverse Transcriptase II (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s protocol. Next, we examined the mRNA expression levels of 18S rRNA, serotonin, BDNF, interleukin (IL)1 β and tumor necrosis factor (TNF) α by quantitative real-time RT-PCR assay using the Applied Biosystems (ABI) Prism 7000 Sequence Detection System (Applied Biosystems Inc., Foster City, CA, USA); 18S rRNA level was used as the internal control. The primers (5-hydroxytryptamine (serotonin) receptor 5B (5HT5B), NM_024395; BDNF, NM_012513; IL-1β, NM_008361; neuroligin (Nlgn)-3, NM_134336) were purchased from Qiagen, Sample & Assay Technologies. The primers for TNF-α (forward: 5’-GGTTCTTTTGTGGCAGTTT-3’, reverse: 5’-TTCTCTTTGAGCCGGAG-3’) were purchased from Hokkaido System Science (Hokkaido System Science, Hokkaido, Japan). Data were analyzed using the comparative threshold cycle method. Then, the relative mRNA expression levels of the memory function-related genes and the related transduction pathway molecules were individually normalized to the expression levels of 18S rRNA in the respective samples and expressed as the mRNA signals per unit 18S rRNA expression.

Measurement of plasma 8-hydroxydeoxyquanosine (8-OHdG)

Twenty-four hours after completion of the social behavioral test, the male and female rats of the control and VPA-induced autism groups were sacrificed under pentobarbital anesthesia and blood samples were collected (n = 6 for each group). The plasma 8OHdG level was measured using high-sensitive 8OHdG Check ELISA kit (Code #KOG-HS10E), according to the manufacturer’s (Nikken Seil Co., Ltd, Fukuroi, Shizuoka, Japan) directions.

Western blot analyses

The left hippocampal tissue (approximately 45 mg) samples of six mice each from the control and VPA-exposed male and female rats were cut and placed on ice in lysis buffer (250 µL/0.01 g of tissue) (RIPA Buffer Halt Protease (Thermo), Phosphate Inhibitor Cocktail, 0.5 M EDTA:100:1:1). The tissue samples were homogenized with an ultrasonicator (Tomy Seiko Co., Ltd., Tokyo, Japan) while the temperature was maintained at 4°C. The homogenates were centrifuged for 15 min at 14000 rpm at 4°C. The supernatant fraction was collected, and the protein concentration in this fraction was determined using the iMarker Microplate Reader (Bio-Rad Laboratories, Hercules, CA, USA). Twenty micrograms of total protein from each rat were subjected to gel electrophoresis using Mini-PROTEAN TGXTM Gels and transferred to a PVDF membrane (Bio-Rad Laboratories). Membranes were blocked for one hour at
room temperature in Can get signal® PVDF blocking reagent (Toyobo Co. Ltd., Osaka, Japan): DW; 1:1). The blots were incubated with the primary antibody for one night at 4°C (Can get signal® Immunoreaction Enhancer solution 1 (Toyobo Co. Ltd.): GAD2 (D5G2) XP® Rabbit mAb (2000:1) and washed with TBST. After further washes, the PVDF membrane was incubated with the secondary antibodies for one hour at room temperature in a dark room (Can get signal® Immunoreaction Enhancer solution 2 (Toyobo Co. Ltd.), anti-rabbit IGg 10000:1, IR Dye® 680 RD Goat anti-mouse 10000:1 Sigma anti-β-actin, 5000:1), and washed with TBST. The chemiluminescence detection was simplified by the use of the Odyssey Fc. Imaging System.

**Histopathological examination**

Two brains each from the control and VPA-exposed male and female rats were removed after the animals had been deeply anesthetized with sodium pentobarbital; the brains were then fixed in 10% formalin. The fixed brains were dehydrated in a graded ethanol series, cleared with xylene, and embedded in paraffin. Coronal paraffin sections were cut at a thickness of 5 μm using a microtome and mounted on 3-aminopropyltriethoxysilane-coated glass slides. Each section was stained with hematoxylin and eosin (H&E) for histopathological examination.

**Immunohistochemical analyses**

Microglia are among the immune cells of the brain, and microglial activation indicates a neurotoxic effect on the brain. To detect microglial activation in the hippocampus, the hippocampal tissue sections were immunostained with microglial marker Iba1. Briefly, the brain sections were immersed in absolute ethanol followed by 10% H2O2 for 10 min each at room temperature. After rinsing in 0.01-M phosphate buffer saline, the sections were blocked with 2% normal swine serum in PBS for 30 min at room temperature and then reacted with goat polyclonal anti-Iba1 (diluted 1:100; abcam: ab5076; Tokyo, Japan) in PBS for 1 hr at 37°C. Thereafter, the sections were reacted with biotinylated donkey anti-rabbit IgG (1:300 Histofine; Nichirei Bioscience, Tokyo, Japan) in PBS for 1 hr at room temperature. After a further rinsed in PBS, Iba1 immunoreactivity was detected using a Dako DAB Plus Liquid System (Dako Corp., Carpinteria, CA, USA). To detect the immunoreactivity of Iba1 in the hippocampus, photomicrographic digital images (150 dpi, 256 scales) of the hippocampal regions were taken using a CCD camera connected to a light microscope.

**Statistical analysis**

All the data were expressed as the means ± standard error (S.E.). The statistical analyses were performed using the StatMate II statistical analysis system for Microsoft Excel, Version 5.0 (Nankodo Inc., Tokyo, Japan). Paired t test was used to analyze the exploration time to the empty cup and stranger 1, followed by stranger 1 and stranger 2. Messenger RNA data and plasma 8-OHdG levels were analyzed by Student’s t test. Differences at P < 0.05 were considered significant.

**RESULTS**

**Effects of VPA exposure in utero on body and brain weights of adult rats**

To detect the general toxicity, the body and brain weights of adult male and female rats from the control and VPA groups (n = 8 each) were measured at the time of sampling of the brain tissues. Although body weight between the male and female rats are different, no significant changes of body weight and brain weight were found between the control and VPA group of male or female rats (data not shown).

**Effects of VPA exposure in utero on the social behavior**

**Sociability**

The control rats spent more time exploring stranger 1 than the empty cup on the other side (Fig. 1A, *P < 0.05). In contrast, the VPA-exposed rats showed no preference for stranger 1, possibly reflecting decreased sociability.

**Social novelty preference**

The control rats spent more time exploring the unfamiliar stranger 2 than the already known rat (stranger 1) (Fig. 1B *P < 0.05). On the other hand, the VPA-exposed rats showed no preference for stranger 2 and rather preferred stranger 1 to stranger 2 (Fig. 1B, *P < 0.05), indicating their impaired preference for social novelty.

**Effects of VPA exposure in utero on the hippocampal expressions of social behavior-related genes**

Serotonin is a monoamine neurotransmitter and hormone. It acts as a critical modulator of neuronal interactions via different specific transporters, receptors and intracellular signaling pathways. We found significantly decreased hippocampal mRNA expression levels of the gene encoding serotonin in the VPA-exposed male rats.
as compared to those in the control male rats (Fig. 2A, *P < 0.05); the mRNA expression levels were also decreased in the VPA-exposed female rats, as compared to those in the control rats, however, the differences were not statistically significant (Fig. 2D).

BDNF plays a role in synaptic plasticity and has been reported to be involved as a neurotrophic factor in the development and functioning of serotonergic neurons (Martinowich and Lu, 2008). In this study, the mRNA expression levels of the gene encoding BDNF in the hippocampus were significantly decreased in the VPA-exposed male rats as compared to those in the control male rats (Fig. 2B, *P < 0.05); in the female VPA-exposed rats, similar to the case for serotonin, the mRNA expression levels of BDNF were decreased as compared to those in the control female rats, however, the differences were not significant (Fig. 2E).

Neuroligins are proteins which take part in synaptic maturation (Kolozsi et al., 2009). Nlgn3-KO rats exhibit a number of ASD-like behavioral phenotypes and are used for investigating the mechanisms underlying the development of ASD. In our present study, the hippocampal mRNA expression levels of Nlgn3 were significantly decreased in both the male and female rats exposed to VPA as compared to those in the control rats (Fig. 2C, 2F, *P < 0.05).

**Effects of VPA exposure in utero on the hippocampal expressions of inflammatory markers**

To detect the inflammatory response in the brain, we investigated the expression levels of potent inflammatory cytokines such as IL-1 β and TNF-α in the hippocampus. The expression levels of IL-1 β and TNF-α were signif-
Fig. 2. Hippocampal expression of social behavior-related genes. Messenger RNA expression level of (A, D) serotonin receptor 5HT5B, (B, E) BDNF and (C, F) Nlgn3 in the hippocampus of 13-week-old male and female rats after in utero exposure to VPA. Each bar represents the mean ± S.E. (n = 6, * P < 0.05).

Fig. 3. Hippocampal expression of (A, C) IL-1β and (B, D) TNF α mRNAs in 13-week-old male and female rats after in utero exposure to VPA. Each bar represents the mean ± S.E. (n = 6, * P < 0.05).
icantly upregulated in the VPA-exposed group as compared with the control group (Fig. 3A and 3C, *P < 0.05). However, no such changes were observed in the female rats (Fig. 3B and 3D).

Effects of VPA exposure in utero on the plasma 8OHdG level

To understand the mechanism underlying the inflammatory response in the hippocampus of the rats exposed to VPA, we also examined the plasma levels of 8OHdG, which is a sensitive marker of oxidative DNA damage (Kim et al., 2004). Increased 8OHdG levels in tissues, blood and urine have been reported under pathological conditions such as cancer, inflammation, diabetes and neurodegeneration. Whereas the hippocampal 8OHdG level was significantly increased in the VPA-exposed male rats as compared to the control male rats (Fig. 4A, *P < 0.05), there was no significant difference in the plasma 8OHdG level between the control and VPA-exposed female rats.

Western blot analysis for GAD65 and GAD67

Glutamic acid decarboxylase (GAD) is the major rate-limiting enzyme that modulates GABA synthesis from the pool of L-glutamate. In the adult brain, GAD exists in two major isoforms, GAD65 (GAD2) and GAD67 (GAD1), which are products of two independently regulated genes located on chromosomes 2 and 10, respectively (Erlander, 1991). We examined the GAD65 and GAD67 protein levels in the hippocampus by Western blot analysis. We found that the GAD67 protein level was reduced in the VPA-exposed male and female rats as compared to the corresponding control rats (Fig. 5).

Histopathological examination

We investigated the morphological changes in the hippocampi of the control and VPA-exposed male and female rats using H & E staining. We did not observe any remarkable morphological differences in the hippocampi between the control and VPA-exposed mice.

Immunohistochemical analyses

We also examined the activation of the major immune cells, microglia, in the hippocampi of the control and VPA-exposed rats using the microglial marker Iba1. We found that microglial activation was pronounced in the hippocampi of the VPA-exposed male rats as compared with that in the corresponding control rats. Representative digital photomicrographs of Iba1-immunostained sections
taken from the hippocampus of the control and VPA-exposed male and female rats are shown in Fig. 6.

**DISCUSSION**

The major findings of our present study were the impaired sociability and preference for social novelty of both male and female rat models of VPA-induced autism. Moreover, the expressions of social behavior-related genes were significantly reduced and those of proinflammatory cytokines were significantly increased in the hippocampi of the rat models of VPA-induced autism. The observed hippocampal GAD deficiency may be associated with abnormalities in the glutamate/GABA balance in the autistic brain. The rat model of VPA-induced autism exhibited features similar to those seen in human autism, suggesting the usefulness of these animal models as valuable experimental models to study the neurodevelopmental alterations induced by exposure to environmental risk factors.

In regard to the results of the social behavioral tests, the VPA-exposed rats spent less time at the cage occupied by stranger 1 rat in the sociability test, and also spent less time at the cage occupied by stranger 2 rat in the test of preference for social novelty. These results suggest that the VPA-exposed rats had no interest in communicating and showed an inappropriate social interest in a stranger rat. Our results are consistent with those reported from other studies which showed impaired social behavior in VPA-exposed animals (Liu et al., 2016; Mony et al., 2016; Barrett et al., 2017).

Human imaging studies have shown that morphological changes were identified in multiple brain regions including the frontal cortex, hippocampus, amygdala and
striatum of ASD patients (DiCicco-Bloom et al., 2006; Langen et al., 2009). It was reported that neural projections have been identified between the amygdala and hippocampus and between the ventral tegmental area and nucleus accumbens and alteration of sociability occurred after stimulation of these projections (Allsop et al., 2014; Calhoon and Tye, 2015). Moreover, over-functioning of mGluR1a signaling in the hippocampus accompanied with autistic-like behavior were found in prenatal VPA-exposed juvenile rats (Peralta et al., 2016). Taken together, these findings show that the hippocampus plays an important role in social deficit in VPA-induced autism model. Therefore, in the present study, we focused on examining the gene expression assay and histopathology, immunohistochemical analyses in the hippocampus of male and female mice.

It has been shown that serotonin (5-HT) regulates the development of the central nervous system (Whitaker-Azmitia, 2001) and is involved in a broad spectrum of behavioral and psychological processes (e.g., social behavior, aggression and anxiety) and in the pathophysiology of various psychiatric disorders (Murphy et al., 2004). In the present study, among the various subtypes of serotonin receptor, we selected 5HT5B because this subtype has been identified in the mouse and rat especially in the hippocampus (Kinsey et al., 2001) and is involved in social behavior such as social isolation stress (Maekawa et al., 2010). A previous experimental study indicated that mice deficient in neuronal tryptophan hydroxylase 2 (Tph2-/−), which lack brain serotonin, showed impairment of social interaction and communication, and also exhibited highly repetitive and compulsive behaviors (Kane et al., 2012). On the other hand, hyperserotonemia associated with immune abnormalities was observed in autistic subjects (Burgess et al., 2006). It was previously demonstrated that the hippocampal 5-HT levels in rats exposed to VPA in utero were decreased by 46% (Dufour-Rainfray et al., 2010), a report that is consistent with the reduced hippocampal serotonin receptor 5HT5B mRNA levels in the VPA-exposed rats observed in our study.

BDNF has been shown to be closely involved in synaptic plasticity, which is crucial for learning and memory functions. It has been shown that infusion of BDNF in the nucleus accumbens of old aged rats restored synaptic plasticity and improved cognition (Li et al., 2012). In our present study, we found decreased hippocampal BDNF mRNA expression levels in both male and female VPA-exposed rats. Diminished BDNF expression may affect synaptic plasticity and also affect the development and function of serotonergic neurons (Martinowich and Lu, 2008).

Neuroligins represent a family of proteins that plays a critical role in synaptic functions. In the present study, we found decreased Nlgn3 mRNA expression in the hippocampi of both male and female rats exposed to VPA in utero. Our result was consistent with results of Kolozsi’s group, which showed that in-utero exposure to VPA caused reduction of Nlgn3 mRNA expression in the CA1 and dentate gyrus of rats (Kolozsi et al., 2009).

Neuroinflammation and synaptic alterations in several brain areas have been suggested to be involved in the physiopathology of ASD. Increased inflammatory markers have been shown in the medial prefrontal cortex and hippocampus in the rat model of VPA-induced autism (Codagnone et al., 2015). In the present study, we found increased mRNA expression levels of the potent inflammatory markers IL-1 β and TNF-α in the hippocampi of male rats exposed to VPA. Moreover, H & E staining showed no histological abnormalities, although microglial activation was observed in the hippocampi of the male VPA-exposed rats. Our findings suggest the occurrence of neuroinflammation in the VPA-induced autism model.

Oxidative stress plays a critical role in neurodegenerative pathologies. It was proposed that mitochondrial oxidative stress is involved in the development of ASD. A recent study indicated that the intracellular reactive oxygen species (ROS) and mitochondrial ROS, and cell apoptosis were increased in the autism lymphoblastic cell lines (Bu et al., 2017). In our present study, the plasma levels of a potent oxidative stress marker, 8OHdG, were significantly increased in male VPA-exposed rats as compared to the control rats.

Previously, VPA was used for the treatment of psychiatric disorders based on its effect of modulating GABA neurotransmission (Haddad et al., 2009). Imbalance between the excitatory amino acid neurotransmitter glutamate and inhibitory amino acid neurotransmitter GABA is observed in neuropsychiatric diseases. The increased ratio of synaptic excitation/inhibition in the amygdala might be associated with the characteristic behavior in VPA-induced rat autism model (Lin et al., 2013). Moreover, increased expression of glutamatergic proteins was found in the prefrontal networks of the postnatal brains of rat offspring following exposure to VPA in utero (Kim et al., 2013). Impairments in the GABAergic system may critically contribute to an increased synaptic excitation/inhibition ratio, an additional mechanism that may also be involved in reduced GABAergic inhibition, as recently shown in the temporal cortex of rat pups exposed to VPA in utero (Banerjee et al., 2013). Autism patients have been reported to show abnormalities
of the glutamate and GABAergic systems in the blood and platelets (Rolf et al., 1993; Moreno et al., 1992; Moreno-Fuenmayor et al., 1996). Downregulation of GAD 67, but not GAD 65, has been demonstrated in several brain areas of subjects with schizophrenia (Guidotti et al., 2000), another neurodevelopmental disorder which shares several biochemical brain abnormalities with autism. Benes and Berretta (2001) also reported decreases in the expressions of both GAD 67 and 65 in the hippocampal tissues of subjects with bipolar disorder. In our study, we found decreased expressions of GAD 67 in the hippocampi of male or female rats exposed to VPA.

Taken together, our present study showed that prenatal exposure to VPA impaired the social behavior of the offspring, associated with reduced expressions of genes related to social behavior and increased expressions of inflammatory markers in the hippocampi of the rats, and downregulation of the GABA synthetic enzymes in the brain. All of these findings were more pronounced in the male rats. In humans, a strong male predominance has been observed in ASD and boys are diagnosed five times more frequently than girls. In this study, the underlying mechanisms of sex specific effect of VPA was not known. It is possible that different hormonal milieu and maturation status of neurotransmitter system in male and female embryos at the time of in utero VPA exposure. Some reports indicate that the GABAergic system in females matures faster than in males, and the developmental shift that switches from excitatory to inhibitory in the brain after birth, seems to occur earlier in females than in males (Galanopoulou, 2006; Perrot-Sinal et al., 2007). Thus, female embryos may be less susceptible to VPA-induced changes at the time of exposure. Moreover, a neuroimaging study showing different metabolic activity and connectivity in the brain of male and female rats after prenatal exposure to VPA (Cho et al., 2017) likely explained sex-specific effect of VPA.

In the present study, we evaluated the postnatal effects of VPA exposure at a defined time-point in utero in rats. Our results showed behavioral abnormalities in the offspring rats, associated with reduced expressions of social behavior-related gene expressions and increased expressions of inflammatory markers in the hippocampi, and activation of the microglia in the brains of the animals exposed to VPA in utero. In addition, the observed alterations of the GABAergic system indicate the possibility of existence of a glutamate-GABA imbalance in the autistic rat brain. We suggest that both environmental and genetic factors contribute to neurodevelopmental disorder like ASD. Environmental factors such as exposure to neurotoxicants during developmental periods and genetic factors such as mutation or deficiency of social behavior-

![Fig. 7](image)

**Fig. 7.** The possible mechanism of interaction of neuroimmune parameters in VPA-induced ASD. Environmental factors such as prenatal exposure to VPA may induce neuroinflammation via inflammatory cytokines and oxidative stress and that may activate microglia, and the activated microglia may lead to dysregulation of synaptic growth factors such as BDNF, thus contributing to autism-like behavior. Genetic factors such as social behavior-related genes like serotonin and Nlgn3 may predispose to induce ASD. The neuroimmune interaction between GABA and immune system may play a role in VPA-induced ASD.
related genes may cause immune system dysregulations or neuroinflammation via interaction of immunological biomarkers like cytokines, T cells and neurological biomarkers like neurotrophins and toxic substances like reactive oxygen species released by activated microglia. On the other hand, neuroinflammation highly contributes to excitatory/inhibitory neurotransmitter imbalance and consequently to the etiology of autism (Ohja et al., 2018).

From our findings, we suggest that environmental factors such as prenatal exposure to VPA may induce neuroinflammation via inflammatory cytokines and oxidative stress and that may activate microglia, and the activated microglia may lead to dysregulation of synaptic growth factors such as BDNF, thus contributing to autism-like behavior. Genetic factors such as social behavior-related genes like serotonin and Nlgn3 may predispose to induce ASD. The neuroimmune interaction between GABA and immune system may play a role in VPA-induced ASD. The possible mechanism of interaction of neuroimmune parameters in VPA-induced ASD is shown in Fig. 7.

Further studies are needed to explore the role of other brain regions like the medial prefrontal cortex and amygdala and other social behavior related hormones such as oxytocin and vasopressin in the mechanism of VPA-induced autism. Our findings suggest that a rat model of autism generated by prenatal exposure to VPA is a useful model for understanding how developmental insults, such as exposure to environmental chemicals, to molecular pathways in the hippocampus may give rise to ASD-related behaviors.

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Conflict of interest— The authors declare that there is no conflict of interest.

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Social behavior in valproic acid-induced autism rats


