*Short Communication***Effect of Prenatal Valproic Acid Exposure on Cortical Morphology in Female Mice**Yuta Hara¹, Yuko Maeda¹, Shunsuke Kataoka¹, Yukio Ago¹, Kazuhiro Takuma¹, and Toshio Matsuda^{1,2,*}¹Laboratory of Medicinal Pharmacology, Graduate School of Pharmaceutical Sciences, Osaka University,
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Abstract. We have recently shown that prenatal valproic acid (VPA) exposure causes autism spectrum disorders—like behavioral abnormalities and Nissl-positive cell loss in both prefrontal and somatosensory cortices in male mice. We have also found that VPA-induced social interaction deficits are observed in male but not female offspring. This study demonstrated that the exposure to VPA at embryonic day 12.5 significantly decreased Nissl-positive cell numbers in the prefrontal cortex, but not in the somatosensory cortex, in female offspring. These findings suggest that VPA-induced morphological abnormalities in the somatosensory cortex may be involved in the sex-dependent social interaction deficits.

Keywords: autism spectrum disorder, valproate, sex difference

Autism spectrum disorders (ASD) are a group of neurodevelopmental disorders defined as pervasive impairments in social interactions and social communication, excessive anxiety or hyperreactivity to stressful experiences, restricted interests and activities, and stereotypical behaviors. It is well established that the incidence of ASD is higher in males than females, with a ratio of approximately 4:1 (1). Although ASD have been correlated with developmental differences in brain structures (2), the exact mechanisms underlying the striking sex difference in ASD remain poorly understood.

Valproic acid (VPA) is widely used to treat epilepsy, bipolar disorders, and migraine. Clinical studies suggest that VPA administration during pregnancy may result in a “fetal valproate syndrome”, which has features similar to ASD (3). Along this line, previous studies in mice showed that prenatal VPA exposure causes not only neural damage but also behavioral deficits later in life that are analogous to the aspects of ASD (4). We have recently shown that male mice exposed to VPA at embryonic day 12.5 (E12.5) display ASD-like behavioral abnormalities including social interaction deficits, anxi-

ety-like behavior, and memory deficits at 4 – 8 weeks of age, and that the behavioral abnormalities are accompanied with Nissl-positive cell loss in the middle and lower layers of the prefrontal cortex and lower layers of the somatosensory cortex (5). We also found that there is a sex difference in VPA-induced social interaction deficits at 8 weeks of age (5); that is, in contrast to male offspring, female offspring did not show VPA-induced social interaction deficits.

In the present study, we examined the effect of prenatal exposure to VPA at E12.5 on morphology in the neocortex of female mice at 8 weeks of age to clarify whether there is a sex difference in VPA-induced morphological effects.

Female ICR (CD1) mice (Japan SLC, Inc., Hamamatsu) were obtained at 8 days of gestation and housed individually in plastic cages under a standard light/dark cycle (12-h light cycle starting at 8:00 AM) at a constant temperature of 22°C ± 1°C. The animals had ad libitum access to food and water, and they were handled in accordance with the guidelines established by the Animal Care and Use Committee of the Graduate School of Pharmaceutical Sciences, Osaka University, the Guiding Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society, and the United States National Institutes of Health Guide for

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The pregnant mice were injected with either 500 mg/kg of VPA (i.p.) or saline on E12.5 (5). VPA was dissolved in 0.9% NaCl solution, and the volume of injection was 10 ml/kg. All animals were returned to their home cages immediately after the injection and left undisturbed until weaning of the offspring. Offspring born from VPA- and saline-treated mothers were weaned, sexed, and caged in groups of 5–6 mice of the same sex at postnatal day 21, and then the offspring were subjected to the experiments.

Nissl staining was performed as previously reported (5). Briefly, mice were deeply anesthetized with pentobarbital and perfused intracardially with 4% paraformaldehyde in phosphate-buffered saline (PBS). The brains were removed, post-fixed with the same fixative, and cryoprotected with 20% sucrose in PBS. Sections (20 μ m) containing the prefrontal cortex and somatosensory cortex were obtained using a cryostat (CM1510; Leica Microsystems GmbH, Wetzlar, Germany) and mounted on slides. The sections were air-dried, stained with 0.1% cresyl violet solution for 5–10 min, and protected with a coverslip. Digitized images of the Nissl-stained sections were obtained with a fluoro phase-contrast microscope system (Biorevo BZ-9000; Keyence Co., Tokyo) using a 10 $\times$ magnification lens. Nissl-positive neuronal cell numbers were manually and rigidly counted within the prefrontal cortex (layers II/III and V) and somatosensory cortex (layers II/III and IV–V) of the scanned digital images. The total cell counts were averaged from at least three sections per animal.

The experimental data were statistically analyzed using StatView® 5.0 for Windows (SAS Institute, Cary, NC, USA). The significance of differences was determined by Student's *t*-test. The criterion for statistical significance was $P < 0.05$.

We first confirmed the previous finding (5) showing

that prenatal VPA exposure induces morphological changes of cortical layers in 8-week-old male offspring (Fig. 1). In agreement with the previous finding (5), the prenatal VPA exposure at E12.5 caused approximately 20%–25% of Nissl-positive cell loss in the middle and lower layers of the prefrontal cortex (Fig. 1A) and in the lower layers of the somatosensory cortex (Fig. 1B), without affecting the thickness of each layer. Under the conditions, we examined the effects of prenatal VPA exposure on Nissl-positive cells in the prefrontal cortex and the somatosensory cortex in 8-week-old female mice (Fig. 2). The prenatal treatment with VPA did not affect the thickness of each layer and caused Nissl-positive cell loss in the middle and lower layers of the prefrontal cortex in female offspring (Fig. 2A). The observation was similar to the case in male offspring, but the prenatal VPA exposure had no effect on Nissl-positive cells in any layers of the somatosensory cortex in female offspring (Fig. 2B). The quantitative analysis on cortical morphology of female mice is shown in Fig. 3. The prenatal VPA exposure at E12.5 significantly decreased the number of Nissl-positive cells in both layers II–III and V of the prefrontal cortex (Fig. 3A), whereas it did not affect the number of Nissl-positive cells in the somatosensory cortex (Fig. 3B).

We have recently demonstrated that male mice prenatally exposed to VPA at E12.5 exhibit significant decreases in the number of Nissl-positive cells not only in the middle and lower layers of the prefrontal cortex but also in the lower layers of the somatosensory cortex (5). The present histochemical comparison between male and female mice reveals that the effect of prenatal VPA on the prefrontal cortex is similar in male and female mice, but there is a difference in the effect of VPA on the somatosensory cortex between male and female mice. These findings indicate that prenatal exposure to VPA causes sex-dependent morphological changes in the so-

Fig. 1. Representative microscopic images of the Nissl-stained brain sections in male mice at 8 weeks of age. Male offspring born to mothers treated i.p. with VPA (500 mg/kg) or saline at E12.5 were subjected to histochemical analysis. Nissl-positive cells were visualized by Cresyl Violet staining. The Nissl-stained prefrontal cortex (A) and somatosensory cortex (B) from VPA-exposed (right) and saline-treated offspring (left). Scale bar, 200 μ m.

Fig. 2. Representative microscopic images of the Nissl-stained brain sections in female mice at 8 weeks of age. Female offspring born to mothers treated i.p. with VPA (500 mg/kg) or saline at E12.5 were subjected to histochemical analysis. Nissl-positive cells were visualized by Cresyl Violet staining. The Nissl-stained prefrontal cortex (A) and somatosensory cortex (B) from VPA-exposed (right) and saline-treated offspring (left). Scale bar, 200 μ m.

Fig. 3. Number of Nissl-positive cells in the brain sections in female mice (8 weeks of age) prenatally exposed to VPA at E12.5. Prenatal VPA exposure decreased the number of Nissl-positive cells in layers II/III and V of the prefrontal cortex (A), but not in layers II/III and IV – V of the somatosensory cortex (B). Data are expressed as the mean $\pm$ S.E.M. ($n = 5$). ** $P < 0.001$ vs. saline-treated mice.

matosensory cortex.

Although it is relatively unlikely that the complex behavioral phenotype of ASD can be explained by abnormalities in a specific brain region, neuropathological studies indicate that abnormalities in the cytoarchitectural organization of the cerebral cortex and subcortical structures are the most consistent findings in postmortem brain tissues from ASD patients (6). The prefrontal cortex has been shown to play a crucial role in decision-making, social cognition, and emotional processing (7). In addition, Rinaldi et al. (8) have shown that the layer V pyramidal neurons of the medial prefrontal cortex are connected to significantly more neighboring neurons in VPA-treated rats as compared to control rats, and that these cells are less excitable. This observation suggests that abnormal structures and function in the prefrontal cortex may contribute to VPA-induced common ASD-like behavioral phenotypes. On the other hand, the somatosensory cortex has been implicated in the processing of social cues to motivation, emotion, and cognition, which finally links to social behaviors in human beings. Soma-

tosensory processing impairment is found in a range of neurodevelopmental disorders including ASD and is associated with deficits in communication, motor ability, and social skills in these disorders (9). In animal studies, Tabuchi et al. (10) showed that ASD-relevant neurotrophin-3 mutation (R451C) knock-in mice exhibited a behavioral phenotype composed of social interaction deficits, increased spatial memory, and increased inhibitory synaptic transmission in the somatosensory cortex. It is also demonstrated that *Fmr1* knockout mice, an excellent model of fragile X syndrome, exhibit behavioral abnormalities, such as learning disabilities and social behavior deficits (11), and circuit and plasticity defects in the developing somatosensory cortex (12). These findings suggest that the somatosensory cortex plays a key role in social interaction deficits. In this relation, prenatal exposure to VPA causes social interaction deficits in male but not female mice, although VPA-induced social anxiety-like behavior and memory deficits are observed in both male and female mice (5). Taken together, we propose that the VPA-induced morphological effect in the soma-

tosensory cortex plays a key role in social interaction deficits and the effect in the prefrontal cortex may be responsible for the anxiety-like behavior and memory deficits.

The exact mechanism for the sex difference in susceptibility of somatosensory cortical cells to VPA remains unclear. Converging evidence supports an important role of serotonin (5-HT) in the refined organization of the somatosensory cortex (13). In addition, it is shown that the brain 5-HT system has a higher potential in female than in male rats (14). On the other hand, there is increasing evidence that estrogens act as direct modulators of cell behaviors in brain development. Interestingly, estrogen receptor β -knockout mice displayed a regional neuronal hypocellularity in the brain, with a severe neuronal deficit in the somatosensory cortex, especially layers II–V at 2 months of age (15). These findings suggest that estrogens play a key role in sex differences regarding susceptibility of somatosensory cortical cells to VPA.

In conclusion, the present study demonstrates that there is a sex difference in the effect of VPA in the somatosensory cortex, but not in the prefrontal cortex. This finding together with the previous behavioral analysis in VPA-treated mice suggests that VPA-induced social interaction deficits may be due to morphological abnormalities in the somatosensory cortex. Furthermore, it is likely that VPA-induced morphological changes in the prefrontal cortex are associated with anxiety-like behavior and memory deficits.

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