Exposure to valproic acid during middle to late-stage corticogenesis induces learning and social behavioral abnormalities with attention deficit/hyperactivity in adult mice

Yuki Sakade¹, Kumiko Yamanaka¹, Hitomi Soumiya¹, Shoei Furukawa¹, and Hidefumi Fukumitsu¹

¹Laboratory of Molecular Biology, Department of Biofunctional Analysis, Gifu Pharmaceutical University, Daigaku-nisi, 1-25-4, Gifu, 501-1196, Japan

(Received 12 July 2019; and accepted 3 August 2019)

ABSTRACT

Single prenatal exposure to valproic acid (VPA) in rodents is a widely used preclinical model of autism spectrum disorder (ASD). Continuous prenatal VPA exposure has been recently proposed as a new ASD model that closely captures the neuropathological features of ASD, including increases in cerebral cortex volume and the number of cortical upper layer neurons. We investigated the influence of prenatal VPA exposure on the behavior of adult offspring of pregnant dams that received intraperitoneal injections of VPA twice on one day during the genesis of cortical upper layer neurons. Mice exposed to VPA at E14 (E14-VPA) showed typical behavior abnormalities including reduced social interaction, hyperactivity, and poor maze learning due to attention deficit/impulsivity relative to healthy controls. Histological analysis revealed that E14-VPA mice had significantly increased neuronal density and impaired neural activity in the prefrontal cortex, but not the somatosensory area, which is likely linked to the observed abnormalities in social behavior. These results suggest that this VPA exposure method is a good model for gaining new insights into the underlying neuropathology of ASD.

Autism spectrum disorder (ASD) is described by the American Psychiatric Association (1) as a group of neurodevelopmental disorders characterized by impairments in social interactions, verbal and nonverbal communication disabilities, repetitive behavioral stereotypy, and limited interest. Symptoms appear during infancy and persist through adulthood. Many factors—both genetic and environmental and their interactions—have been implicated in the neuropathogenesis of ASD (5, 7). Impaired social interaction is a core symptom of ASD and dysfunction in various brain regions has been reported, including the prefrontal cortex and somatosensory area (11, 42). The pathophysiological mechanism of the symptoms of ASD, however, remains unknown.

Valproic acid (VPA) is widely used as a drug for epilepsy, bipolar disorder, and migraine. However, children born to mothers who used VPA during pregnancy are at an increased risk of neurodevelopmental disorders, including ASD, and lower intelligence quotient scores (fetal valproate syndrome) (13, 43). Based on such clinical reports, the effects of fetal VPA exposure have been examined in rodents, which result in behavioral abnormalities similar to the symptoms of ASD, such as low social interaction and repetitive affective behaviors (12, 18, 35).

The development of neuro-cytoarchitecture and neurocircuits is strictly spatiotemporally regulated in the embryonic brain, including the cerebral cortex which plays an important role in social interactions and communication (4, 26). Thus, it is reasonable to expect the effect of prenatal VPA exposure to vary depending on the fetal developmental stage. In fact,
single intraperitoneal (i.p.) injection of high-dose VPA to a pregnant rodent dam (embryonic day 12, E12) has been identified as the most vulnerable time point to induce social behavioral abnormalities and reduce the number of deep layer cortical neurons. Such behavioral abnormalities are not observed when VPA exposure occurs at E9 or E14 (23, 25). Recent reports have suggested that the number of cortical neurons in mice offspring is significantly increased when embryos are repeatedly (34) or continuously (17) exposed to lower dosages of VPA from E12 to postnatal day 23 or from E1 to birth. There are no data available regarding the behavior of mice in these models, although their neuropathological features may better represent those of ASD than the single i.p. injection models.

In the present study, we reevaluated the time-dependent influence of prenatal VPA exposure on social-related behaviors of mice offspring exposed to i.p. lower-dose VPA (200 mg/kg) twice a day during the middle to late stage of corticogenesis, when the cortical upper layer neurons important for cortical–cortical and cortical–subcortical neural circuits and social behaviors are generated. Thus we found that the adult mice offspring after VPA exposure at E14 (E14-VPA mice) showed typical behavior abnormalities including reduced social interaction, hyperactivity, and poor maze learning due to attention deficit/impulsivity. We also examined neural activity in the related brain regions of the E14-VPA mice.

MATERIALS AND METHODS

Animals. All experiments were approved by the Animal Research Committee of Gifu Pharmaceutical University, Gifu, Japan, and conducted in accordance with the National Institutes of Health guidelines for animal care. All efforts were made to minimize the number of animals used and their suffering.

Pregnant ddY mice (embryonic days 13–17, i.e., E13–E17) were obtained as previously reported (39, 40). In brief, eight-week-old female and male ddY mice were purchased from Japan SLC (Shizuoka, Japan). They were then mated at around 6:00 PM and separated at 9:00 AM the next morning. If a vaginal plug was found, then 12 PM of that day was defined as embryonic day 0.5 (E0.5).

Drug administration. Pregnant dams bearing E14 embryos received either i.p. injection of VPA or vehicle (0.9% sodium chloride solution, saline) in the morning and evening (at 9:00 AM and 4:00 PM of the 14th day of pregnancy). VPA (sodium salt) was obtained from Wako (Osaka, Japan) and freshly dissolved in sufficient saline to obtain the desired dosage (200 mg/kg, calculated on the basis of the pure form of VPA). All animals were immediately returned to their home cage after the injection procedure. Pups were allowed to mature without further intervention except for weekly cage cleaning. Female pups were killed by pentobarbital overdose (100 mg/kg) at 3 weeks (P3W), and only age-matched P8W–P9W male mice were used in all experiments.

Behavioral tests. Behavioral test batteries are described in the order that were conducted. On the first day of the test battery, the open-field test was performed. The three-chamber social interaction and elevated plus maze tests were carried out on the third and fifth days, respectively. Individual mice were only used once in each behavioral test (Fig. 1A). 1. Open-field test.

The open-field test measures fear- and anxiety-related behaviors. It was performed as described previously with minor modifications (22, 39). Briefly, the testing apparatus consisted of a white Plexiglas box with a 40 × 40 cm² arena and 20 cm high walls; the floor was divided into 16 squares by parallel and intersecting black lines. The box was placed in a dark and quiet room, and the four squares at the center of the box floor were illuminated (~100 lux) by shining a light from each side of the box. The 12 squares along the walls were defined as the periphery. Mice were placed at the center of the field and allowed to explore freely for 5 min. Their activity was recorded using a video camera positioned above the open-field. The number of entries into the center area and the time spent in the center area were manually recorded by counting the number of times they crossed the black lines.

2. Social interaction and elevated plus maze tests. The three-chamber social interaction and elevated plus maze tests were conducted as described previously (22, 39, 41).

3. Wire-net cued eight-arm radial maze.

The eight-arm radial maze test cued with wire nets was performed as described previously (41). Briefly, following habituation and shaping, an individual animal was placed at the center of the maze and trained once a day for 12 consecutive days. The inner wall surfaces of the four arms (numbers 1, 3, 5, and 7) were covered with a wire-net and the food cups in these four arms were baited with a single 10 mg food pellet per cup for each daily training trial; an empty food cup was placed at the end of the other
four arms without wire nets (numbers 2, 4, 6, and 8). Each mouse was allowed to freely explore until it had found all pellets or 5 min elapsed. The number of correct selections (entering into net-covered/baited arms in the first four entries), the number of reference memory errors (entering an arm that was not baited), and the number of working memory errors (revisits to net-covered/baited arms previously entered) were recorded. Two hours after the task, the brains of the mice were prepared for immunocytochemistry.

**Tissue preparation, immunostaining, and Nissl staining.** Brain tissue was excised and processed for immunohistological analyses as described previously (41). In brief, free-floating serial coronal sections (30 μm) were made by a cryostat and collected in phosphate-buffered saline. Every third section was taken and processed for c-Fos immunohistochemistry (anti-c-Fos antibody; Santa Cruz SC-52). The adjacent series of sections was used for Nissl staining.

**Quantitative studies of c-Fos-positive cells.** Three to five sections from each stained series were chosen for the quantification of c-Fos-positive neurons in prefrontal areas. Five sections were chosen for the quantification of stained c-Fos-positive neurons in barrel field of the primary sensory cortex (S1BF) and secondary somatosensory cortex (S2). The chosen sections were those closest to the dorsal–ventral interaural level (6.02 mm for the prefrontal area and 2.86 mm for S1BF and S2) according to Paxinos and Franklin’s anatomic atlas (33). Only the left hemisphere from these sections was quantified. For each brain section, the number of c-Fos-immunopositive cells in a given brain structure was counted, divided by the area occupied by that structure (in mm²), and expressed as a positive-cell density. For cortical areas, the entire depth of the cortical field was included in a particular section. The borders of the cortical areas and subcortical nuclei were determined using adjacent Nissl-stained sections. These borders were drawn by an investigator who was blinded to the experimental group assignment of the animals and then reviewed by a second investigator. The area was measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

**Statistical analyses.** Data are represented as the mean ± standard error of the mean (SEM). Group means from behavioral experiments and histological analyses were compared using Student's \( t \)-test (Figs. 1, 3, 5B–D, and 6A–C). Multiple group means were compared using two-way analysis of variance (ANOVA) with Sidak’s post hoc analysis (Figs. 4, 5E, 5F, 6D and 6E). The social interaction test (Fig. 2) was analyzed using Wilcoxon’s test.

**RESULTS**

**Prenatal VPA injection causes abnormal behaviors in adult mice**

In order to examine the effects of prenatal VPA exposure from middle to late corticogenesis (the timing of upper layer [IV-II/III] neurogenesis (23, 40)) on the behavior of adult mice, we administered VPA (200 mg/kg) to dams by i.p. injection twice a day for one day between E13 and E17 and then examined whether treatment affected social behaviors and fear/anxiety-related behaviors in adulthood. As the control, an equal volume of saline was injected twice a day at E14, and the born mice were used for analysis in adulthood. Hereafter, we refer to adult offspring given i.p. injection of VPA twice a day at E14 as “E14-VPA” mice. The behavioral test battery was performed in the order of open-field test, social interaction test, and elevated plus maze test to avoid unnecessary stress on mice (for the schedule of the behavioral test battery, see Fig. 1A).

Preliminary data by using small number of mice revealed that E14- and E15-VPA mice tended to show abnormalities in social behavior with hyper activities (E13-, E14-, E15-, and E17-VPA mice born to two individual dams were tested; data not shown). Because of typical behaviors, we further investigated E14-VPA mice for all subsequent experiments.

In the open-field test, compared to controls, E14-VPA showed significant hyperactivity (total number of crossings of the lines; Fig. 1B). Mice tend to avoid brightly lit and wide open places. An increase in the number of entries into the central area is thought to reflect less sensitivity to mild stressful conditions (27). Although E14-VPA mice tended to enter into the central portion of the well-lit open-field more frequently, and spent there longer time, that may be related to the locomotor activity of each group, because the differences were slight and not significant (Figs. 1C and D). In the three-chamber social interaction test, controls mice spent more time in the “social” side chamber containing a conspecific probe mouse than in the “nonsocial” empty side chamber; whereas adult E14-VPA mice showed no preference for the social chamber (Fig. 2). In the elevated plus maze, E14-VPA mice tended to enter the open arm more frequently and to spend more
task under conditions requiring the detection of whisker cues for reward. In this maze, four arms were cued with wire nets and baited at the ends, whereas the other four arms contained no tactile cues and were never baited. In this apparatus, the mice had to learn and memorize the relationship between the tactile cue and reward and the spatial relationships among cued/baited and uncued/unbaited arms.

Both groups showed selective entering into the net-covered arms across trials (1 trial per day for 12 consecutive days). The ratio of net arm choice also increased over training days from the “by-chance” level and plateaued by day 10 in both groups (data not shown). However, the average number of times E14-VPA mice chose a different bait/net arm in the first four entries was significantly less frequent than that of control mice in the last three days of the trial (Fig. 4A). Furthermore, although the number of...
Fetal VPA exposure — ADHD mice

in the PrL and LO (Figs. 6A–C) of E14-VPA mice, but not in the somatosensory cortex (Figs. 6D and 6E). These data suggest that prenatal VPA exposure at E14 impairs the functional development of prefrontal circuits, resulting in learning and behavioral abnormalities with attention deficit hyperactivity, without affecting the whisker-tactile perception system.

DISCUSSION

In the present study, we investigated the effects of prenatal VPA exposure during the middle to late stages of cortical neurogenesis and found that mice exposed to VPA at E14 showed several typical behavior abnormalities: E14 is the stage for genesis of layer II/III neurons in the mice cerebral cortex (23, 40). Compared with previous reports in which rodents were exposed to a single i.p. VPA injection between E10 and E13, typically at E12.5 (23, 25, 31), our mice exposed to VPA at E14 showed a similar reduction in social interactions. By contrast, our E14-VPA mice did not show any anxiety-like behaviors in the elevated plus maze test, although anxiety is a common symptom in previous VPA-exposed rats models (10, 29, 36). Poor maze learning performance in E14-VPA mice likely resulted from their attention deficit and impulsivity.

Complex behavioral abnormalities in ASD are difficult to explain solely by dysfunction of a specific brain area. Malformations in the cytoarchitectonic cerebral cortex (7, 24) and dysfunction in cortical circuits (32, 44) are the most commonly observed findings in autopsied brains and in clinical studies of patients with ASD. After the last radial maze learning trial of this study, the density of c-Fos-positive cells in the prefrontal cortex (PrL, VO and LO), which correlates with neural activity, was clearly reduced, but not in the barrel field of the primary somatosensory cortex (S1BF) and secondary somatosensory cortex (S2) of E14-VPA mice compared with controls (Fig. 5). Although selective entering into the net-cued baited arm increased in E14-VPA mice in the last three trial days (long-term memory), it was still significantly lower than in controls (Fig. 4A). This is consistent with reports that the prefrontal cortex plays an important role in short- and long-term memory formation with goal-directed behavior during radial maze learning (2, 37). Dysfunction of the prefrontal cortex is also known to impair social behaviors and emotion control in mice (6, 21). Therefore, the poor social interactions in E14-VPA (Fig. 2) may also be due to prefrontal cortex dys-
Fig. 3  E14-VPA mice showed a trend of reduced anxiety (or higher impulsivity) in the elevated plus maze test. (A) Number of entries into the open arm (counts). (B) Time spent in the open arm (s). (C) Number of total entries into any arm (counts). Note that E14-VPA mice tended to explore maze arms more frequently and to spend longer time in open arms than control mice did. All values are expressed as the mean ± SEM. Student’s t-test; 

\[ N = 4 \text{ cohorts}, \ n = 15 \text{ mice for control and} \ N = 4 \text{ cohorts}, \ n = 14 \text{ mice for E14-VPA mice (for behavioral data of individual mice, see Supporting Data 3).} \]

Fig. 4  E14-VPA mice showed reduced exploring behavior and poor learning in a wire-net guided eight-arm radial maze. (A) Number of mice choosing different arms baited and covered with a net in the first four entries (counts). (B) Number of mice entering arms without a net in the first and last three trial days (counts). (C) Number of mice reentering previously entered arms in the first and last three trial days (counts). (D) Time spent in each arm (s). Note that E14-VPA mice explored each arm for less time on the first trial day and showed worse learning than controls in the radial maze task. All values are expressed as the mean ± SEM. *\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.005 \), and #\( P < 0.05 \). Two-way ANOVA with Sidak’s post hoc multiple comparisons test; \( N = 2 \) cohorts, \( n = 8 \) mice for control and \( N = 1 \) cohort, \( n = 5 \) mice for E14-VPA mice.
Fig. 5 Prenatal exposure to VPA altered radial maze task learning-related c-Fos expression in the frontal, but not in somatosensory, cortex of E14-VPA mice. A. Photographs of c-Fos-expression in PrL (prelimbic cortex) of mice 2 h after the last trial of the eight-arm radial maze task. Quantitative analysis revealed that c-Fos-positive density in the prefrontal cortex (B, PrL: prelimbic cortex; D, LO: lateral orbitofrontal cortex) of E14-VPA mice was significantly decreased following the eight-arm radial maze task compared with control mice. The expression patterns were unchanged in other regions of the prefrontal cortex (C, VO: ventral orbitofrontal cortex) and somatosensory cortex (E, S1BF: barrel field of the primary sensory cortex; F, S2: secondary somatosensory cortex). Values are expressed as the mean ± SEM. *P < 0.05 versus control mice, **P < 0.01 versus control mice, Student’s t-test; N = 1 cohort, n = 4 mice for control and N = 1 cohort, n = 5 mice for E14-VPA mice.
In the first trial of radial maze learning, E14-VPA mice seemed to pay less attention to the tactile cue, as the average time spent exploring each arm was significantly shorter and the number of memory errors was significantly larger than in controls (Figs. 4B and 4C). In addition, in the elevated plus maze test and open-field test, E14-VPA mice showed slight hyperactivity with a decrease in anxiety-like behaviors (or an increase in impulsivity). These changes may be similar to the inattention and hyperactivity commonly seen in both ASD patients (28, 38) and children with fetal valproate syndrome (9).

How do differences in prenatal VPA-induced behavioral abnormalities arise? Although the influences of prenatal VPA exposure differ in mice strains with different genetic backgrounds (3, 16), we believe that differences in the VPA administration method—not in the used mouse strain—may explain the underlying mechanism; in this study, we used 200 mg/
kg i.p. injection twice a day at E14 ddY mice, while previous typical studies used 500 mg/kg single i.p. injection at E12.5 ICR mice (23, 31). VPA is known to affect various biological processes during corticogenesis, including histone deacetylase activity, gamma aminobutyric acid concentration, and the glycogen synthetase kinase-3β/β-catenin pathway (19). The half-life of VPA in rodents is 1–2 h (30) and its histone deacetylase inhibitory effect is reported to completely disappear 6–12 h after administration (23). Therefore, the influence of VPA exposure on corticogenesis is dependent on the timing of administration. In agreement with this, in the E14-VPA model, we found that the density of Nissl-positive neurons in the prefrontal cortex was significantly increased but was unchanged in the somatosensory area (Fig. 6), whereas neuronal density was significantly reduced in both the frontal cortex and somatosensory area in mice subjected to high-dose VPA exposure (single i.p. injection of 500 mg/kg at E12.5) (20, 23). In addition, neuronal density was significantly increased in both the prefrontal cortex and somatosensory area in mice subjected to a long-term low-dose VPA exposure: 20 or 100 mg/kg daily i.p. injection from E12.5 to postnatal day 23 (34); 0.4% VPA peroral administration from E1 until birth (17).

In the present study, we found that adult mice exposed to VPA administered by i.p. injection twice at E14 showed reduced social interaction with hyperactivity and inattention/impulsivity. Although genesis and development of neural cells are generally delayed for 1–3 days in the rat nervous system compared to the mouse (14, 15), E12.5 is the most vulnerable time point in both species to induce social behavioral abnormalities using the high-dose single i.p. VPA injection model; by contrast, such behavioral abnormalities do not occur in E9 and E15 (23, 25). Taken together, transient prenatal VPA exposure impairs the genesis and development of neural (stem/progenitor) cells that constitute any of brain regions related to social behavior, and the influence on the neural cells or the neural cell lineages is sustained. Such impairments in the development of the related nervous system are increased over threshold, and lead to abnormalities in social behaviors. Considered with peripheral symptoms, the neural subtypes which have been impaired should vary depending on the timing and duration of VPA exposure, although the social behavioral symptoms are similar. In fact, recent studies have revealed that a single VPA injection at E12.5 impairs glutamate, cholesterol/isoprenoid, and endocannabinoid metabolism as well as intracellular signaling pathways in glia and neurons of various brain regions throughout life (8, 10, 36). Although further neuropharmacological and neuropathological studies are necessary, we believe that this study describes an important model for assessing the complex neuropathological mechanisms underlying fetal valproate syndrome and ASD.

REFERENCES

1. American Psychiatric Association (2013) Diagnostic and Statistical Manual of Mental Disorders (DSM-5). 5th edn., VA: American Psychiatric Association, Arlington.
2. Auger ML and Floresco SB (2014) Prefrontal cortical GABA modulation of spatial reference and working memory. Int J Neuropsychopharmacol 18, pyu013.
3. Beck SL (2001) Does genomic imprinting contribute to valproic acid teratogenicity? Reprod Toxicol 15, 43–48.
4. Bell SB and DeWall N (2018) Does transcranial direct current stimulation to the prefrontal cortex affect social behavior? A meta-analysis. Soc Cogn Affect Neurosci 13, 899–906.
5. Betancur C (2011) Etiological heterogeneity in autism spectrum disorders: more than 100 genetic and genomic disorders and still counting. Brain Res 1380, 42–77.
6. Bicks LK, Koike H, Akbarian S and Morishita H (2015) Prefrontal cortex and social cognition in mouse and man. Front Psychol 6, 1805.
7. Bolte S, Girdler S and Marschik PB (2019) The contribution of environmental exposure to the etiology of autism spectrum disorder. Cell Mol Life Sci 76, 1275–1297.
8. Bristot Silvestrin R, Bambini-Junior V, Galland F, Daniele Bobermmi L, Quincozes-Santos A, et al. (2013) Animal model of autism induced by prenatal exposure to valproate: Altered glutamate metabolism in the hippocampus. Brain Res 1495, 52–60.
9. Bromley RL, Mawer GE, Briggs M, Cheyne C, Clayton-Smith J, et al. (2013) The prevalence of neurodevelopmental disorders in children prenatally exposed to antiepileptic drugs. J Neurol Neurosurg Psychiatry 84, 637–643.
10. Cartocci V, Catallo M, Tempestilli M, Segatto M, Pfrieger FW, et al. (2018) Altered brain cholesterol/isoprenoid metabolism in a rat model of autism spectrum disorders. Neurosci 372, 27–37.
11. Cheng W, Rolls ET, Gu H, Zhang J and Feng J (2015) Autism: reduced connectivity between cortical areas involved in face expression, theory of mind, and the sense of self. Brain 138, 1382–1393.
12. Chomiak T, Turner N and Hu B (2013) What we have learned about autism spectrum disorder from valproic acid. Pathol Res Int 2013, 712758.
13. Christensen J, Gronborg TK, Sorensen MJ, Schendel D, Parner ET, et al. (2013) Prenatal valproate exposure and risk of autism spectrum disorders and childhood autism. JAMA 309, 1696–1703.
14. Clancy B, Darlington RB and Finlay BL (2001) Translating developmental time across mammalian species. Neuroscience 105, 7–17.
15. Clancy B, Finlay BL, Darlington RB and Anand KJ (2007) Extrapolating brain development from experimental species to humans. Neurotoxicology 28, 931–937.
16. Downing C, Biers J, Larson C, Kimball A, Wright H, et al. (2010) Genetic and maternal effects on valproic acid teratogenesis in C57BL/6J and DBA/2J mice. Toxicol Sci 116, 632–639.
Supporting Data 1 open field test

|        | number of total crossings (counts) | number of entries to central area (counts) | time spent in central area (secs) |
|--------|-----------------------------------|-------------------------------------------|----------------------------------|
| **control** |                                   |                                           |                                  |
| 1 cont_E14_I-1 | 58                     | 2                                          | 14.1                             |
| 2 cont_E14_I-2 | 144                    | 13                                         | 23.4                             |
| 3 cont_E14_I-3 | 167                    | 12                                         | 15.5                             |
| 4 cont_E14_II-1 | 144                     | 7                                          | 10.4                             |
| 5 cont_E14_II-2 | 83                     | 3                                          | 5.7                              |
| 6 cont_E14_II-3 | 123                    | 2                                          | 12.2                             |
| 7 cont_E14_II-4 | 175                    | 6                                          | 7.6                              |
| 8 cont_E14_II-5 | failure of the video recording |                                           |                                  |
| 9 cont_E14_III-1 | 90                    | 4                                          | 16.4                             |
| 10 cont_E14_III-2 | 110                   | 6                                          | 11.2                             |
| 11 cont_E14_III-3 | 128                   | 7                                          | 8.0                              |
| 12 cont_E14_III-4 | 160                   | 10                                         | 12.9                             |
| 13 cont_E14_III-5 | 115                   | 9                                          | 7.7                              |
| 14 cont_E14_IV-1 | 210                   | 11                                         | 20.8                             |
| 15 cont_E14_IV-2 | 126                   | 9                                          | 11.9                             |
| 16 cont_E14_IV-3 | 208                   | 5                                          | 4.9                              |

| **E14-VPA** | number of total crossings (counts) | number of entries to central area (counts) | time spent in central area (secs) |
|-------------|-----------------------------------|-------------------------------------------|----------------------------------|
| 1 VPA_E14_I-1 | 111                   | 1                                          | 11.4                             |
| 2 VPA_E14_I-2 | 151                   | 4                                          | 4.6                              |
| 3 VPA_E14_I-3 | 133                   | 2                                          | 10.8                             |
| 4 VPA_E14_I-4 | 107                   | 6                                          | 19.1                             |
| 5 VPA_E14_I-5 | 189                   | 6                                          | 10.8                             |
| 6 VPA_E14_II-1 | 158                   | 6                                          | 10.5                             |
| 7 VPA_E14_II-2 | 208                   | 12                                         | 13.4                             |
| 8 VPA_E14_II-3 | 154                   | 10                                         | 17.2                             |
| 9 VPA_E14_II-4 | 163                   | 6                                          | 4.5                              |
| 10 VPA_E14_II-5 | 171                  | 14                                         | 16.0                             |
| 11 VPA_E14_III-1 | 118                  | 9                                          | 17.4                             |
| 12 VPA_E14_III-2 | 285                  | 10                                         | 20.5                             |
| 13 VPA_E14_III-3 | 248                  | 16                                         | 21.3                             |
| 14 VPA_E14_III-4 | 159                  | 7                                          | 16.1                             |
| 15 VPA_E14_III-5 | 262                  | 14                                         | 37.2                             |
| 16 VPA_E14_III-6 | 262                  | 16                                         | 16.1                             |
| 17 VPA_E14_III-7 | 174                  | 8                                          | 10.4                             |
| 18 VPA_E14_IV-1 | 119                  | 7                                          | 17.2                             |
| 19 VPA_E14_IV-2 | 172                  | 8                                          | 19.1                             |
| 20 VPA_E14_IV-3 | 137                  | 5                                          | 11.1                             |

E14N-n: N = cohort identification number; n = individual identification number, respectively
Supporting Data 2 social interaction test

|        | control | non-social room | social room |
|--------|---------|-----------------|-------------|
| 1 cont_E14_I-1 | 105.3 | 156.8 |
| 2 cont_E14_I-2 | 82.1 | 185.9 |
| 3 cont_E14_I-3 | 182.9 | 63.4 |
| 4 cont_E14_II-1 | 87.7 | 153.2 |
| 5 cont_E14_II-2 | 81.3 | 121.8 |
| 6 cont_E14_II-3 | 53.9 | 216.7 |
| 7 cont_E14_II-4 | 93.1 | 155.0 |
| 8 cont_E14_II-5 | 50.5 | 153.7 |
| 9 cont_E14_III-1 | 163.9 | 125.6 |
| 10 cont_E14_III-2 | 97.6 | 181.6 |
| 11 cont_E14_III-3 | 53.4 | 215.2 |
| 12 cont_E14_III-4 | 72.1 | 169.2 |
| 13 cont_E14_III-5 | 51.3 | 231.1 |
| 14 cont_E14_IV-1 | 140.6 | 130.4 |
| 15 cont_E14_IV-2 | 170.6 | 75.1 |
| 16 cont_E14_IV-3 | 97.9 | 157.7 |

|        | E14-VPA | non-social room | social room |
|--------|---------|-----------------|-------------|
| 1 VPA_E14_I-1 | 176.3 | 68.9 |
| 2 VPA_E14_I-2 | 71.6 | 162.6 |
| 3 VPA_E14_I-3 | 231.7 | 37.2 |
| 4 VPA_E14_I-4 | 180.8 | 60.5 |
| 5 VPA_E14_I-5 | 201.1 | 89.7 |
| 6 VPA_E14_II-1 | 93.2 | 102.4 |
| 7 VPA_E14_II-2 | 106.7 | 116.3 |
| 8 VPA_E14_II-3 | 44.8 | 146.2 |
| 9 VPA_E14_II-4 | 126.7 | 144.1 |
| 10 VPA_E14_II-5 | 93.7 | 133.7 |
| 11 VPA_E14_III-1 | 255.0 | 13.1 |
| 12 VPA_E14_III-2 | 90.4 | 175.9 |
| 13 VPA_E14_III-3 | 82.6 | 180.8 |
| 14 VPA_E14_III-4 | 41.2 | 221.3 |
| 15 VPA_E14_III-5 | 68.9 | 141.4 |
| 16 VPA_E14_III-6 | 96.0 | 144.5 |
| 17 VPA_E14_III-7 | 64.3 | 201.2 |
| 18 VPA_E14_IV-1 | 119.5 | 128.2 |
| 19 VPA_E14_IV-2 | 50.8 | 191.4 |
| 20 VPA_E14_IV-3 | does not move from the center room |

E14-n: N = cohort identification number; n = individual identification number, respectively
## Supporting Data 3 elevated plus maze test

### Fetal VPA exposure — ADHD mice

| control          | number of entries to open arm (counts) | time spent in open arm (secs) | number of total crossings (counts) |
|------------------|----------------------------------------|------------------------------|-----------------------------------|
| cont_E14_I-1     | 0                                      | 0.0                          | 3                                 |
| cont_E14_I-2     | 2                                      | 16.5                         | 16                                |
| cont_E14_I-3     | 6                                      | 36.0                         | 16                                |
| cont_E14_II-1    | 2                                      | 7.0                          | 10                                |
| cont_E14_II-2    | 0                                      | 0.0                          | 2                                 |
| cont_E14_II-3    | 0                                      | 0.0                          | 5                                 |
| cont_E14_II-4    | 3                                      | 17.9                         | 16                                |
| cont_E14_II-5    | 1                                      | 1.6                          | 20                                |
| cont_E14_III-1   | 0                                      | 0.0                          | 2                                 |
| cont_E14_III-2   | 0                                      | 0.0                          | 1                                 |
| cont_E14_III-3   | 0                                      | 0.0                          | 1                                 |
| cont_E14_III-4   | 1                                      | 1.8                          | 8                                 |
| cont_E14_III-5   | 1                                      | 0.9                          | 6                                 |
| cont_E14_IV-1    | 0                                      | 0.0                          | 3                                 |
| cont_E14_IV-2    | 1                                      | 9.7                          | 5                                 |
| cont_E14_III-6   | dropping from the elevated plus maze device |                        |                                   |
| cont_E14_III-7   | 3                                      | 13.6                         | 9                                 |
| cont_E14_III-8   | 1                                      | 3.4                          | 5                                 |
| cont_E14_IV-1    | 1                                      | 1.6                          | 12                                |
| cont_E14_IV-2    | dropping from the elevated plus maze device |                        |                                   |

### E14-VPA

| VPA_E14_I-1      | number of entries to open arm (counts) | time spent in open arm (secs) | number of total crossings (counts) |
|------------------|----------------------------------------|------------------------------|-----------------------------------|
| VPA_E14_I-2      | dropping from the elevated plus maze device |                        |                                   |
| VPA_E14_I-3      | 0                                      | 0.0                          | 5                                 |
| VPA_E14_I-4      | 0                                      | 0.0                          | 7                                 |
| VPA_E14_I-5      | 7                                      | 36.3                         | 13                                |
| VPA_E14_II-1     | 0                                      | 0.0                          | 3                                 |
| VPA_E14_II-2     | 0                                      | 0.0                          | 4                                 |
| VPA_E14_II-3     | 0                                      | 0.0                          | 5                                 |
| VPA_E14_II-4     | dropping from the elevated plus maze device |                        |                                   |
| VPA_E14_II-5     | 0                                      | 0.0                          | 13                                |
| VPA_E14_III-1    | dropping from the elevated plus maze device |                        |                                   |
| VPA_E14_III-2    | dropping from the elevated plus maze device |                        |                                   |
| VPA_E14_III-3    | 6                                      | 22.3                         | 15                                |
| VPA_E14_III-4    | 1                                      | 1.4                          | 6                                 |
| VPA_E14_III-5    | 18                                     | 101.7                        | 34                                |
| VPA_E14_III-6    | dropping from the elevated plus maze device |                        |                                   |
| VPA_E14_III-7    | 3                                      | 13.6                         | 9                                 |
| VPA_E14_IV-1     | 1                                      | 3.4                          | 5                                 |
| VPA_E14_IV-2     | dropping from the elevated plus maze device |                        |                                   |
| VPA_E14 IV-3     | 1                                      | 1.6                          | 12                                |

E14N-n: $N =$ cohort identification number; $n =$ individual identification number, respectively