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CELLULAR AND MOLECULAR BIOLOGY

Brain and plasma amino acid concentration in infant rats prenatally exposed to valproic acid

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Abstract: Autism spectrum disorder is associated with alterations in GABAergic and glutamatergic neurotransmission. Here, we aimed to determine the concentration of GABA, glutamate, glutamine, aspartate, taurine, and glycine in brain tissue and plasma of rats prenatally exposed to valproic acid (VPA), a well-characterized experimental model of autism. Pregnant rats were injected with VPA (600mg/Kg) during the twelfth-embryonic-day. Control rats were injected with saline. On the fourteen-postnatal-day, rats from both groups (males and females) were anesthetized, euthanized by decapitation and their brain dissected out. The frontal cortex, hippocampus, amygdala, brain stem and cerebellum were dissected and homogenized. Homogenates were centrifuged and supernatants were used to quantify amino acid concentrations by HPLC coupled with fluorometric detection. Blood samples were obtained by a cardiac puncture; plasma was separated and deproteinized to quantify amino acid concentration by HPLC. We found that, in VPA rats, glutamate and glutamine concentrations were increased in hippocampus and glycine concentration was increased in cortex. We did not find changes in other regions or in plasma amino acid concentration in the VPA group with respect to control group. Our results suggest that VPA exposure *in utero* may impair inhibitory and excitatory amino acid transmission in the infant brain.

Key words: Amino acid, autism, developing brain, valproic acid.

INTRODUCTION

Autism spectrum disorder (ASD) is characterized by persistent deficits in social communication and interaction, as well as restricted, repetitive patterns of behavior, interests, or activities present in the early developmental period (American Psychiatric Association 2013). It has been proposed that the core clinicopathology of ASD is characterized by an accelerated or early brain tissue overgrowth in regions involved in socialization, communication, and emotional functions, which are regularly affected in these patients (Chomiak 2013, Courchesne et al. 2007). Evidence suggests that ASD may be linked with abnormalities in inhibitory and excitatory neurotransmission systems, mediated by gamma-aminobutyric acid (GABA) (Fatemi et al. 2002, 2009, Harada et al. 2011, Zieminska et al. 2018) and glutamate (GLU) (Hassan et al. 2013, Moreno-Fuenmayor et al. 1996, Page et al. 2006, Shimmura et al. 2011, Shinohe et al. 2006, Zieminska et al. 2018, Wang et al. 2018), respectively.

A reduction in protein levels of glutamic acid decarboxylase (GAD_{65} and GAD_{67}), the enzyme responsible for the synthesis of GABA from GLU, has been reported in the cerebellum and parietal cortex from ASD patients (Fatemi et al. 2002), as well as a reduction in GAD_{67} mRNA in the cerebellar Purkinje cells (Yip et al. 2007). Also, an abnormality in the chromosome 15q11–q13,

that contains a $GABA_A$ receptor subunit gene, has been detected in ASD patients (Cook et al. 1998). In addition, there are higher levels of GLU in the plasma from patients with ASD (Hassan et al. 2013, Moreno-Fuenmayor et al. 1996, Shimmura et al. 2011, Shinohe et al. 2006). Magnetic resonance spectroscopy performed in ASD patients detected significantly higher levels of GLU in the bilateral anterior cingulate, the left striatum, the left cerebellar hemisphere, the left frontal lobe (Hassan et al. 2013) and the amygdala-hippocampal region (Page et al. 2006) than in control individuals. Furthermore, postmortem brain samples from ASD patients show lower density of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors and alterations in GLU transporters (Purcell et al. 2001).

There are three types of experimental models for studying autism: genetic, environmental (induced by chemicals) or associated with infection and inflammation. Genetic models of ASD mimic a specific genetic alteration as observed in human syndromes like fragile X (FMR1 gene) and Rett´s (MECP2 mutations). Environmental models are induced by exposure to chemical substances during prenatal or early postnatal period. Those models include valproic acid (VPA) and thalidomide. ASD also can be modeled by producing viral infection in pregnant rodents (for example influenza virus) or by maternal inflammation triggered by the injection of immunogens such as IL-1 and IL-6 during pregnancy (Ergaz et al. 2016). Evidence show that children born from mothers that use VPA as anticonvulsant medication (*in utero* exposure to VPA) have a higher risk for the development of ASD (Christianson et al. 1994, Christensen et al. 2013). Based on that information, an environmental animal model of autism was developed by injecting VPA to pregnant rats and studying the offspring (Rodier et al. 1996). Rats prenatally exposed to VPA have similar cerebral anomalies (Rodier et al. 1996, Ingram et al. 2000, Mychasiuk et al. 2012) and behaviors than those displayed by ASD patients (Mychasiuk et al. 2012, Schneider & Przzewlocki 2005, Schneider et al. 2008). Prenatally VPA exposed rats show impaired hippocampal pre- and post-synaptic inhibitory transmission (Banerjee et al. 2013) and a reduced GAD expression (Kim et al. 2013). They also have changes in hippocampal GLU uptake, glutamine (GLN) synthetase activity, GLU transporters expression (Silvestrin et al. 2013), and an overexpression of N-methyl-D-aspartate (NMDA) receptor subunits (Rinaldi et al. 2007). These alterations might be associated with hyperexcitability or possibly with an increased ratio of synaptic excitation/inhibition at an early developmental age. In rats prenatally exposed to VPA an increase of presynaptic efficacy of excitatory transmission as well as of long-term potentiation in the lateral nucleus of the amygdala have been reported (Lin et al. 2013).

Altogether, these studies suggest that abnormalities in GABAergic and glutamatergic neurotransmission may play an important role in the pathophysiology of autism. In addition, an increase in the ratio between synaptic inhibition and excitation during a critical period may trigger a dysfunctional development of the neural circuits that in turn may cause the core symptoms of ASD, and could also determine the higher prevalence of epilepsy in those patients (Rubenstein & Merzenich 2003, Gogolla et al. 2009). In this study, we aimed to determine whether prenatal exposure to VPA modifies brain and plasma amino acid concentration. We quantify amino acid concentrations in fourteen-day-old rat pups (P14) that were prenatally exposed to VPA. This age was chosen considering that autistic-like behaviors are observed during early childhood. Our results suggest that VPA exposure *in utero* may impair inhibitory and

excitatory amino acid transmission in the infant brain.

MATERIALS AND METHODS

Animals

We controlled the fertility cycles of female Wistar rats from our local breeding colony (Centro de Investigaciones Cerebrales, Universidad Veracruzana) by conducting daily vaginal smears. We placed fertile females overnight with a sexually experienced male. Vaginal smears were collected the following morning and, if spermatozoa were found, this was designated as the first day of pregnancy. During the study, pregnant females were housed individually and maintained in a vivarium on a 12:12 h circadian cycle (lights on at 08:00), at 23-25° C temperature and 60-70% relative humidity, with free access to water and food (Rismart). This study was carried out following the Mexican guidelines on the care and use of laboratory animals (NOM-062-ZOO-1999) and was approved by the Internal Committee for the Care and Use of Laboratory Animals (ICCULA) of the Universidad Veracruzana (protocol Number: CICUAL-CICE 2017-002-c).

Administration of VPA to pregnant rats

On the twelfth embryonic day (E12), rats received a single intraperitoneal injection of 600 mg/kg of VPA (sodium valproate Sigma-Aldrich, St. Louis, MO) dissolved in 0.9% saline for a concentration of 250 mg/mL. VPA dose and exposure window were similar to those used in other studies (Schneider & Przewlocki 2005, Perez-Pouchoulen et al. 2016, Puig-Lagunes et al. 2015, 2016). Control rats received an injection of physiological saline during the same embryonic day. Rats were housed individually and were allowed to care for their litters. Brain amino acid determination was performed in male and female rat pups from both prenatal VPA-exposed (n=16; 10 females, 6 males) and control groups (n=15; 5 females, 10 males). Plasma amino acid quantification was performed in both gender rat pups from prenatal VPA-exposed (n=16; 4 females, 12 males) and control groups (n=8; 3 females, 5 males). Prenatal exposure to VPA produced congenital crocked tail with different grades of severity. No abnormalities were detected in rats from the control group.

Brain tissue processing

P14 rat pups were anesthetized with pentobarbital sodium (60 mg/kg, i.p.), decapitated and their brains were immediately removed to be dissected on an ice-cooled Petri-dish. The following brain regions were collected: frontal cortex (FC), hippocampus (HI), amygdala (AM, amygdala complex containing the piriform cortex), brain stem (BS) and cerebellum (vermis [VE] and hemispheres [HE]). These brain areas were chosen since they have been regularly associated with ASD. Brain tissue from each area was weighed and mechanically homogenized with a wireless pellet pestle (Sigma-Aldrich, Z359971) by adding 30 µl of perchloric acid (0.1 M, $HClO_4$, Baker) containing 4 mM sodium bisulfite (Baker, S-1516) per 10 mg tissue (López-Meraz et al. 2012). The homogenized tissue was centrifuged at 10'000 rpm for 20 min to 4° C. Subsequently, the supernatant was collected and filtered (filters-HV of 0.45 µm, Millipore). The filtrates and the pellet were placed in Eppendorf tubes, frozen in liquid nitrogen and stored at -76° C until amino acids and protein analyses by high-performance liquid chromatography (HPLC) and Bradford method, respectively, were performed.

Collection and processing of blood plasma

A different group of P14 rats was anesthetized with pentobarbital sodium (60 mg/kg, i.p.) and placed in the supine position on a base

to prevent movement. Skin was removed and the abdominal wall was exposed to show the heart (approximately 1 cm caudal to the last rib). Blood (0.2-0.4 ml) was collected by intra-cardiac puncture and placed in EDTA tubes. Samples were gently shaken to homogenize blood with the anticoagulant and then centrifuged at 4'000 rpm for 15 min at 4° C. Plasma was separated from the cell pack, deproteinized with 0.5 M $HClO_4$ and centrifuged again under the same conditions. The supernatant was separated, filtered (filters-HV, 0.45 µm, Millipore) and stored at -76° C until processing for amino acid quantification by HPLC.

Determination of brain tissue and plasma amino acids by HPLC

Amino acid quantification was performed according to a slightly modification of the procedure described by Luna-Munguía et al. (2012). An HPLC system coupled to a fluorescence detector (Waters® model 474), a 3.9 x 20 mm pre-column (Nova-Pack, Waters®) and a reversed-phase 3.9 150 mm column (Nova-Pack, 4 mm, C18, Waters®) were used. For quantification of the brain amino acid concentrations (dilutions fluctuated from 1:200 to 1:1000 depending on brain region), we mixed 20 µl of perfusate with 6 µl of the derivatization agent containing O-phthalaldehyde (OPA; Sigma) and 2-beta-mercaptoethanol (Sigma). Two minutes later, samples were injected manually to the HPLC system. Chromatographic separation was performed using a binary gradient system. The mobile phase A consisted of sodium acetate (40 mM) dissolved in 90% milli-Q water and 10% methanol (pH 6.7); the mobile phase B was a solution of 20% sodium acetate (8 mM) and 80% methanol (pH 5.7). Amino acid concentration was calculated by a linear regression analysis using the Millennium system (Waters®) from a external standard technique using a calibration curve of GABA, GLU, GLN, aspartate (ASP), glycine (GLY) and taurine (TAU) (50, 100, 300 and 500 ng/ml).

The plasma concentration of amino acids was determined using the same HPLC system, but the derivatization agent was a mixture of OPA and N-Acetyl Cysteine (NAC, Sigma). Injection of samples was carried out by an autosampler. In each vial, 15 µL of plasma was mixed with 10 µL of buffer OPA-NAC and let stand in the dark for 2 hours at 5°C; 2 h later samples were injected to the chromatograph. A calibration curve was performed with standard amino acids (200, 100, 50 and 10 ng/ml).

Protein quantification by Bradford method

Protein quantification was performed in the pellet remaining from brain homogenates. Samples were reconstituted with 250 µl of a solution containing 0.1 M $HClO_4$ and 4 mM sodium metabisulfite. Quantification was performed with a microplate reader system (Spectra Max 190) by adding 20 µl of the sample (diluted with ultrapure water Milli-Q, 1:1 or 1:10, depending the brain area analyzed) and 180 µl of Bradford solution (1x, BIO-RAD). A standard curve was prepared with bovine serum albumin (0.05-0.6 µg/µl; Baker).

Statistical analysis

Data were analyzed by an unpaired *Mann Whitney U-test* (MWU) and expressed as the median and the interquartile range. Statistical significance for all comparisons was considered when $p<0.05$. All analyses were performed using the GraphPad Prism version 6.00.

RESULTS

Prenatal VPA exposure increases GLU, GLN and GLY brain concentration

Statistical significant differences in brain amino acid concentration (ng/mg protein) were found in rats prenatally exposed to VPA with respect to controls (Table I). VPA rats showed an increase in the concentration of GLU [51.2 (38.5); MWU = 41.5, p=0.02] and GLN [31.4 (21.4); MWU = 49.5, p=0.04] in the HI, compared to that of control rats [31.2 (28.3) and 18.9 (22), respectively]. In addition, GLY levels were augmented in the FC [6.5 (3.3); MWU = 51, p = 0.03] of rats prenatally exposed to VPA compared to control rats [3 (3.7)]. No statistically significant differences were found between groups for the concentration of other amino acids in any brain region analyzed.

When sex was considered in the evaluation of amino acid concentration, results showed that GLU [14.5 (2.9), MWU=4, p<0.05] and ASP levels in VE [2.8 (2.6); MWU=3, p<0.05] were lower in male rats prenatally exposed to VPA than in control males [22.8 (15.6) and 5.5 (10), respectively]. In the case of female rats, GLU levels in HI [57.3 (28.1); MWU = 4, p<0.03] and BS [32.8 (22.3); MWU = 4, p<0.02] were higher in female rats prenatally exposed to VPA than in control females [29.6 (33.6) and 12.5 (12.8), respectively]. Similarly, GABA [7.9 (3.5); MWU = 1, p<0.03] and ASP concentration [9.1 (2.2); MWU = 1, p<0.01] in BS, was augmented in prenatally VPA exposed females when compared to control female rats [1.4 (4.9) and 4.1 (4.2), respectively]. GABA levels in AM were lower in females prenatally exposed to VPA [4.1 (4.2); MWU = 0, p<0.01] than in control female rats [7.3 (10,9)]. We did not find statistically significant differences associated with sex between treatments for any other brain region or amino acid (Table I).

Prenatal VPA exposure does not modify plasma amino acid concentration

We did not find statistically significant differences in amino acid plasma concentrations between prenatal VPA-exposed and control groups [GABA: MWU = 30, p=0.05; GLU: MWU = 45, p =0.47; GLN: MWU = 46, p = 0.37; ASP: MWU = 37, p =0.14; TAU: MWU = 55, p = 0.75] (Figure 1). In addition, no statistically significant differences associated with sex were observed for any amino acid.

DISCUSSION

Our results show that prenatal VPA exposure increases the concentration of GLU and GLN in HI and the concentration of GLY in FC in P14 rats when compared to control rats. A more detailed analysis shows that specific changes in those concentrations of amino acid are sex- and brain structure-dependent. However, despite the prenatal VPA exposure, the concentration of amino acids in plasma remains unchanged. Our data suggest that VPA exposure *in utero* may impair inhibitory and excitatory amino acid concentration in the infant rat brain, which may contribute to changes in excitability observed in autism models.

Previous studies have quantified changes in brain and blood amino acid levels in ASD patients (Harada et al. 2011, Hassan et al. 2013, Moreno-Fuenmayor et al. 1996, Page et al. 2006, Shimura et al. 2011, Shinohe et al. 2006, Aldred et al. 2003) and in experimental rat models (Meurs et al. 2008, Cavalheiro et al. 1994, Zieminska et al. 2018). However, results are inconsistent and therefore, difficult to interpret. Differences found among published results may be due to different analytical methods used in each study (for example HPLC, magnetic resonance (MR) or nuclear magnetic resonance (NMR) spectroscopy). Our results showed that VPA rats

Table I. Effect of valproic acid prenatal exposure on brain tissue and plasma amino acid levels in rats.

Brain tissue (ng/mg protein)						
Males and females						
Brain region/ Group	**GABA**	**GLU**	**GLN**	**ASP**	**GLY**	**TAU**
FC						
SS	3.9 (7.7)	24.4 (11.6)	18.6 (24)	8 (12.3)	3 (3.7)	34.4 (26.1)
VPA	2.9 (2.3)	29.4 (22.8)	26.8 (24.4)	13.8 (11.2)	6.5 (3.3)	50.8 (27.8)
	MWU=47, p=0.09	MWU=65, p=0.33	MWU=90.5, p=0.74	MWU=79, p=0.57	MWU=51, p=0.03*	MWU=57, p=0.10
HI						
SS	4.4 (6.8)	31.2 (28.3)	18.9 (22)	6.6 (9.4)	3.5 (3.8)	30.8 (33.6)
VPA	3.1 (4.4)	51.2 (38.5)	31.4 (21.4)	12.4 (10.7)	5.5 (3.3)	50.6 (34)
	MWU=63, p=0.61	MWU=41.5, p=0.02*	MWU=49.5, p=0.04*	MWU=61.5, p=0.15	MWU=72.5, p=0.25	MWU=46, p=0.05
AM						
SS	6 (5.5)	45.7 (59.4)	25 (35.5)	12.9 (20.4)	5.2 (4.2)	43.7 (58.7)
VPA	4.4 (2.2)	41.9 (27.8)	23.6 (14)	11 (6.6)	3.7 (3.7)	40.2 (23.8)
	MWU=46.5, p=0.05	MWU=66.5, p=0.36	MWU=76, p=0.48	MWU=84, p=0.74	MWU=65, p=0.49	MWU=70, p=0.34
BS						
SS	3.1 (9.6)	18 (22.6)	15.5 (21.6)	8 (10.8)	3.8 (5.5)	21.4 (29.1)
VPA	7.2 (3)	32.3 (13.4)	21.2 (11.6)	9.1 (3.7)	4.6 (2.9)	33.1 (18.8)
	MWU=47, p=0.09	MWU=49, p=0.12	MWU=67, p=0.38	MWU=68, p=0.41	MWU=75, p=0.65	MWU=55.5, p=0.14
VE	1.8 (4.2)	21.5 (37.6)	14.4 (11)	6.1 (12)	3.4 (5.2)	21.4 (8.5)
SS	2.4 (1.3)	15.2 (16.9)	13.8 (8.2)	3.7 (4)	3.5 (1.7)	21.6 (11.1)
VPA	MWU=61, p=0.36	MWU=55.5, p=0.79	MWU=81.5, p=0.65	MWU=54.4, p=0.07	MWU=99, p=0.80	MWU=81.5, p=0.65
HE						
SS	1.8 (2.4)	22.8 (36)	25.8 (18.2)	7.2 (9.5)	3.8 (2.6)	38.6 (29.6)
VPA	2.3 (2.4)	25.3 (10.5)	20.7 (6.7)	7.1 (3.7)	3.3 (2.5)	29.5 ± 4.5
	MWU=60, p=0.34	MWU=79, p=0.79	MWU=91, p>0.99	MWU=91, p>0.99	MWU=105, p=0.88	MWU=81, p=0.45
Males						
FC						
SS	4.6 (7.8)	27.5 (41.7)	18.6 (20.6)	8 (11.2)	3 (3.2)	35.2 (27.8)
VPA	2.9 (2.3)	40.5 (21.5)	29.1 (25.1)	12 (10.7)	6.4 (5)	51.4 (22.1)
	MWU=10, p=0.10	MWU=20, p=0.59	MWU=17, p=0.35	MWU=18, p=0.60	MWU=10, p=0.07	MWU=22, p=0.76
HI						
SS	4.3 (7.2)	31.2 (28.7)	18.9 (23.4)	6.6 (18.3)	3.8 (4.5)	35.2(31.2)
VPA	2.5(4.7)	34.7 (65)	27 (41.1)	8.3 (27.6)	5.2 (3.8)	42.9 (45)
	MWU=13.5, p=0.53	MWU=14, p=0.60	MWU=12.5, p=0.31	MWU=116 p=0.82	MWU=17, p=0.73	MWU=11, p=0.33
AM						
SS	5.8 (7.5)	43 (81.6)	22.9 (47.8)	11.8 (18.4)	4.5 (7.4)	36 (79)
VPA	5.6 (3.1)	34.1 (18.8)	16.7 (14.7)	9.9 (4.8)	3 (2.4)	35 (19.2)
	MWU=22, p=0.81	MWU=19, p=0.54	MWU=21, p=0.71	MWU=21, p=0.71	MWU=14.5, p=0.63	MWU=20, p=0.90

BS						
SS	4.8 (10.4)	28.3 (38)	19 (23.4)	13 (12.9)	3.6 (5.8)	25.3 (33.6)
VPA	6.9 (2.1)	24.4 (20)	16.8 (10.5)	9.3 (7.1)	4.6 (2.7)	34.5 (11.1)
	MWU=17, p=0.93	MWU=22, p=0.93	MWU=17, p=0.67	MWU=23, p=0.65	MWU=17, p=0.51	MWU=16, p=0.34
VE						
SS	1.7 (1.9)	22.8 (15.6)	16.6 (10.1)	5.5 (10)	3.7 (6.1)	22.5 (8.4)
VPA	2.4 (0.6)	14.5 (2.9)	13.7 (2.2)	2.8 (2.6)	3.4 (1.9)	19.9 (8.5)
	MWU=8.5, p=0.32	MWU=4, p=0.04*	MWU=13, p=0.48	MWU=3, p=0.02*	MWU=23, p=0.80	MWU=12, p=0.37
HE						
SS	1.6 (0.5)	19.7 (37.8)	20.3 (20.4)	7.2 (14.2)	3.2 (2.7)	24.7 (28.1)
VPA	2.7 (1.3)	25.3 (14.6)	23.5 (7.7)	8.8 (5.6)	4.1 (1.9)	26.7 (19.7)
	MWU=6.5, p=0.11	MWU=22, p=0.93	MWU=19, p=0.67	MWU=23, p=0.68	MWU=23, p=0.85	MWU=26, p=0.90
Females						
FC						
SS	1.9 (7.9)	24.7 (35.2)	27.1 (44.2)	12.4 (18.2)	5.5 (6.4)	26.9 (27.1)
VPA	2.9 (2.1)	42.7 (33.4)	25.4 (24.4)	15.7 (14.4)	6.7 (4)	50.3 (37.5)
	MWU=12, p>0.99	MWU=6, p=0.24	MWU=14.5 p=0.63	MWU=18, p>0.99	MWU=17, p=0.85	MWU=6, p=0.18
HI						
SS	5 (6.1)	29.6 (33.6)	13.1 (21.6)	5.3 (6.9)	2.7 (3.5)	34.1 (35.1)
VPA	3.7 (4.3)	57.6 (28.1)	31.4 (17.5)	14.6 (10.6)	5.8 (3.5)	55.7 (33.8)
	MWU=8, p=0.49	MWU=4, p=0.03*	MWU=6, p=0.07	MWU=7, p=0.07	MWU=8, p=0.09	MWU=9, p=0.13
AM						
SS	7.3 (10.9)	48.4 (51.7)	28.4 (33)	22.1 (19.9)	5.7 (2.3)	43.7 (42.1)
VPA	4.1 (2.2)	48.4 (33)	26.8 (15.2)	12.7 (12.2)	4.1 (4.6)	40.1 (43.2)
	MWU=0, p=0.001*	MWU=12.5, p=0.45	MWU=13, p=0.35	MWU=15, p=0.49	MWU=15, p=0.92	MWU=14, p=0.29
BS						
SS	1.4 (4.9)	12.5 (12.8)	12.3 (30.3)	4.1 (4.2)	4.7 (7.1)	12 (17.4)
VPA	7.9 (3.5)	32.8 (22.3)	22.7 (10.6)	9.1 (2.2)	4.5 (3.9)	33 (39.5)
	MWU=1, p=0.03*	MWU=2, p=0.02*	MWU=9, p=0.19	MWU=1, p=0.01*	MWU=16, p=0.85	MWU=10, p=0.26
VE						
SS	2.3 (6.5)	18 (64.3)	12.8 (29.2)	3.2 (18)	2.4 (5.7)	21.2 (50.2)
VPA	2.3 (2.1)	15.7 (18.6)	15.3 (11.2)	3.7 (5.2)	3.4 (1.9)	22.5 (20)
	MWU=19.5, p=0.97	MWU=17.5, p=0.54	MWU=19, p=0.86	MWU=223, p=0.83	MWU=17, p=0.66	MWU=20, p>0.99
HE						
SS	2.5 (4.4)	31.9 (30.7)	26.1 (13.6)	7.6 (7)	3.7 (2.8)	40.6 (22.5)
VPA	2.1 (1.6)	25.5 (10.9)	20.6 (10.4)	6.1 (3.6)	3.2 (2.4)	30.2 (14.9)
	MWU=16, p=0.59	MWU=13, p=0.65	MWU=14, p=0.60	MWU=13, p=0.68	MWU=14, p=0.60	MWU=12, p=0.18

Abbreviations: GABA: gamma-aminobutyric acid; GLU: glutamate; GLN: glutamine; ASP, aspartate; GLY: glycine; TAU: taurine; FC: frontal cortex; HI: hippocampus; AM: amygdala; BS: brain stem; VE: cerebellar vermis; HE: cerebellar hemispheres. Amino acid concentration is shown as the median and the interquartile range and was analyzed with a Mann Whitney U-test. *p<0.05 vs control group.

had higher GLU and GLN levels in the HI when compared to control rats. The latter is consistent with the finding of a high GLU concentration observed in the amygdala-hippocampal region from humans with ASD (Page et al. 2006). An increase in the concentration of GLU and GLN in brain tissue is also seen in male rat hippocampus in the VPA and thalidomide-induced models of autism (Zieminska et al. 2018). Interestingly, Kim and collaborators found an increase in the glutamatergic neuronal marker VGluT1 in the hippocampus from rats prenatally exposed to VPA during E12 (Kim et al. 2013). GLU plays a key role in brain development, neurotransmission and neurotoxicity. GLU stimulates neurite outgrowth, synaptogenesis and maturation of synapsis in the developing brain (Richards et al. 2005, Saneyoshi et al. 2010, Kwon & Sabatini 2011). These data suggest that prenatal VPA exposure enhances glutamatergic neurotransmission in the rat hippocampus. Furthermore, male rats prenatally exposed to VPA show an increase in the glutamatergic synaptic transmission in the dorsal raphe nucleus (Wang et al. 2018).

We did not find statistically significant differences in blood amino acid levels between prenatal VPA-exposed and control groups. These results are consistent with previous studies reporting no changes in plasma or platelet amino acid concentrations in ASD patients (Arnold et al. 2003, Elbaz et al. 2014). However, other studies have found higher levels of GLU in plasma from ASD patients (Hassan et al. 2013, Shinohe et al. 2006, Aldred et al. 2003). Discrepancies could be due to the age of the patients with ASD (Dhossche et al. 2002) and the broad range of signs and symptoms observed in those patients (Moreno-Fuenmayor et al. 1996). We focused on P14 rat pups, because at that age the brain rat development resembles that of the human infant/toddler (Sengupta 2013). However, ASD patients included in the studies mentioned above have a broader range of age (Spence & Schneider 2009).

GABA is the main inhibitory neurotransmitter in the adult brain, but it is excitatory in immature neurons (Ben-Ari 2001, 2002). This is due to changes in the concentration gradient of Cl^-, which depends on the expression of the K-Cl (KCC2) and Na-K-2Cl cotransporters (NKCC1) (Ben-Ari 2006, Kahle et al. 2008). The change of GABA from excitatory to inhibitory is observed in rats during the second week of life and until the end of the first month (Ben Ari 2002, Perrot-Sinal et al. 2003). It is important to take into account this information considering that we studied P14 rats. However, our results show no changes in GABA brain concentration in rats prenatally exposed to VPA when compared to control rats. Our data differ from that reported recently in rats prenatally exposed to VPA and thalidomide (Zieminska et al. 2018). Those authors found that levels of GABA in HI are significantly increased in both ASD models. A possible difference between both studies may be the age of the rats; we performed our analysis specifically during the 14-postnatal day, whereas the study by Zieminska et al. (2018) was carried out in one-month-old rats. A previous study show that rats prenatally exposed to VPA have reduced GAD expression in the cortex and the HI (Kim et al. 2013). Application of VPA to cortical neurons in culture reduces GAD expression (Fukuchi et al. 2009). Furthermore, a decrease in the expression and function of GAD is seen in the parietal cortex and cerebellar Purkinje cells from ASD patients (Fatemi et al. 2002, Yip et al. 2007). A decrease in GABA concentration has been reported in plasma, platelet, and brain from patients with ASD (Harada et al. 2011, Cochran et al. 2015, Dhossche et al. 2002). We did not find such decrease in our study.

A caveat to our study is the fact that we used the barbiturate pentobarbital as

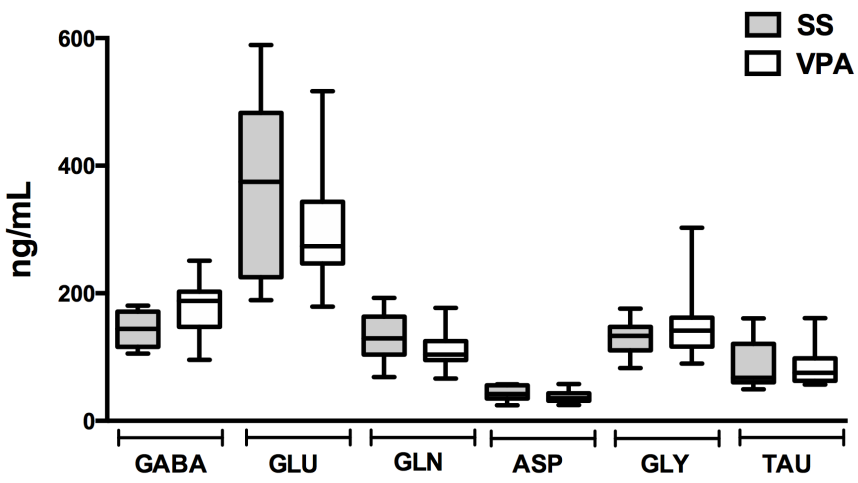

Figure 1. Brain plasma amino acid levels in infant rats prenatally exposed to VPA or Saline. GABA: gamma-aminobutyric acid; GLU: glutamate; GLN: glutamine; ASP: aspartate; GLY: glycine; TAU: taurine. No differences were observed between groups when data were analyzed with a *Mann Whitney U-test.*

anesthetic. In rat CA3 neurons, pentobarbital decreases excitatory synaptic transmission by activating $GABA_A$ receptors and inhibiting voltage-dependent Na^+ and Ca^{++} channels, affecting GABA and glutamate release (Shin et al. 2013). To our knowledge, the dose of pentobarbital that we used does not modify the concentration of amino acids in the brain. However, phenobarbital, another barbiturate, at doses higher than 100 mg/kg produces a slight decrease in GABA concentration (Battistin et al. 1984).

We found an increase in GLY concentration in the cortex of rats prenatally exposed to VPA. It is known that GLY is the main inhibitory neurotransmitter in the spinal cord and the BS (Legendre 2001). However, GLY is also a co-agonist of glutamate for NMDA receptors, which mediate excitatory neurotransmission. High concentrations of GLY cause hyperexcitability and neurotoxicity in hippocampal brain slices (Newell et al. 1997). Thus, it is also possible that a high GLY concentration in FC after prenatal VPA exposure facilitates GLU-mediated excitability. Since our study is merely descriptive, additional experiments should be carried out to characterize the possible role of GLY in the VPA model of ASD.

ASD is more common in male than in female children (Christensen et al. 2013). When we considered sex, our results showed differences in the concentration of some brain amino acids in prenatally VPA exposed male and female rats compared to control rats. These differences were observed in VE, BS, and AM. A recent study using VPA and thalidomide models shows increased levels of GLU, GLN and GABA in male´s hippocampus (Zieminska et al. 2018); however, in that study, no other brain regions were evaluated. Our results showed that male rats prenatally exposed to VPA have decreased concentrations of GLU and ASP in VE, but not changes in the HI. Female rats prenatally exposed to VPA showed higher concentrations of GLU in HI and BS than their matched controls. This latter result is similar to the findings of Zieminska et al. (2018). Females prenatally exposed to VPA also had increased concentrations of GABA and ASP in BS, but a lower concentration of GABA in AM.

Epidemiologic studies have shown a high prevalence and incidence of epilepsy in ASD patients (Bolton et al. 2011, Tuchman et al. 2010, Canitano 2007, Hara 2007). Both diseases are characterized by an excitatory/inhibitory imbalance (Hassan et al. 2013, Lam et al. 2006, Tebartz van Elst et al. 2014).

The increased glutamatergic activity or the suppression of the GABAergic system support the hyperglutamatergic hypothesis in the pathogenesis of autism (Fatemi et al. 2002, Harada et al. 2011, Hassan et al. 2013, Page et al. 2006, Shimmura et al. 2011, Lam et al. 2006, Tebartz van Elst et al. 2014). Data from our study support that prenatal VPA exposure can produce a deregulation in the neurochemistry of glutamatergic system, which may explain the higher seizure susceptibility observed in Rats prenatally exposed to VPA (Kim et al. 2011, Puig-Lagunes et al. 2016).

In conclusion, in two-week-old rat pups prenatally exposed to VPA, there is an increase in the concentration of GLU and GLN in hippocampus and in the concentration of GLY in frontal cortex. These changes may be the cause of the imbalance between inhibition and excitation proposed as pathogenesis of ASD. To our knowledge, this is one of the first studies that evaluates the effect of prenatal VPA exposure in amino acid concentration in different brain regions of the infant rat. This approach could help to understand the neurochemical and behavioral changes associated with ASD.

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