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Research report

A single low dose of valproic acid in late prenatal life alters postnatal behavior and glutamic acid decarboxylase levels in the mouse

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ABSTRACT

Rationale: Rodents exposed to valproic acid (VPA) in prenatal life exhibit post-natal characteristics analogous to autism spectrum disorder (ASD). Many previous studies used relatively high doses of VPA during early pregnancy, potentially confounding interpretation because the offspring are the 'survivors' of a toxic insult. Low dose or late gestation exposure has not been widely studied.
Objectives: We examined the behavioral sequelae of late gestation exposure to low dose VPA in the mouse. We also examined postnatal levels of glutamic acid decarboxylase (GAD65 and GAD67) as markers for GABA neurons, because GABA pathology and subsequent excitatory/inhibitory imbalance is strongly implicated in ASD.
Methods: Pregnant C57BL/6N mice received a single subcutaneous injection of 100 or 200 mg/kg on gestation day 17. The control group received a saline injection on the same day. The offspring were tested in a battery of behavioral tests in adolescence and adulthood. Six brain regions were harvested and GAD65 and GAD67 were measured by western blotting.
Results: Compared to saline-exposed controls, adult mice exposed to prenatal VPA had impaired novel object exploration and fear conditioning anomalies. GAD67 was decreased in midbrain, olfactory bulb, prefrontal cortex and increased in cerebellum, hippocampus and striatum; GAD65 was decreased in all 6 regions.
Conclusions: Our results suggest that a low dose of VPA in late pregnancy has persistent effects on brain development, and in particular the GABA system, which may be relevant to ASD. Further attention to the impact of gestation time and dose of exposure in VPA-induced ASD models is encouraged.

1. Introduction

Exposure to valproic acid (VPA) in pregnancy is associated with a higher incidence of autism spectrum disorder (ASD) by case reports and population studies [1–9]. Rodents exposed to VPA prenatally simulate a number of behavioural traits found in ASD [10,11]; eg. lower sensitivity to pain, increased repetitive/stereotypic-like activity, higher anxiety, decreased level of social interaction [12]. They have also been reported to recapitulate neurochemical and neuroanatomical features of ASD such as impaired inhibitory gamma amino butyric acid (GABA)-ergic synaptic transmission [13], increased frontal cortical serotonin [14], altered excitatory post-synaptic development [15]; and differences in brain structure and gene expression [16–19].

One of the challenges with this model is that it is difficult to translate the doses of VPA used in humans to the eqiuvalent in rodent. In humans, VPA is used within its relatively narrow therapeutic range, for example 20–30 mg/kg in epilepsy [20]. In contrast, rodents have been reported to be relatively resistant to VPA toxicity [21] and the majority of the reports that studies that link VPA exposure in pregnancy to ASD-like outcomes in rodents, have used either a moderate dose of VPA (200 mg/kg) over consecutive days, or a single high dose of VPA (500–800 mg/kg). However, these regimes can induce physical malformations and even death

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of offspring [11,22,23]. Specifically, the usual rate of perinatal loss before the postnatal day 6 in rats is 8.9%; but this increases to 15.6% following administration of 300 mg/kg VPA, and to 48.7% following administration of 500 mg/kg VPA [24]. This raises a question about how best to interpret results from survivors of such significant insults. Despite, this potential confound, few studies have examined low dose exposure to VPA in pregnancy in experimental settings [25–27].

The gestational timepoint of exposure may also influence outcome following prenatal environmental exposures. For example, we and others have reported that exposure to maternal immune activation (MIA) in early or late gestation precipitates a distinct phenotype in terms of both nature and severity [28–30]. In the VPA model, the embryotoxicity of VPA initially came to light because it induced neural tube defects. As a result, many animal studies focused on the time of neural tube closure in early pregnancy [3,31]. However, despite initial evidence that embryotoxicity following VPA exposure may be even higher in late gestation [32], the effects of late exposure VPA have received scant attention since.

Finally, another over-looked factor which may influence the outcome of exposure to VPA in prenatal life, is the sex of the offspring. The majority of VPA studies have focused on male off-spring only, by convention and/or the reported higher incidence of males with ASD [33].

Therefore, in this series of experiments we aimed to investigate the effects of low dose VPA (100 mg/kg or 200 mg/kg) administered to pregnant mice in the late gestation. We examined juvenile and adult mice of both sexes in paradigms which measure behaviours relevant to ASD, namely open field activity, prepulse inhibition of startle (PPI) measure of sensorimotor gating, olfactory discrimination (adults only) and fear conditioning (adults only) paradigms. In addition, using western blot, we measured regional brain levels of Glutamic acid decarboxylase (GAD), the rate-limiting enzyme in the conversion of excitatory neurotransmitter glutamate to inhibitory GABA in the brain [34]. This is because brain excitatory/inhibitory imbalance is increasingly linked to ASD [35–38], and both GAD65 and GAD67 isoforms, have been reported to be altered in ASD [39], and also in preclinical models of ASD such as the BTBR mouse [40] and the MIA model [41]. However, it is unknown whether prenatal VPA exposure alters GAD level postnatally.

2. Materials and methods

2.1. Animals and sample collections

Twelve pregnant female C57BL/6N mice were supplied by HKU Laboratory Animal Unit (LAU) breeding colony – the day that a vaginal plug was identified was taken as embryonic day 0 (GD0). They were provided to the behavior unit within the LAU on GD15. The mice were maintained in a normal light cycle (lights on at 7:00; off at 19:00) through-out the entire experiment. The temperature and humidity in the holding room was controlled at $21 \pm 1\,^{\circ}C$, and $55 \pm 5\%$ respectively, and a conventional *ad libitum* food and water diet was supplied by the LAU. All experiments were approved by the Committee on the Use of Live Animals in Teaching and Research at The University of Hong Kong, and every effort was made to minimize the number of animals used and their suffering.

Valproic acid was purchased as sodium valproate (Sigma, St Louis, USA) and was dissolved in 0.9% saline to achieve a concentration of 20 mg/ml. Twelve pregnant mice were divided equally into 3 weight-matched groups and injected with 0.9% saline control (CON-0), 100 mg/kg VPA (VPA-100), or 200 mg/kg VPA (VPA-200) on GD17. Pregnant mice were housed individually and allowed to raise their own litters. Eighty mice were born in total. There were 13 female and 17 male in CON-0 group, 14 female and 13 male in VPA-100 group, and 8 female and 15 male in VPA-200 group. Offspring were weaned on postnatal day 21 and mice of the same sex from the same litter were group housed in standard cages. No more than four animals were kept to a cage. All the mice were generally healthy and careful visual inspection revealed no abnormal physical features.

The mice were weighed twice, once as juveniles in week 4 right after weaning and once in week 12 as adults. The open field test, the novel object recognition test and the prepulse inhibition (PPI) test were conducted at ages 5–6 weeks and 12–15 weeks. Olfactory testing was conducted only in adulthood (week 16) because juvenile mice are known to respond unpredictably to odors [42]. The fear conditioning test was also only conducted once at the end of behavior tests (week 17), to avoid any residual influence of the aversive nature of that test. MRI scan of mouse brain was conducted in 7 female and 7 male from each treatment group in week 19 (data under analysis and not reported here). After an undisturbed period all mice were sacrificed in week 22. Their brains were harvested and rapidly dissected to collect tissue samples from cerebellum (CB), hippocampus (HP), midbrain (MB), olfactory bulb (OB), prefrontal cortex (PFC) and striatum (STR).

2.2. Behavioral tests

2.2.1. Open field test

The open field test was used to measure locomotor activity in the dim light and general anxiety levels in rodents in the bright light [43,44]. The experimental setting comprised four identical square arenas (40×40 cm) surrounded on all sides by a 40-cm white plastic wall. A central area (15×15 cm) was drawn in the center of each arena. A digital camera was mounted above, capturing images from all four arenas at the same time and the videos were analyzed by the EthoVision tracking system (Version 3.1, Noldus Information Technology). The mice were tested in squads of four. They were gently placed in the center of the appropriate arena in the dim light and allowed to explore undisturbed for 30-min. Locomotor activity was indexed by distance travelled recorded in successive 5-min bins and the anxiety-related behaviors were measured by distance travelled in the central area.

2.2.2. Novel object recognition test

The procedure was adapted from previous protocols [45,46]. A plastic cage ($30 \times 30 \times 30$ cm) with white walls was used in 3 sessions. In the habituation session on the first day, the mouse was placed into the empty cage to habituate for 10-min. On the second day, a 10-min training session preceded the 5 min test session by 1 h. During the training session, two identical objects A (blue cylinder, 3 cm diameter $\times$ 1 cm height,) were placed near the corners of one wall of the cage (5 cm from each adjacent wall). The mouse was placed near the opposite wall, facing it, then allowed to explore both objects. During the test session, a fresh "familiar" object A' and a novel object B (pink cuboid, $3 \times 1 \times 1$ cm,) were placed in the same place as objects in the training session and the mouse was allowed to explore. To avoid odor clues, the cage and objects were thoroughly cleaned with 70% ethanol between mice. The videos of the test phase were analyzed using the EthoVision tracking system and 'Exploration' was defined as the nose of mouse placed within 1 cm of the object A (T_A) and the object B (T_B). A discrimination index (DI) was calculated as: $DI = (T_B - T_A)/(T_B + T_A) \times 100$

2.2.3. Prepulse inhibition (PPI) test

The procedures and testing parameters for the PPI test used here followed those described previously [29]. Two standard acoustic startle chambers for mice (SR-LAB, San Diego Instruments, San Diego, CA, USA) generated the continuous background noise of 65 dB and white noise stimuli. The white noise stimuli was set to be