Prefrontal Cortex, Hippocampus, and Basolateral Amygdala Plasticity in a Rat Model of Autism Spectrum

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KEY WORDS   valproic acid; prefrontal cortex; hippocampus; amygdala; cortical thickness; autism

ABSTRACT   We aimed to investigate the effect of prenatal administration of valproic acid (VPA) (500 mg/kg) at embryonic day 12.5 on the anatomical properties of the prefrontal cortex, hippocampus, and basolateral amygdala, at three different ages: immediately after weaning (postnatal day 21 [PD21]), prepubertal (PD35), and postpubertal (PD70) ages in a rat model of autistic spectrum disorder. Quantitative analysis of the thickness of the prefrontal cortex revealed a reduced size at all study ages in the cingulate 1 area of the prefrontal cortex and CA1 of the dorsal hippocampus in prenatally exposed animals compared to controls. At the level of the basolateral amygdala, a reduction in the size was observed at PD35 and PD70 in the VPA group. In addition, a reduced thickness was observed in the prelimbic region of the prefrontal cortex in VPA animals at PD35. Interestingly, no differences in cortical thickness were observed between control and VPA animals in the infralimbic region of the prefrontal at any age. Our results suggest that prenatal exposure to VPA differentially alters cortical limbic regions anatomical parameters, with implication in the autistic spectrum disorder. **Synapse 68:468–473, 2014.**   © 2014 Wiley Periodicals, Inc.

INTRODUCTION

Autism spectrum disorder (ASD) is a severe behavioral disorder that develops in the first 3 years of life with an incidence of two to five cases in 10,000 births. It is characterized by pervasive impairments in social interaction, deficits in verbal and nonverbal communication, and stereotyped and repetitive patterns of behaviors and interests. While all individuals diagnosed as autistic are grossly impaired in the area of social relatedness, there is a high level of variability with respect to the severity and specific nature of cognitive impairments (Bachevalier, 1994; Szatmari and Jones, 1991). Interestingly, it is now generally believed that autism involves alterations in several brain regions, as opposed to being psychodynamically determined (Rapin, 1991). In addition, 85–90% of autistic subjects show some indication of underlying brain dysfunction (Steffenburg, 1991). A considerable number of neuro-imaging studies have suggested that cortical inter-connectivity is dysfunctional in ASD (for review, see Wass, 2011). Therefore, several researches are presently going on to gain understanding on specific neurological substrates of ASD.

The prenatal exposure to VPA has been proposed as a neurodevelopmental model of ASD-like behavioral abnormalities in the rat (Ingram et al., 2000; Markram and Markram, 2010; Rodier et al., 1997). Altered behaviors include hyper-responsiveness to novel environment (Schneider and Przewłocki, 2005), deficits in sensory gating and social interaction (Schneider et al., 2006), and stereotyped or repetitive behaviors (Schneider and Przewłocki, 2005). Interestingly, prenatal VPA exposure induces neuronal rearrangements at the level of cortical subregions (Bringas et al., 2013; Mychasiuk et al., 2012; Snow et al., 2008). In addition, our recent report suggests

that prenatally VPA exposed rats show rearrangement of dendritic morphology in limbic cortical regions at different ages, supporting the underconnectivity theory (Bringas et al., 2013). This theory suggests that ASD is a cognitive and neurobiological disorder marked—and possibly caused by—underfunctioning of long-distance integrative circuitry, eventually resulting in a deficit of integration of information at the neural and cognitive levels (for review, see Wass, 2011).

In order to assess whether or not limbic cortical sub-regions are altered in VPA prenatally exposed rats during the postnatal development, we analyzed the thickness of the cingulate1 (Cg1), Cg3 and infralimbic regions of the PFC, of the CA1 area of the dorsal hippocampus and of the BLA in VPA animals and control animals at three different ages: immediately after weaning (postnatal day 21 [PD21]), prepubertal (PD35), and postpubertal (PD70) ages.

MATERIAL AND METHODS
Animals

Pregnant Sprague-Dawley rats were obtained at gestational day 10 from our facilities (University of Puebla). Rats were individually housed in a temperature and humidity controlled environment on a 12-12 h light-dark cycle with free access to food and water. All procedures described in this study were in agreement with the "Guide for Care and Use of Laboratory Animals" of the Mexican Council for Animal Care (Norma Oficial Mexicana NOM-062-ZOO-1999) and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and the number of animals used in this study.

Prenatal VPA exposure

Valproic acid (Sigma-Aldrich, St. Louis, MO) was purchased as sodium salt and dissolved in 0.9% saline at a concentration of 250 mg/mL. Females received a single intraperitoneal injection of 500 mg/kg sodium valproate (VPA) or physiological saline (vehicle) on E12.5 as previously described (Bringas et al., 2013; Hara et al., 2012; Kataoka et al., 2013; Rinaldi et al., 2007; Schneider and Przewlocki, 2005). Unchanged litter size, pup body weight, and general

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Abbreviations

ASD	autism spectrum disorder
BLA	basolateral amygdale
DA	dopamine
EEG	electroencephalography
MRI	magnetic resonance imaging
PD	postnatal day
PFC	prefrontal cortex
VPA	valproic acid

health of the mothers and pups (data not shown) were indications of normal rearing conditions for treated rats. Females raised their own litters. Male offspring were weaned on postnatal day 21 (PD21). No more than three siblings were housed together in cages. Vehicle rats and VPA-treated rats were housed in separate cages. Animals had free access to food and water.

Cortical thickness quantification

To evaluate possible changes in the limbic cortical thickness in autism-related regions including PFC, hippocampus and BLA, we used the Golgi-Cox stain samples that were previously used to analyze dendritic arborization and neuronal spine density at PD21, PD35, and PD70 (Bringas et al., 2013). Briefly, rats from the two treatments were anesthetized with sodium pentobarbital (60 mg/kg body weight, i.p.) and perfused intracardially with saline solution. Brains were removed and stained by modified Golgi-Cox method as described previously (Alcantara-Gonzalez et al., 2010; Bringas et al., 2012; Flores et al., 2005; Gibb and Kolb, 1998; Juarez et al., 2008). Coronal sections of 200-μm thickness from the PFC, hippocampus and BLA were obtained using a vibratome (Campden Instruments, MA752, Leicester, UK). These sections were collected on clean gelatin-coated microscope slides and treated with ammonium hydroxide for 30 min, followed by 30 min in Kodak film fixer and subsequently washed with distilled water, dehydrated and cleared in successive baths of 50% (1 min), 70% (1 min), 95% (1 min), and 100% (2 × 5 min) ethanol followed by 15 min in a xylene solution. Subsequently, the slides were covered with balsam resinous medium.

The cortical thickness (in mm) was measured in the Golgi-Cox-stained slides using a Zeiss-Jena projector at a magnification of X 10 (Mychasiuk et al., 2012). Three PFC subregions were analyzed, Cg1, prelimbic and infralimbic areas, which were measured for four different plates (3.7, 3.2, 2.7, and 2.2 mm from Bregma; Paxinos and Watson, 1998) in each hemisphere. The CA1 area of the dorsal hippocampus was analyzed. This region was measured for six different plates (-2.6, -3.14, -3.3, -3.6, -3.8, and 4.16 mm from Bregma; Paxinos and Watson, 1998). Finally, the area of BLA was measured for nine different plates (-1.6, -1.8, -1.88, -2.12, -2.3, -2.56, -2.8, -3.14, and -3.3 mm from Bregma; Paxinos and Watson, 1998) by using a software developed by BUAP (with the help of the software MATLAB Versión 5), using which the separate plates of each region were collapsed to get a representative analysis of each cortical thickness region and the area of the total BLA (Fig. 1).

Statistical analysis

The mean values for each brain region were treated as a single measurement for the data analysis
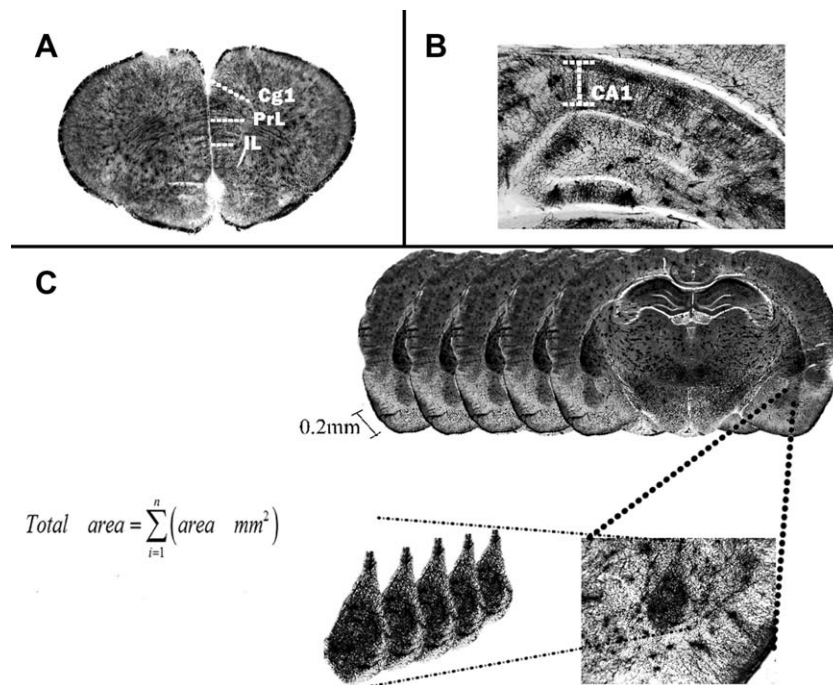
Fig. 1. (**A**) Three PFC subregions were analyzed, Cg1, prelimbic and infralimbic areas, in order to measure cortical thickness (3.7, 3.2, 2.7, and 2.2 mm from Bregma; Paxinos and Watson, 1998). (**B**) Dorsal hippocampus CA1 area was measured for six different plates (−2.6, −3.14, −3.3, −3.6, −3.8, and 4.16 mm from Bregma; Paxinos and Watson, 1998). (**C**) The areas of the BLA were measured for nine different plates (−1.6, −1.8, −1.88, −2.12, −2.3, −2.56, −2.8, −3.14, and −3.3 mm from Bregma; Paxinos and Watson, 1998) and averaged for total area.

($n = 7$–9). The data were analyzed by two-way ANOVA, followed by the Sidak test for post hoc comparisons, with prenatal VAP exposure and age as independent factors. $P < 0.05$ was considered significant in all tests.

RESULTS

The effect of prenatal VPA exposure on cortical thickness of the infralimbic, prelimbic and Cg1 region of the PFC and CA1 of the dorsal hippocampus were analyzed (Figs. 2A–2E). In addition the basolateral amygdala was analyzed by measuring the area (Fig. 2E).

Consistent with previous report (Hara et al., 2012; Mychasiuk et al., 2012), a two-way ANOVA revealed that cortical thickness of the Cg1 of the PFC was significantly affected by VPA prenatal exposure ($F_{1,47} = 14$, $P < 0.001$), by age ($F_{2,47} = 26$, $P < 0.01$), and by VPA and age interactions ($F_{2,47} = 45$, $P < 0.001$). Post hoc tests showed that the cortical thickness of the Cg1 of the PFC of VPA animals at PD21 ($P < 0.001$) and PD35 ($P < 0.001$) was significantly reduced compared to their corresponding control group (Fig. 2A). In contrast, at PD70, cortical thickness of the Cg1 of the PFC of VPA animals was significantly increased ($P < 0.001$) compared to its corresponding control group (Fig. 2A). In addition, the two-way ANOVA analysis (age, $F_{2,47} = 5.4$, $P < 0.01$,

VPA and age interaction, $F_{2,47} = 8.3$, $P < 0.001$) of the prelimbic (Cg3) region of the PFC from VPA animals showed a decrease in cortical thickness only at PD35 ($P < 0.001$) (Fig. 2B) compared to its corresponding control group. Further analysis of the infralimbic cortex suggested that no differences between VPA and control rats take place at any age (Fig. 2C). Interestingly, the effect of prenatal VPA exposure on the CA1 region of the dorsal hippocampus was a reduction in the cortical thickness at all ages ($P < 0.01$) (Fig. 2D, two-way ANOVA, VPA, $F_{1,47} = 68$, $P < 0.001$, age, $F_{2,47} = 8.4$, $P < 0.01$).

Finally, the analysis of the area of the basolateral amygdala revealed that prenatal VPA animals show a significant reduction in the area of the BLA at PD21 ($P < 0.05$) and PD35 ($P < 0.01$) compared to their corresponding control group (Fig. 2E, two-way ANOVA, VPA, $F_{1,34} = 30$, $P < 0.001$) without change at PD70.

DISCUSSION

This study demonstrated that the antiepileptic agent VPA administered prenatally reduces cortical thickness in the CA1 dorsal hippocampus at all analyzed ages as well as in Cg1 area of the PFC at PD21 and PD35 and in prelimbic area of the PFC at PD35. On the contrary, the thickness of the Cg1 region of the PFC increases at P70. No changes were observed
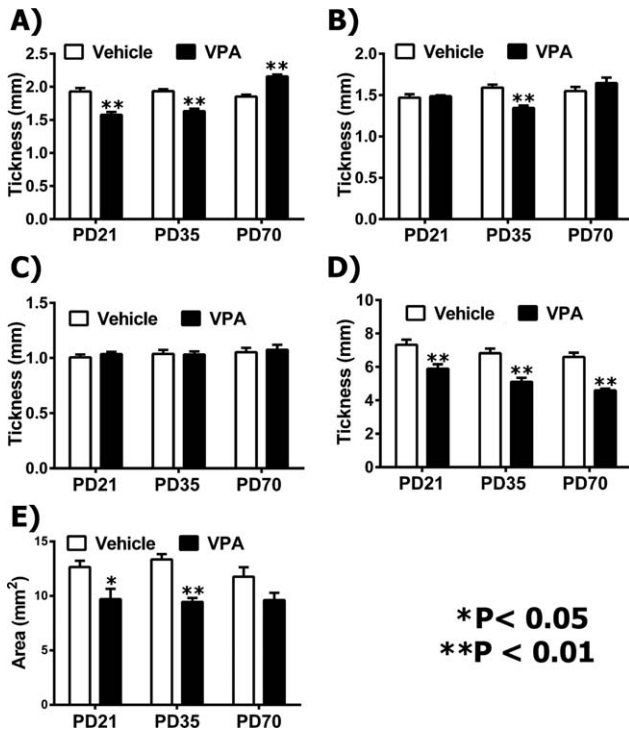
Fig. 2. Effect of prenatal VPA injection on cortical thickness of the prefrontal cortex (PFC) and hippocampus and on the basolateral amygdala (BLA) area size at PD21, PD35, and PD70. (**A**) Cg1 of the PFC, (**B**) prelimbic area of the PFC, (**C**) infralimbic region of the PFC, (**D**) CA1 of the dorsal hippocampus, and (**E**) area size of the BLA.

in the infralimbic area of the PFC. Interestingly, measurements of the surface area of the BLA showed for the first time that VPA exposure also reduces the size of the amygdala at PD21 and PD35. The morphological alterations in cortical thickness reported here are consistent with our previous report using the same animal model (Bringas et al., 2013). We previously found reduced dendritic arborization at P21 and number of dendritic spines at all analyzed ages (PD21, PD35 and PD70) with an increase in the dendritic arborization at PD 70 in the PFC of VPA rats (Bringas et al., 2013). In addition, our previous report showed a reduction of the dendritic arborization at PD35 and PD70 and dendritic spines density at PD70 in dorsal hippocampus of the VPA animals (Bringas et al., 2013). Our data are consistent with a recent report suggesting that prenatal VPA exposed animals display a reduction in cortical thickness (Mychasiuk et al., 2012)—in unspecified subregions—but only at PD106.

Animal studies using prenatal exposure to VPA have been conducted to model the core signs of autism and identify the brain regions linked to the deficits. These animals exhibit the three core signs of autism; impaired social behavior, stereotypic/repetitive behaviors, and sensory/communication deficits (for review, see Roullet et al., 2013). However, the

mechanism by which prenatal administration of VPA induces different long term changes in the thickness of PFC and dorsal hippocampus and in the size of the BLA are not clear. The fact that VPA animals show decreased number of Nissl-positive cell in the PFC (Hara et al., 2012), reduced dendritic arborization in the hippocampus and reduced dendritic spine density in the PFC and BLA (Bringas et al., 2013; Mychasiuk et al., 2012), together with higher hippocampal cells density in the CA1 hippocampus field (Edalatmanesh et al., 2013) support the hypothesis that an abnormal extent of connectivity of PFC, Hippocampus and BLA characterizes this animal model. In addition, this animal model also shows enhanced NMDA receptor expression, larger NMDA synaptic currents in neocortical pyramidal neurons (Rinaldi et al., 2007, 2008), hyperreactivity to electrical stimulation with enhanced synaptic plasticity, as well as a deficit in inhibition at cellular level in the BLA (Markram et al., 2008). In the present report, we also found that in some regions, the changes in cortical thickness were opposite at early age than after puberty. All together, these results suggest that homeostatic plasticity or compensatory mechanism(s) contribute to maintain stability and functionality of neural circuits in the face of challenges posed by developmental events in this animal model. Changes in local and distant connectivity in the brain have been proposed as a possible cause of autistic behavior (Geschwind and Levitt, 2007). In the prenatal VPA exposure model, local changes in the connectivity and excitability have been found in the PFC at prepuberal age (Rinaldi et al., 2008), which switches from hyper to hypofunction with the age (Martin and Manzoni, 2014).

Interestingly, neuroanatomical abnormalities similar to those reported in prenatal VPA animals have been detected in many brain areas of autistic patients (Edalatmanesh et al., 2013). For example, in some human studies, abnormalities in packing density of neurons in the hippocampus and the amygdala have also been reported (Aylward et al., 1999; Bailey et al., 1998; Jacot-Descombes et al., 2012; Schumann and Amaral, 2006). In postmortem autistic amygdala, Schumann and Amaral (2006) found, by using a stereological analysis, that the number of cells in the BLA is diminished. However, Courchesne et al., (2001) and Palmen et al., (2004) found that BLA neurons of autistic patients are smaller, more numerous, and tightly packed, whereas Jacot-Descombes et al., (2012) reported that pyramidal neurons of the Brodmann areas 44 and 45 of postmortem autistic frontal cortex showed a reduction in somatic size. In addition, some postmortem studies have revealed small and densely packed neurons in the CA1 hippocampus fields (Aylward et al., 1999; Bailey et al., 1998). All these impairments lead to changes in the density,

size and form of packing of the cells as the underlying cause of neuroanatomical alterations in cortical thickness.

Finally, several neuroimaging studies have revealed that neuro-psychiatric disorders are associated with various types of abnormalities in the hippocampus, PFC, and amygdala (for review, see Calderoni et al., 2012; Charman et al., 2011; Kim et al., 2011). For example, in agreement with our data, a recent report that analyzed the cerebral morphometry and structural connectivity by using multimodal imaging has shown reduced frontal gyrification with altered connectivity in children/adolescents with ASD (Schaer et al., 2013). In addition, a Golgi study from postmortem ASD patients suggests that pyramidal neurons of the CA1 hippocampus display less arborization compared to control samples (Raymond et al., 1996). Our study allows appreciating the range of developmental and region-specific alterations in a rat model of autism.

In conclusion, our results suggest that prenatal VPA exposure produces changes in the cortical thickness at level of the PFC, hippocampus and BLA, consistent with the data reported in patients with ASD and with the hypothesis that autism is associated with synaptic dysfunction.

ACKNOWLEDGMENTS

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