Brain activity and connectivity in rat model of autism induced by prenatal exposure to valproate

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Brain activity and connectivity in rat model of autism induced by prenatal exposure to valproate

Directed by Professor Chul Hoon Kim

The Doctoral Dissertation
submitted to the Department of Medical Science,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

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December 2016

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December 2016

ACKNOWLEDGEMENTS

박사 학위 과정을 돌이켜보면 많은 분들께서 여러 가지로 많은 도움을 주셨기 때문에 이 과정을 잘 마칠 수 있었다는 생각이 듭니다. 그 동안 미처 전하지 못한 감사하다는 마음을 지면을 통하여 표현하기에는 부족하지만, 도움을 주셨던 많은 분들께 이렇게 짧게나마 감사하다는 말씀을 드리고 싶습니다.

학위 기간 동안 글로 표현하기 어려울 정도로 많은 가르침을 주신 김철훈 교수님께 감사드립니다. 언제나 제가 보지 못하던 것을 볼 수 있게 해주시고 새로운 생각을 할 수 있도록 많은 도움을 주신 김동구 교수님께 감사드립니다. 학생 때부터 지금까지 많은 시간 동안 여러 가지로 이끌어 주신 박해정 교수님께도 감사드립니다. 논문을 진행하는 동안 여러 가지로 조언해 주시고 도움을 주신 정호성 교수님과 김어수 교수님께 감사드립니다. 약리학교실에 있는 동안에 많은 가르침을 주셨던 김경환 교수님, 안영수 교수님, 이민구 교수님, 박경수 교수님, 김주영 교수님, 김형범 교수님께 이 자리를 빌려 감사의 마음을 드립니다. 전공의 때부터 지금까지 많은 도움을 주시고 이 자리에 있을 수 있도록 이끌어 주신 이종두 교수님, 윤미진 교수님, 강원준 교수님께 감사드립니다.

약리학교실에 있는 동안에 부족한 저에게 많은 도움을 주신 선·후배님들과 동료들에게 감사드립니다. 처음 실험실에 들어왔을 때부터 많은 도움을 주었던 헌영이 형, 진세 형, 같이 실험실에 들어와서 많은 힘이 되었던 홍인이 형, 은석이, 형순이, 정환이, 그리고 같은 실험실에서 많은 시간을 함께한 실험실 동료들, 특히

정호, 제호, 그리고 조아련 박사님께 감사한 마음을 전하고 싶습니다. 처음 실험이라는 세계를 접할 때 많은 도움을 주신 최민아 박사님, 그리고 바쁜 와중에도 많은 시간을 힘든 내색 없이 도와주신 오맹근 선생님, 무리하면서까지 많은 도움을 주었던 혜원이에게도 감사하다는 말을 전하고 싶습니다. 그리고 짧지 않은 시간 동안 함께하면서 언제나 든든한 힘이 되어준 태윤이 형, 준상이, 윤대, 호준이에게도 평소에 미처 말하지 못했지만 항상 감사하고 있었다는 마음을 전하고 싶습니다.

끝으로 지금까지 늘 그랬듯이 항상 곁에서 지켜주고 흔들리지 않도록 도와준 가족에게 감사한 마음을 전하고 싶습니다.

다시 한 번 모든 분들께 진심으로 감사드립니다.

2016년 12월

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Brain activity and connectivity in rat model of autism induced by prenatal exposure to valproate

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Autism spectrum disorder (ASD) is a severe lifelong neurodevelopmental disorder characterized by early-onset impairments in social communication and social interaction, and restrictive and repetitive behavior, interests, or activities. Currently no biological markers are available to make a reliable diagnosis of ASD. I used valproic acid (VPA) rat model to explore the possible alterations in metabolic brain activity and connectivity. Female Sprague Dawley rats were given a single intraperitoneal injection of sodium valproate or normal saline on embryonic day 12.5. To evaluate autistic-like behaviors in pups, social interaction was examined during postnatal weeks 4-6 using three-chamber social approach test. Resting-state ^{18}F-fluorodeoxyglucose (FDG) positron emission tomography (PET) scans were acquired at postnatal weeks 6 or 62. To assess the changes in metabolic brain activity by VPA treatment, Statistical Parametric Mapping (SPM) was performed. I also examined if change in metabolic brain connectivity was

accompanied with alterations of regional metabolic activity. Metabolic connectivity was modeled using both conventional correlation analysis, and sparse inverse covariance estimation (SICE), a partial correlation analysis. VPA-treated rats exhibited impairments in social behaviors, and this difference was more pronounced in male than female rats. Preference for social novelty was impaired in VPA-treated male rats, while sociability was diminished in VPA-treated female rats. I found that metabolic activity and connectivity was significantly changed by VPA treatment. Changes in metabolic connectivity was revealed by SICE while conventional correlation analysis did not show any difference. VPA-treated male rats had significantly decreased metabolic activity in the olfactory bulb, and had decreased metabolic connectivity between the left insular cortex and left amygdala, which constitute the salience network. There were no brain regions with decreased metabolic activity in VPA-treated female rats. In contrast, VPA-treated female rats had reduced metabolic connectivity between the thalamus and midbrain, and between the right medial prefrontal cortex and left caudoputamen. Such alterations in metabolic activity and connectivity may represent neurobiological substrates of autistic-like behavior, particularly in males, and may serve as a pathognomonic sign in VPA rat models of ASD. As such this study supports the idea that non-invasive brain imaging may serve as an imaging endophenotype that could aid diagnosis of ASD, classification of severity, and possibly reveal insights to neurobiological underpinnings in autistic-like behavior.

Key words : autism spectrum disorder, rat, valproic acid, metabolic connectivity, fluorodeoxyglucose, positron emission tomography, sparse inverse covariance estimation

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I. INTRODUCTION

Autism spectrum disorder (ASD) is a severe lifelong neurodevelopmental disorder characterized by early-onset impairments in social communication and social interaction, and restrictive and repetitive behavior, interests, or activities.[1] ASD is one of the most common disorders among children. According to estimates released by the Centers for Disease Control and Prevention, ASD affects one in 68 children. ASD is five times more prevalent in boys than in girls.[2] Early diagnosis is essential for early intervention, which provides the opportunity for optimal development and greatly improves prognosis.[3,4] However, ASD comprises a highly heterogeneous set of disorders, which poses a considerable challenge to make an accurate diagnosis, especially in preverbal children.[5] The variable manifestations of ASD stem from the level of functioning and comorbidity, also renders the diagnosis difficult.[6] As there are no reliable biological markers for ASD, the diagnosis is made based on behavioral signs and

symptoms. There is no single known cause for ASD. Both the genetic and environmental factors are thought to play roles in the development of ASD.[7]

Valproic acid (VPA) is one of the most commonly prescribed teratogenic drugs in women of child bearing potential. It is used for the treatment of epilepsy and other neuropsychological disorders. VPA has been shown to increase in brain concentration of gamma-aminobutyric acid (GABA), the major inhibitory neurotransmitter.[8] The mechanism of action of VPA has also been attributed to the direct inhibition of voltage-gated sodium channel and negative modulation of glutamatergic signaling. It was also demonstrated that VPA acts as a nonselective histone deacetylase (HDAC) inhibitor, and this contributes to the teratogenic effects of VPA.[9]

Prenatal exposure to VPA is known to be associated with increased risk of ASD in human.[10,11] Fetal valproate exposure in rodent showed brain abnormalities similar to those found in patients with ASD: reduced in neuron number in the oculomotor, trigeminal, abducens, and hypoglossus nerves; and small cerebellum with decreased number of Purkinje cells. Rats prenatally exposed to VPA displayed reduced social interaction, increased repetitive/stereotyped behaviors, and early signs of neurodevelopmental delay.[12] The VPA rat model has been shown to have construct validity, face validity, and predictive validity.[13] However, it remains unclear how VPA exposure leads to neurobiological alterations responsible for the development of autistic-like behaviors.

A growing body of evidence supports the notion that ASD is associated with altered brain connectivity.[14] Decreases in white matter integrity and impaired long-range connectivity are well known findings in humans with ASD.[15,16] However, the connectivity changes associated with such findings and their relation to the behavioral phenotypes of ASD are still uncertain.[17-19] Furthermore, findings from neuroimaging studies are largely inconsistent and even contradictory across studies.[18,20] This may be due to the heterogeneous nature of

subtypes and origins of the disorders studied. Thus, a well-controlled study investigating changes in neural circuitry in a homogeneous type of ASD would reveal a more concrete relation between autistic-like behaviors and neurobiological alterations.

In addition to heterogeneity, a strong male predominance has been observed in ASD, which affects boys five times more frequently than girls.[2] This difference in prevalence between boys and girls is thought to arise from less pronounced repetitive and stereotyped behaviors in girls and dissimilar levels of functioning between boys and girls, or male biased diagnostic criteria, and social and cultural expectations.[21,22] Sex-specific differences are thought to potentiate risk in males and/or attenuate risk in females induced by genetic and environmental factors.[2] However, little attention has been paid to how ASD in males differs from that in females, particularly with how differences in neurobiology may underlie differences in behavior. Although VPA is known to affect rats in a sex-specific way,[23] no clear neurobiological mechanism underlying this difference has been identified.

In the present study, I explored alterations in the brain connectivity of VPA-treated and control rats using resting-state ^{18}F-fluorodeoxyglucose (FDG) positron emission tomography (PET). Functional connectivity refers the temporal correlation of the activities among distinct brain regions. It is generally inferred from blood oxygenation level-dependent (BOLD) functional magnetic resonance imaging (fMRI) or electro- or magnetoencephalogram (EEG/MEG).[24] In particular, BOLD fMRI has received much attention after the discovery of spatially organized low-frequency spontaneous fluctuations in the absence of explicit task.[25,26] Recent investigations regarding brain circuitry have begun to utilize resting-state functional connectivity mapping with fMRI in animals.[27] However, unlike studies involving humans, animal studies using resting-state fMRI inevitably require sedation of animals, which limits exploration of resting-state functional connectivity.[28] In contrast, ^{18}F-FDG uptake reflects activity in the

awake state since animals are sedated after the completion of ^{18}F-FDG distribution during the resting state. Furthermore, regional ^{18}F-FDG uptake can be considered a more direct measure of neuronal activity BOLD signals in fMRI, as neurons exhibit preferential uptake of glucose in an activity-dependent manner.[29] In contrast, the BOLD signal is an indirect measure of neural activity. It depends on the change in concentration of deoxyhemoglobin in the microvasculature, and is affected by cerebral blood flow (CBF), cerebral blood volume (CBV), and cerebral blood oxygen consumption ($CMRO_2$) in relation to neural activity.[30,31] Although ^{18}F-FDG PET images do not provide time series data for the conventional evaluation of functional connectivity, introduction of cross-sectional analysis of ^{18}F-FDG PET imaging allows researchers to evaluate differences in both regional activity and connectivity between groups. As ^{18}F-FDG PET reveals neuronal activity at steady state in hourly terms, PET-based metabolic connectivity can be estimated by measuring regional variations of metabolic activity in the brain.[32,33]

First I performed resting-state ^{18}F-FDG PET in VPA-treated rats and applied Statistical Parametric Mapping (SPM) to explore if there were any brain regions with altered metabolic activity in VPA-treated rats. I also examined if change in metabolic connectivity was accompanied with alterations of regional metabolic activity. I adopted a recently proposed analysis approach for estimating inverse covariance, called sparse inverse covariance estimation (SICE), a group-level partial correlation analysis of metabolic activity, which enables a more comprehensive understanding of brain connectivity, as compared to conventional intensity-based analysis.[34,35] I tested whether the metabolic connectivity estimated by both conventional correlation analysis and SICE can identify the altered brain connectivity in VPA rat model of ASD in relation to autistic-like behaviors.

II. MATERIALS AND METHODS

1. Animals

Animal protocols were approved by the Yonsei University College of Medicine Institutional Animal Care and Use Committee (IACUC). Pregnant Sprague-Dawley (SD) female rats (Orient Bio Inc., Gyeonggi-do, South Korea) were randomly assigned to receive a single subcutaneous injection of sodium valproate (Sigma) dissolved in saline (400 mg/kg), or saline alone on embryonic day 12.5 (E12.5), as previously described.[36] Dams were housed individually and were allowed to raise their own litters. The rats were divided into two groups (adolescence and adulthood) based on the age at imaging. Litters in adolescent group were culled to four male and four female pups on postnatal day 2. Litters in adult group were not culled. Pups were weaned at postnatal day 21 (P21), and were housed with 2-3 rats in same-sex and same-treatment cages. Standard plastic laboratory cages were used with bedding and *ad libitum* access to food and water, handled twice a week. Animals were kept in a 12-hr light-dark schedule with lights on at 8:00 am, in rooms under controlled humidity and temperature.

2. Pup body weight

Body weight gain of the pup was measured weekly for 5 wk for adolescent group (postnatal week (PNW) 1-5), and for 31 wk for adult group (PNW 2-32). I used a mixed effect model to analyze the effect of VPA treatment on pup body weight over time. The model was built using R (version 3.2.2) and the *lmer()* function in the lme4 package (version 1.1-10).

3. Three-chamber social approach test

Three-chamber social approach test was performed as described previously with minor modifications.[37] The test consisted of three phases. In the first phase (habituation), a test rat was placed in the center of the empty three-chamber apparatus with two small wire cages in the left or right chamber

to habituate for 5 min. In the second phase (sociability test), an age- and sex-matched rat that had never been exposed to the test rat, was placed in one of the two wire cages. The empty wire cage was presented as a novel object. Then the two entrances were opened to allow the test rat in the center to freely explore each of the three chambers for 10 min. In the third phase (preference for social novelty test), the test rat was gently guided to the center chamber, and another age- and sex-matched novel rat was placed in the empty wire cage. Then the two entrances were opened to allow the test rat in the center to freely explore for another 10 min. All tests were performed between 9 AM and 6 PM. Time spent in each chamber was measured by the video tracking software (EthoVision XT 11.5, Noldus Information Technology, Wageningen, The Netherlands). In adolescent group, the test was performed on PNW4, and adult group test was performed on PNW6.

4. ^{18}F-FDG PET acquisition

PET scans were performed on PNW6 in adolescent group, and on PNW62 in adult group. All rats were deprived of food for 12-18 hr before the scanning, but had access to water at all times. The rats were injected intraperitoneally with 37 MBq of ^{18}F-FDG under light isoflurane anesthesia. Then the rats were moved back to the plastic cages, and woke up immediately from anesthesia. The cage was placed in a quiet room with dim light for 50 min. PET scans were acquired on Siemens Inveon scanner (Siemens, Knoxville, TN, USA). Static emission scan was started at 60 min after injection under isoflurane anesthesia. Emission scan was acquired for 20 min, followed by 20 min of transmission scan. All PET scans were performed between 9 AM and 6 PM. The images were reconstructed using the ordered subsets expectation maximization (OSEM) algorithm with attenuation, scatter, and random correction. The voxel size was 0.776 × 0.776 × 0.796 mm.

5. Image processing

Spatial processing was performed using Statistical Parametric Mapping (SPM8, Wellcome Department of Cognitive Neurology, London, UK). All reconstructed images were spatially normalized to the ^{18}F-FDG rat brain template (PMOD 3.6, PMOD Technologies Ltd, Zürich, Switzerland).

As the core symptoms of ASD and comorbidities tend to persist throughout the lifetime,[38,39] I assumed that if there are distinct features in the brain which represent autistic-like behavior, the features do not vary with age as long as the symptoms persist. Therefore, I did not consider the effect of aging in further imaging analysis.

6. Voxel-based statistical analyses

Voxel-based statistical analyses were performed with SPM. Spatially normalized images were smoothed with a 1.6 × 1.6 × 1.6 mm^3 full-width-half-maximum (FWHM) Gaussian kernel. Proportional scaling was used for global normalization. For statistical inferences, an P-value < 0.05 (family-wise error (FWE) corrected) with an extent threshold of 50 continuous voxels was considered significant.

7. Node definition

The images were segmented using predefined volume of interests (VOIs) in PMOD software. I chose 58 anatomical VOIs that served as nodes throughout the brain (Table 1). ^{18}F-FDG uptake in the VOIs was normalized to global brain activity.

Table 1. Volume of interest for connectivity estimation

Name	Abbreviation
Motor cortex left	MO-L
Somatosensory cortex left	SS-L
Auditory cortex left	AUD-L
Visual cortex left	VIS-L
Cingulate cortex left	CG-L
Frontal association cortex left	FA-L
Medial prefrontal cortex left	MPF-L
Orbitofrontal cortex left	OF-L
Insular cortex left	INS-L
Retrosplenial cortex left	RSP-L
Parietal association cortex left	PA-L
Olfactory bulb left	OB-L
Anterodorsal hippocampus left	HA-L
Posteroventral hippocampus left	HP-L
Entorhinal cortex left	ENT-L
Amygdala left	AMG-L
Caudoputamen left	CP-L
Nucleus accumbens left	ACB-L
Septum left	S-L
Thalamus left	TH-L
Hypothalamus left	HY-L
Superior colliculus left	SC-L
Inferior colliculus left	IC-L
Midbrain left	MB-L
Periaqueductal gray left	PAG-L
Ventral tegmental area left	VTA-L
Pons left	P-L
Medulla left	M-L

Cerebellum left	CB-L
Motor cortex right	MO-R
Somatosensory cortex right	SS-R
Auditory cortex right	AUD-R
Visual cortex right	VIS-R
Cingulate cortex right	CG-R
Frontal association cortex right	FA-R
Medial prefrontal cortex right	MPF-R
Orbitofrontal cortex right	OF-R
Insular cortex right	INS-R
Retrosplenial cortex right	RSP-R
Parietal association cortex right	PA-R
Olfactory bulb right	OB-R
Anterodorsal hippocampus right	HA-R
Posteroventral hippocampus right	HP-R
Entorhinal cortex right	ENT-R
Amygdala right	AMG-R
Caudoputamen right	CP-R
Nucleus accumbens right	ACB-R
Septum right	S-R
Thalamus right	TH-R
Hypothalamus right	HY-R
Superior colliculus right	SC-R
Inferior colliculus right	IC-R
Midbrain right	MB-R
Periaqueductal gray right	PAG-R
Ventral tegmental area right	VTA-R
Pons right	P-R
Medulla right	M-R
Cerebellum right	CB-R

8. Connectivity estimated by correlation

For each pair of VOIs, Pearson's correlation coefficient was calculated in an inter-subject manner. Then, correlation matrix was generated for each group, VPA-treated and control rats in each sex. Correlation matrices for each group were transformed to Z scores using Fisher transformation. Permutation test was performed to test statistical differences between groups, followed by Fisher transformation. The subject labels for each group were permuted 10,000 times. Significant differences were determined by applying a false-discovery rate (FDR) threshold of 0.05.

9. Connectivity estimated by sparse inverse covariance estimation

The SICE was used to estimate metabolic connectivity structure, and stability approach to regularization selection (StARS) was applied to determine the optimal network.[34,35] I adopted a constrained optimization algorithm to estimate the strength of connections in the graph obtained by SICE.[40] To test statistical differences between VPA-treated and control rats, I obtained a null distribution by permuting subject labels and the optimal network was re-estimated for each permuted groups. The entire subject labels were permuted 10,000 times. A FDR threshold of 0.05 was used as significance cutoff. I compared the sparsity and connectivity strength in the network between groups.

10. Statistical analysis

Statistical analyses were performed using R (version 3.2.2). Unless otherwise specified, data were analyzed with two-tailed unpaired Student's t-test or Mann-Whitney U test as appropriate. I also used one-way analysis of variance (ANOVA) with injected ^{18}F-FDG activity as a between-group factor. The null hypothesis was rejected for alpha greater than 5%. Details on statistical method applied in imaging analyses are described in each section.

III. RESULTS

1. Pup body weight gain

Rats treated with VPA had lower body weight both in adolescent and adult groups. I found a statistically significant effect of VPA treatment both in adolescent (*t*-value = -6.71, P < 0.001) and adult (*t*-value = -2.97, P = 0.004) groups. *Post hoc* analysis using two-tailed unpaired Student's t-test or Mann-Whitney U test showed lower body weight in VPA-treated rats on PNWs from 1 to 5 in adolescent group in both sexes (*n*=8 except for VPA-treated male rats (*n*=7)) (Figure 1A). In adult group, lower body weight was seen in VPA-treated male rats (*n*=15) compared to control male rats (*n*=9) on PNWs 12, 21, and 28. VPA-treated female rats (*n*=11) had lower body weight compared to control female rats (*n*=11) on PNWs 11, 12, 19, 20, 22-24, 27, 31, and 32 (Figure 1B).

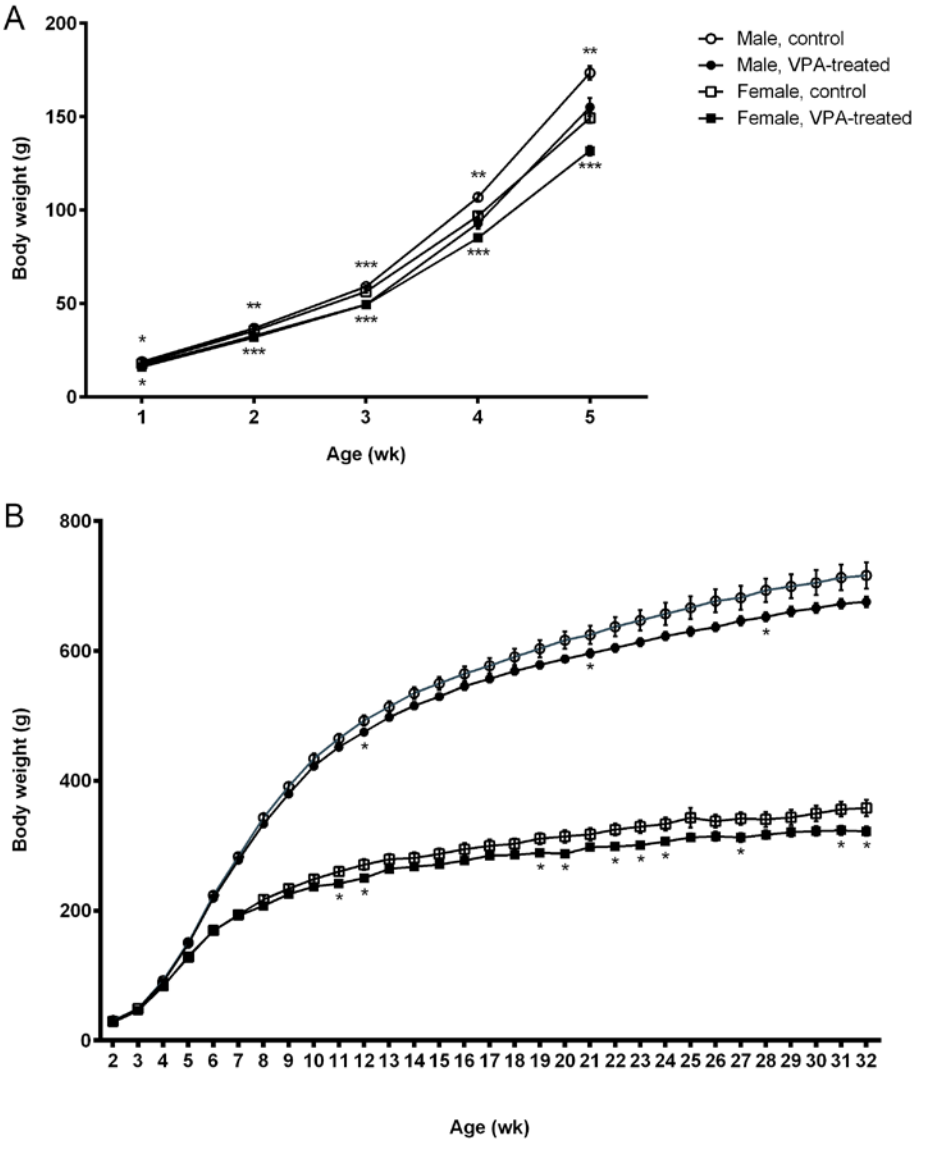

Figure 1. Prenatal exposure to VPA result in reduced body weight in rat pups. (A) Weight gain in adolescent group. (B) Weight gain in adult group. There was a significant effect of VPA treatment on body weight. VPA-treated male (*n*=7, adolescent VPA-treated male; *n*=15, adult VPA-treated male) and female rats (*n*=8, adolescent VPA-treated female; *n*=11, adult VPA-treated female) were lighter than controls (*n*=8, adolescent control male; *n*=9, adult control male; *n*=8,

adolescent control female; *n*=11, adult control female) both in adolescent and adult groups. Female rats were lighter than male rats. Data shown as mean and standard error of the mean (SEM). In each time point, data were analyzed with two-tailed unpaired Student's t-test or Mann-Whitney U test as appropriate. VPA, valproic acid; *P < 0.05, **P < 0.01, ***P < 0.001.

2. Three-chamber social approach test

VPA treatment found to have no significant effect in sociability test. Both in adolescent and adult group, all rats preferred to explore the first stranger (S1) over an empty wire cage presented as a novel object (O), irrespective of VPA treatment or sex (Figures 2A, B). I found that VPA-treated male rats displayed reduced social preference for novelty. In adolescent group, there was a trend that VPA-treated male rats spent less time with the second stranger (S2) compared to controls, however VPA-treated male rats still spent significantly more time with the S2 than with the S1, as controls do (Figure 2C). In adult group, control male rats preferred to spend more time with the S2 over S1. In contrast, the preference to the S2 over S1 was absent in VPA-treated male rats (Figure 2D). VPA-treated female rats in adolescent group showed preference to the S1 over S2, while control female rats did not show preference to the S1 (Figure 2C). As all adult female rats had preference to the S2 over S1, there was no significant effect of VPA treatment (Figure 2D). In brief, although VPA-treated adolescent male rats maintained preference for the S2 over S1, VPA-treated male rats showed autistic-like behaviors in general.

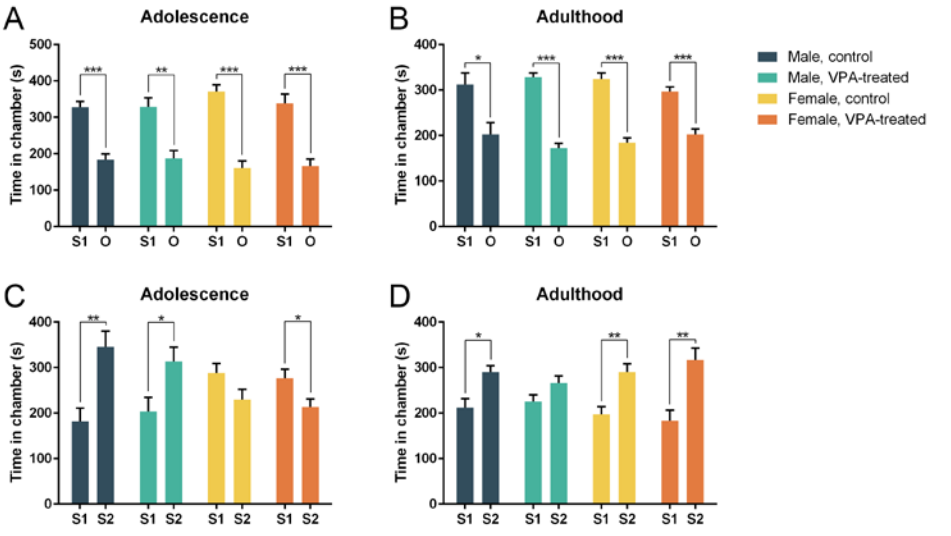

Figure 2. Sociability or social preference for novelty was impaired by VPA treatment. (A) Sociability test in adolescent group (*n*=8, adolescent control male; *n*=7, adolescent VPA-treated female; *n*=8, adolescent control female; *n*=8, adolescent VPA-treated female). (B) Sociability test in adult group (*n*=9, adult control male; *n*=15, adult VPA-treated male; *n*=11, adult control female; *n*=11, adult VPA-treated female). (C) Social preference for novelty test in adolescent group. (D) Social preference for novelty test in adult group. VPA treatment had no effect on sociability test. VPA-treated adult male rats lost preference to the S2 over S1. VPA-treated female rats spent more time with the S1 over S2. Data represent mean ± SEM. Data were analyzed with two-tailed unpaired Student's t-test or Mann-Whitney U test as appropriate. VPA, valproic acid; O, object; S1, stranger 1; S2, stranger 2; SEM, standard error of the mean. *P < 0.05, **P < 0.01, ***P < 0.001.

3. Injected ^{18}F-FDG activity

Injected ^{18}F-FDG activity in control males (*n*=17, 40.52 ± 0.40 MBq), VPA-treated males (*n*=22, 40.12 ± 0.34 MBq), control females (*n*=19, 40.48 ± 0.35 MBq), and VPA-treated females (*n*=19, 40.48 ± 0.31 MBq) were not differed across groups. All values are mean ± standard error of the mean (SEM).

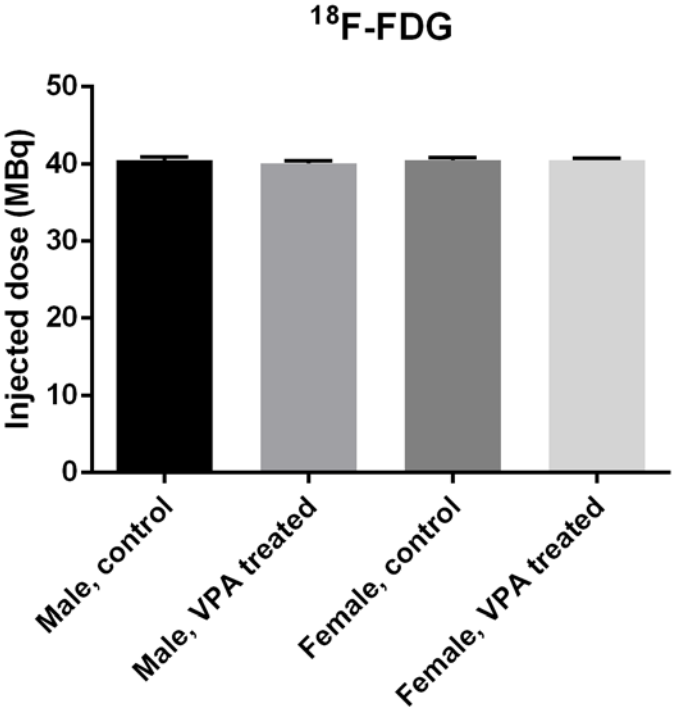

Figure 3. Injected ^{18}F-FDG activity did not differ between all four subgroups. Injected dose in control males (*n*=17), VPA-treated males (*n*=22), control females (*n*=19), and VPA-treated females (*n*=19) were not different across groups. Data represent mean ± SEM. Data were analyzed with one-way ANOVA. VPA, valproic acid; SEM, standard error of the mean; ANOVA, analysis of variance.

4. Voxel-based statistical analyses

VPA-treated male rats (*n*=22) had reduced metabolism in the olfactory bulb compared to control male rats (*n*=17) at $P < 0.05$ corrected for family-wise error (FWE) (Figure 4A). The metabolism in the bilateral somatosensory cortices, thalami, and cerebellum was increased in VPA-treated male rats than control male rats (Figure 4B). In VPA-treated female rats (*n*=19), no region was decreased in metabolism compared to control female rats (*n*=19). Increased metabolism was observed in the left caudoputamen and left medulla in VPA-treated female rats compared to control female rats (Figures 4C, D). Peak coordinates in Paxinos space for each cluster are shown in Table 2.

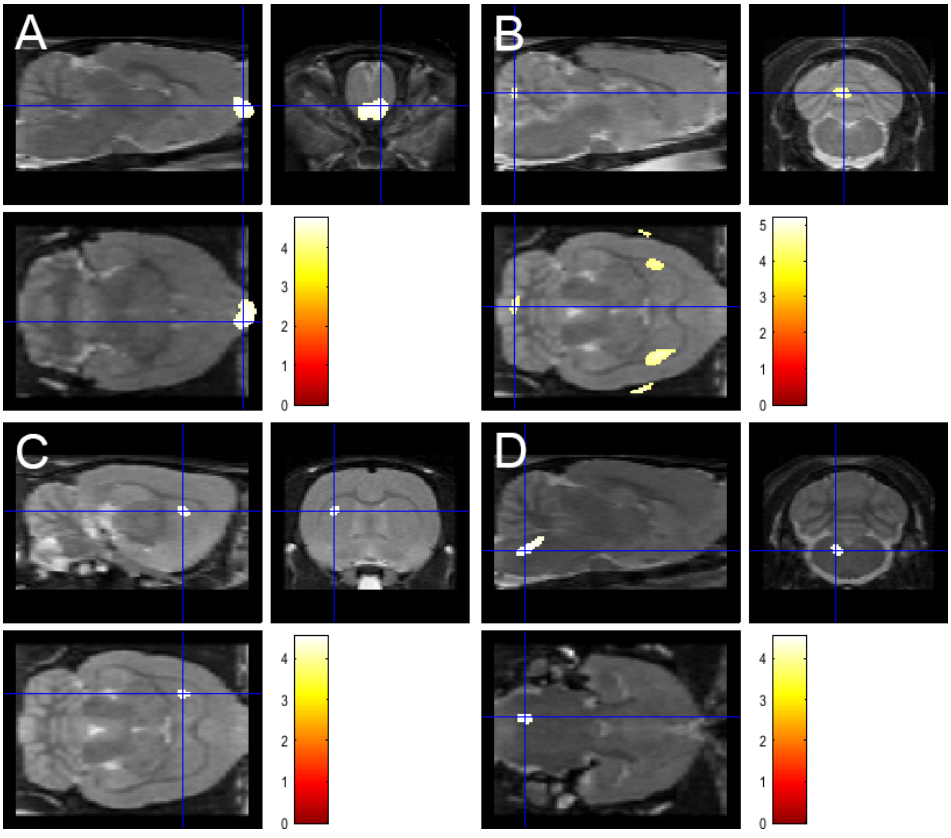

Figure 4. Statistical parametric mapping revealed altered brain activity by VPA treatment. (A) Metabolism in the olfactory bulb was decreased in VPA-treated male rats (*n*=22) compared to control male rats (*n*=17). (B) Metabolism in the bilateral somatosensory cortices, caudoputamen, and cerebellum was increased in VPA-treated male rats. (C-D) VPA-treated female rats (*n*=19) had increased metabolism in the left caudoputamen and left medulla compared to control female rats (*n*=19). Color bar indicates *t*-values. Voxels survived at family-wise error (FWE) corrected $P < 0.05$ were shown. VPA, valproic acid.

Table 2. Brain regions with significant metabolic differences in VPA-treated rats compared to control rats

Region	Paxinos coordinates			*t*-value	*k*
	x	y	z		
VPA-treated male					
Decreased metabolism*					
Olfactory bulb, bilateral	1.1	5.2	-5.8	4.7	962
Increased metabolism*					
Somatosensory cortex, left	-6.7	-2.0	-3.0	5.2	597
Somatosensory cortex, right and caudoputamen, right	6.7	-1.4	-3.4	5.1	1160
Cerebellum, bilateral	-0.3	-14.0	-4.6	4.7	108
Caudoputamen, left	-4.3	-1.0	-4.6	4.5	187
VPA-treated female					
Increased metabolism*					
Medulla, left	-1.1	-13.0	-8.2	4.5	198
Caudoputamen, left	-3.3	-0.4	-4.6	4.4	64

*Survived at family-wise error (FWE) corrected $P < 0.05$. VPA, valproic acid.

5. Connectivity estimated by correlation

Correlation matrices for each group in both sexes were generated: control males (*n*=17), VPA-treated males (*n*=22), control females (*n*=19), and VPA-treated females (*n*=19) (Figures 5A-D). Permutation test revealed that no connection was significantly different to show the effect of VPA treatment in both sexes.

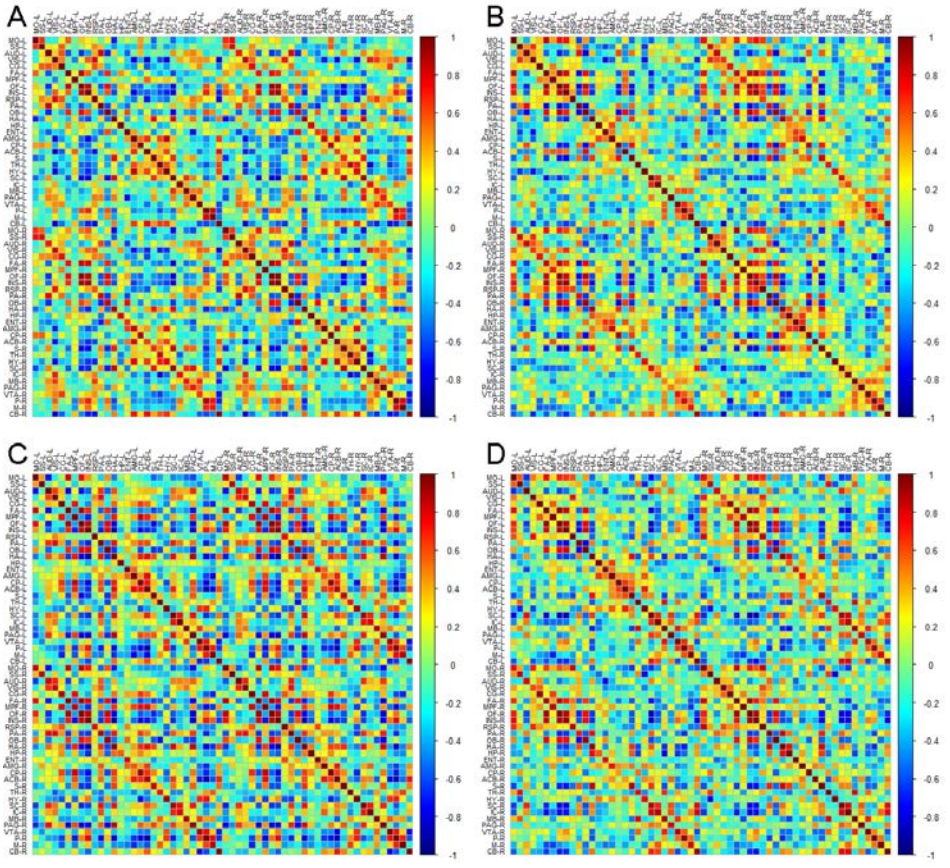

Figure 5. Correlation analysis did not show any connectivity changes by VPA treatment. (A) Control males (*n*=17). (B) VPA-treated males (*n*=22). (C) Control females (*n*=19). (D) VPA-treated females (*n*=19). VPA, valproic acid.

6. Connectivity estimated by sparse inverse covariance estimation

I estimated the most robust and stable connections using SICE for each group, VPA-treated and control rats in both sexes (Figures 5A-D). There was no significant difference in total number of connections in the brain between VPA-treated and control rats in both sexes. The number of connections within each hemisphere and between hemispheres were also not differed between VPA-treated and control rats in both sexes (Table 3).

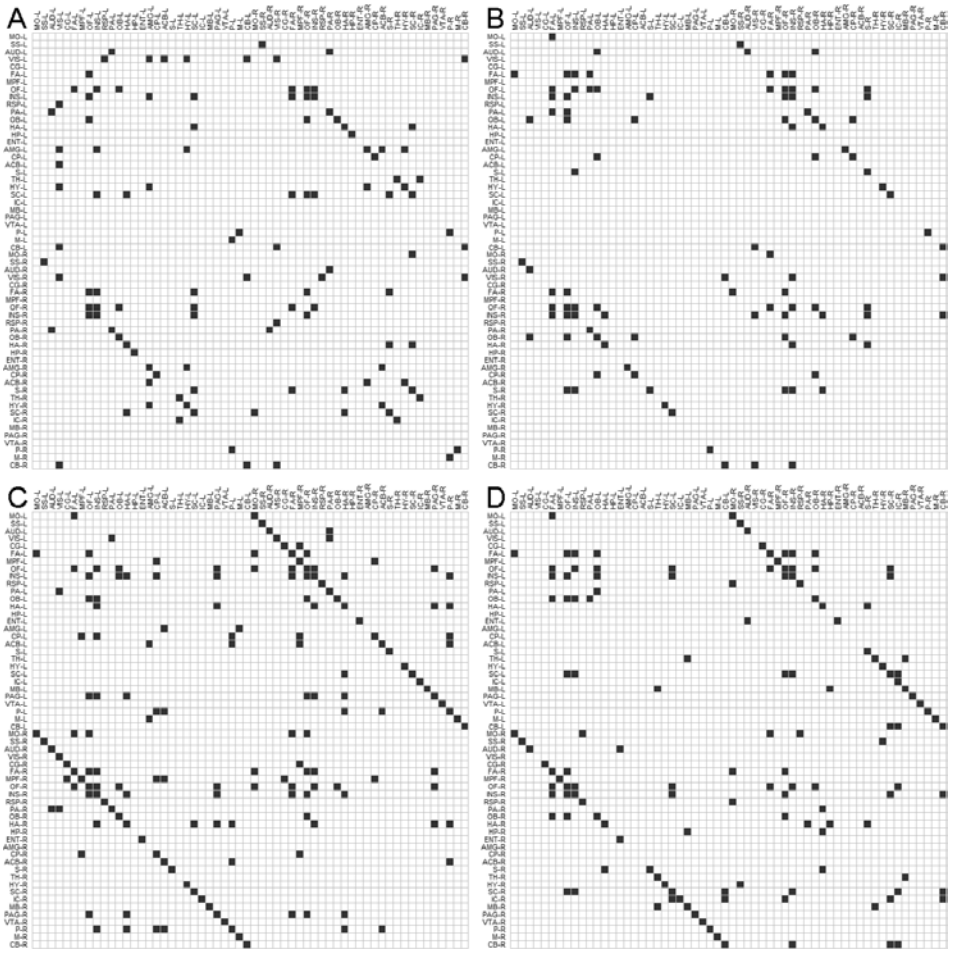

Figure 6. SICE identified connectivity changes by VPA treatment. (A) Control males (*n*=17). (B) VPA-treated males (*n*=22). (C) Control females (*n*=19). (D) VPA-treated females (*n*=19). SICE, sparse inverse covariance estimation; VPA, valproic acid.

Table 3. Number of connections in whole brain, within each hemisphere, and between hemispheres

	Number of connections		P
	Control	VPA-treated	
Male			
Whole brain	60	51	0.515
Within left cerebral hemisphere	8	10	0.284
Within right cerebral hemisphere	7	10	0.242
Between cerebral hemispheres	18	24	0.174
Female			
Whole brain	84	69	0.310
Within left cerebral hemisphere	10	8	0.401
Within right cerebral hemisphere	9	8	0.438
Between cerebral hemispheres	33	24	0.151

Data were analyzed using permutation test. VPA, valproic acid.

7. Node degree

VPA-treated male rats and control male rats were not differed in node degree. In VPA-treated female rats, the number of connections involving the left thalamus was significantly increased than in control female rats (Table 4 and Figure 7). No node with decreased number of connections was found in VPA-treated female rats compared to control female rats.

Table 4. Changes in node degree in VPA-treated female rats

Node	Number of connections		P
	Control female	VPA-treated female	
Thalamus, left	0	3	<0.001

Data were analyzed using permutation test. VPA, valproic acid.

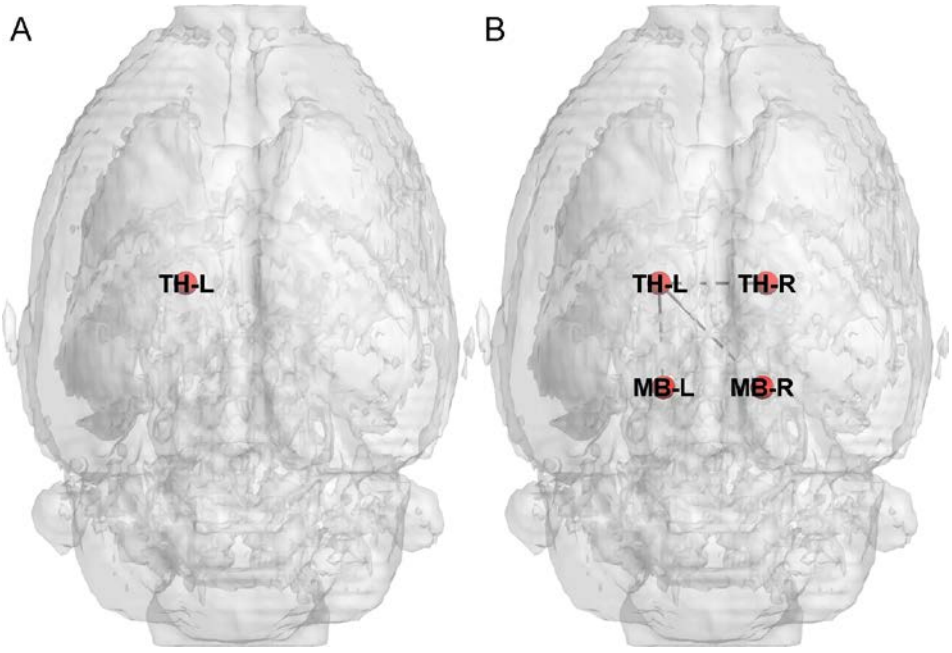

Figure 7. Aberrant number of connections was found in VPA-treated female rats. (A) Control females (*n*=19). (B) VPA-treated females (*n*=19). Compared to control females, VPA-treated female rats had an increased number of connections in the left thalamus. Data were analyzed using permutation test. MB, midbrain; TH, thalamus; VPA, valproic acid; L, left; R, right.

8. Connectivity strength

In VPA-treated male rats, the connectivity strength between the left insular cortex and left amygdala was significantly reduced than in control male rats, which is important in salience processing.[41] The connectivity strength between the left pons and left medulla, and between the right nucleus accumbens and right hypothalamus were increased in VPA-treated male rats compared to control male rats. VPA-treated female rats had decreased connectivity strength between the thalamus and midbrain, bilaterally, and between the right medial prefrontal cortex and left caudoputamen compared to control female rats. The connectivity strength between the right medial prefrontal cortex and right caudoputamen, and between the right somatosensory cortex and right hypothalamus were increased in VPA-treated female rats (Table 5 and Figure 8)

Table 5. Changes in connectivity strength by VPA treatment

Comparisons Connections	Connectivity strength		P
	VPA-treated	Control	
VPA-treated males < Control males			
Insular cortex, left ↔ Amygdala, left	0	1.96	0.001
VPA-treated males > Control males			
Pons, left ↔ Medulla, left	0	-2.61	<0.001
Nucleus accumbens, right ↔ Hypothalamus, right	0	-2.55	<0.001
VPA-treated females < Control females			
Thalamus, right ↔ Midbrain, right	-1.61	0	<0.001
Thalamus, left ↔ Midbrain, left	-0.93	0	0.001
Medial prefrontal cortex, right ↔ Caudoputamen, left	0	3.33	0.001
VPA-treated females > Control females			
Medial prefrontal cortex, right ↔ Caudoputamen, right	0	-4.52	<0.001
Somatosensory cortex, right ↔ Hypothalamus, right	2.08	0	0.002

VPA, valproic acid.

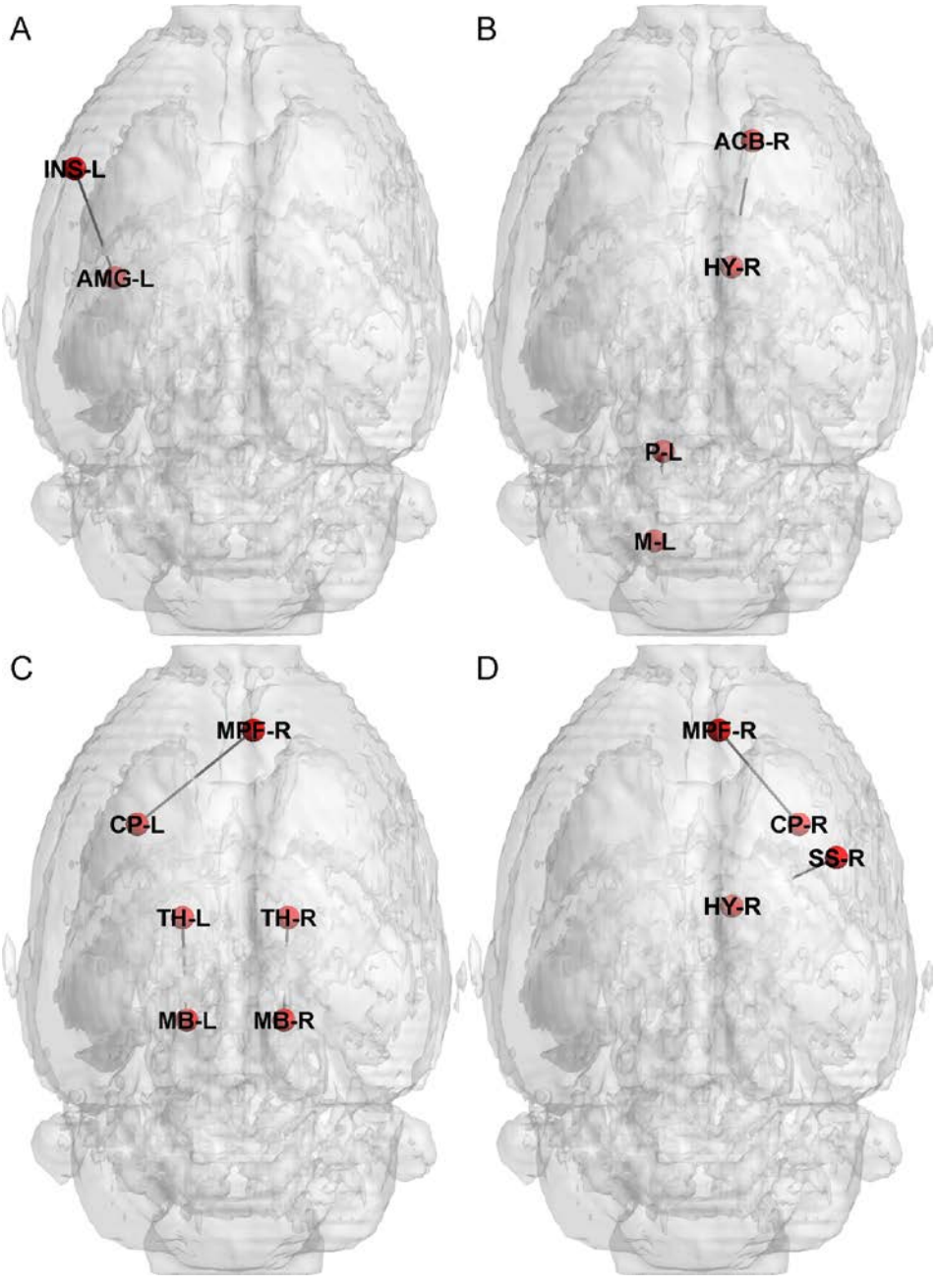

Figure 8. VPA treatment results in significant changes in connectivity strength. (A) In VPA-treated male rats (*n*=22), the connection between the left insular cortex and left amygdala was impaired compared to control males (*n*=17). (B) The

connections between the left pons and left medulla, and between the right nucleus accumbens and right hypothalamus were decreased in control male rats than in VPA-treated male rats. (C) VPA-treated female rats (*n*=19) had decreased connections between the thalamus and midbrain, bilaterally, and between the right medial prefrontal cortex and left caudoputamen compared to control female rats (*n*=19). (D) The connections between the right medial prefrontal cortex and right caudoputamen, and between the right somatosensory cortex and right hypothalamus were decreased in control female rats than in VPA-treated female rats. Data were analyzed using permutation test, followed by false discovery rate corrections (corrected $P \leq 0.05$). ACB, nucleus accumbens; AMG, amygdala; CP, caudoputamen; HY, hypothalamus; INS, insular cortex; M, medulla; MB, midbrain; MPF, medial prefrontal cortex; P, pons; SS, somatosensory cortex; TH, thalamus; VPA, valproic acid; L, left; R, right.

IV. DISCUSSION

ASD is heterogeneous in nature, and common pathognomonic signs remain elusive. Many neuroimaging efforts aimed at identifying commonalities in patients with ASD are also hampered by the vast diversity of ASD cases. Longitudinal follow-up studies have shown that abnormally increased brain volumes and accelerated rates of brain growth during early childhood were observed in only a small minority of children with ASD.[42,43] Also, there was no significant difference between ASD and typical development in terms of the growth curve of total corpus callosum volume.[44] Moreover, according to a recently conducted study with a large sample size, corpus callosum size was not decreased in ASD.[18] Among the few studies that have investigated resting-state brain glucose metabolism in humans with ASD, there has been no consensus across ^{18}F-FDG PET investigations. Indeed, some studies have observed no differences between patients with ASD and controls,[45] while others have reported fewer positive correlations between frontal and parietal regions,[46] or widespread hypermetabolism in individuals with ASD.[47] These studies are mostly based on regional metabolic differences, with the exception of one study that explored resting-state metabolic connectivity in humans with ASD.[46] In that study, correlation analysis was used to estimate the metabolic connectivity among frontal cortices, parietal cortices, and subcortical structures, and patients with ASD showed impaired interactions between frontal/parietal regions and the neostriatum and thalamus.

I suspected that these inconsistent results may stem from the heterogeneous nature of ASD cases. Thus, I focused on a homogeneous type of ASD induced by prenatal exposure to VPA. I hypothesized that alterations in functional connectivity would provide insight into the neurobiological basis of ASD, particularly that caused by VPA exposure. I also explored sex-specific changes in behavior and metabolic networks following homogeneous VPA treatment. I demonstrated that VPA-treated male rats displayed reduced social behavior, had decreased metabolic

activity in the olfactory bulb, and had impaired metabolic connectivity between left insular cortex and left amygdala compared to control male rats.

Prenatal VPA exposure causes changes in neurodevelopmental trajectory. *In vitro* VPA exposure of chick neural crest cells resulted in loss of N-cadherin expression, leading to neural tube defect.[48] The neural tube normally closes during 4th weeks in the human.[49] In the rat, E12.5 corresponds to this period, in which the neurogenesis occurs. VPA exposure in this vulnerable period results in abnormal neural development.[50] As VPA act as a nonselective HDAC inhibitor, it can induce increase in synapse numbers and a robust facilitation of excitatory synapse maturation.[51] VPA also enhance neural proliferation and promotes neurite growth, which in turn contributes to abnormal neural development.[52,53]

VPA treatment caused reduced body weight gain in the pups. Both the VPA-treated male and female rats had less body weight than controls. It is not clear whether the body weight gain is associated with autistic-like behaviors. Few studies have explored the body weight gain in human with ASD,[54] and results from VPA-treated rat are mixed.[12,55] Further study is needed to clarify the relationship between VPA treatment and body weight gain.

VPA-treated male rats in adult group lost the preference to the novel rat over familiar rat, while VPA-treated male rats in adolescent group maintained the preference, although the degree of the preference was less than that of controls. It is not clear whether the male rats in adolescent group will lose the preference if the three-chamber social approach test is performed more later. In contrast, VPA-treated female rats in adult group had the preference to the novel rat over familiar rat, as control rats do, while the preference was absent in VPA-treated female rats in adolescent group. The loss of preference for social novelty has not been considered as robust a social phenotype of ASD as the loss of sociability. Nevertheless, the loss of preference for social novelty may be one indicator of functionally significant impairment in social interaction, a core deficit in patients with ASD.[1] In this regard, loss of preference for social novelty could be one possible aspect of this functionally

significant impairment. It is also of note that there are occasions when time spent in each chamber is not significant but sniffing time is significant.[56] The behavioral discrepancy between VPA-treated adolescent males and adult males is needed to be validated, as there are multiple measures to test autistic-like behaviors in two symptom domains of ASD.[57]

VPA-treated male rats had significantly decreased metabolism in the olfactory bulb. Diminished function of olfactory system has implicated both in rat and human with ASD. In VPA-treated male rats, nest-seeking response was delayed.[12] Children with ASD did not showed the preference to pleasant odorants over unpleasant odorants. In contrast, typically developing children had the preference to pleasant odorants over unpleasant odorants.[58] Increasing evidence supports that social chemosignaling is an important mediator of social interaction both in human and rodent, as olfaction fundamentally influences social behaviors.[59,60] Given that the sniff response is similar between humans and rodents,[61] decreased metabolism in the olfactory bulb may serve as a potential biomarker for ASD. However, whether this finding is specific to ASD or common across various neurodevelopmental disorders remains to be elucidated.

Metabolism in the bilateral somatosensory cortices, caudoputamen, and cerebellum was increased in VPA-treated male rats. Longitudinal studies have shown that an increase in growth rate of striatum, which was specific to caudate nucleus, was correlated with repetitive behavior in humans with ASD.[62,63] Right caudate volume also implicated in repetitive behaviors in ASD.[64] Increased metabolism in the caudoputamen in VPA-treated male rats is in line with these observations. Increasing evidence supports the notion that the cerebellar region not only mediates motor function but also influences social processing.[65] In humans with ASD, the cerebellum was shown to have defects early in life as core ASD deficits appear, especially in the vermis, which were correlated with social impairments.[66] However, whether increase in metabolism in the cerebellum is

correlated with cerebellar defects in relation to ASD remains unclear. The role of somatosensory cortex in ASD is also needed to be explored.

There was no significant effect of VPA in node degree in male rats. In VPA-treated rats, the connectivity strength between the left insular cortex and left amygdala was significantly reduced than in control males, which suggests that VPA-treated male rats had impaired salience processing as compared to controls. Salience network processes multiple stimuli perceived by the brain and determines which stimulus is stands out than others, and allows to show relevant behaviors. The insular cortex plays a central role in salience processing. The insular cortex is an information processing hub, and the anterior insular cortex interprets external sensory cue with internal bodily states. The insular cortex has extensive structural connections with the amygdala, orbitofrontal cortex, olfactory cortex, anterior cingulate cortex, and superior temporal sulcus. In particular, the activity of the insular cortex, often together with amygdala activity, reflects one's interoception, as well as the bodily perceptions of external stimuli.[41] Previous studies revealed that altered salience network connectivity or reduced salience network integrity were associated with ASD core symptoms.[67,68] Reduced salience processing may serve as a pathognomonic sign in VPA rat model of ASD. The significance of increased connections between the left pons and left medulla, and between the right nucleus accumbens and right hypothalamus, which were shown in VPA-treated males, is needed to be clarified.

VPA-treated female rats had increased metabolism in the left caudoputamen and left medulla, had increased metabolic connectivity between the right medial prefrontal cortex and right caudoputamen, and between the right somatosensory cortex and right hypothalamus, but had decreased metabolic connectivity between the thalamus and midbrain, bilaterally, and between the right medial prefrontal cortex and left caudoputamen. Whether these regions play a significant role in the development of autistic-like behavior is not clear.

VPA-treated female rats showed less prominent behavioral derangement and associated changes in brain connectivity, and these findings are in line with those of a previous study, which indicated that VPA affects rats in a sex-specific manner.[23] Whereas women are less affected by ASD than men, women show a broad spectrum of clinical presentation. While women with low-functioning ASD exhibit more severe cognitive impairment, women with high-functioning ASD may be underdiagnosed or misdiagnosed.[69] Such behavioral variability makes group comparison following VPA treatment difficult.

The changes in brain activity and connectivity correlated with autistic-like behavior in the present study were lateralized rather than symmetric. Previous studies have also recognized abnormal brain lateralization in patients with ASD. Atypical cerebral asymmetry and the absence of the left hemispheric dominance for language has been reported as a feature in patients with ASD, although this alteration is not specific to ASD.[70] In this study, I could not find shared features between changes in brain activity and connectivity. However, a number of studies have already shown that changes in brain activity and connectivity often accompany one another in patients with ASD.[15] Subtle changes were possibly obscured by strict statistical criteria.

My results indicated that multiple brain regions were involved in the development of autistic-like behavior in VPA-exposed rats. The olfactory bulb processes social chemosignaling.[59,60] The insular cortex and amygdala constitute the salience network and are involved in social cognition.[71,72] The cerebellum has also been implicated in social and emotional processing, language, and cognition.[65] Taken together, these findings suggest that inappropriate responses to social stimuli from deficient salience processing and abnormal social processing in the cerebellum contribute to autistic-like behavior. Although VPA exposure represents only one possible risk factor for ASD, the VPA rat model may shed light on brain alterations that occur during the development of ASD.

In a methodological aspect, brain connectivity estimated by SICE revealed more differences among groups compared to the estimation using conventional correlation analysis, as group comparison based on Pearson's correlation analysis failed to show any significant differences. A fundamental issue is that the Pearson's correlation method cannot factor out latent effects of a third and/or fourth node that may modulate connectivity between the two nodes. This makes interpretation of the correlative activities unclear, whether they are from the intrinsic structural connection or from polysynaptic induction, common modulatory effects, or common feed-forward projections via the thalamus.[73] By its definition, inverse covariance measures connection between two brain regions while controlling for the effects from other brain regions.[74] Thus, inverse covariance measures are more efficient in revealing direct associations between brain areas. If a covariance between two brain regions is considerably large and is shared by both groups, comparison using conventional correlation analysis may not capture the group differences. However, as SICE models brain connectivity at the group level, it is not possible to estimate brain connectivity at the individual level, limiting its use in aiding the diagnosis of ASD. Another limitation is that, as the number of subjects was not sufficient to model brain connectivity in each age group, it remains to be validated whether the robust brain connectivity observed in VPA-treated rats changes with age. Another limitation is that nodes were empirically defined based on anatomy. A data-driven method called independent component analysis (ICA), which does not require prior knowledge, may provide more appropriate chance to explore connectivity of the entire brain, especially in terms of substructures.[75]

V. CONCLUSION

VPA-treated rats exhibited impairments in social behaviors, and this difference was more pronounced in male than female rats. Preference for social novelty was impaired in VPA-treated male rats, while sociability was diminished in VPA-treated female rats. I found that metabolic activity and connectivity was significantly changed by VPA treatment. Changes in metabolic connectivity was revealed by SICE while conventional correlation analysis did not show any difference. VPA-treated male rats had significantly decreased metabolic activity in the olfactory bulb, and had decreased metabolic connectivity between the left insular cortex and left amygdala, which constitute the salience network. There were no brain regions with decreased metabolic activity in VPA-treated female rats. In contrast, VPA-treated female rats had reduced connectivity between the thalamus and midbrain, and between the right medial prefrontal cortex and left caudoputamen. Such alterations in metabolic activity and connectivity may represent neurobiological substrates of autistic-like behavior, particularly in males, and may serve as a pathognomonic sign in VPA rat models of ASD. As such this study supports the idea that non-invasive brain imaging may serve as an imaging endophenotype that could aid diagnosis of ASD, classification of severity, and possibly reveal insights to neurobiological underpinnings in autistic-like behavior.

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ABSTRACT (IN KOREAN)

산전 발프론산 노출 자폐 백서 모델에서의 뇌 활성 및 연결성

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조 호 진

자폐스펙트럼장애는 일생 동안 지속되는 심한 신경발생학적 장애로, 조기에 사회적 의사소통과 사회적 상호작용에 저하가 발생하고, 제한적이고 반복적인 행동이 나타나는 것이 특징이다. 현재 진단에 유용한 생물학적 표지자는 알려진 것이 없다. 발프론산 백서 모델을 사용하여 뇌 대사 및 연결성에 변화가 존재하는지 알아보았다. 암컷 백서(female Sprague Dawley rat)에 임신 후 12.5일에 발프론산 또는 생리식염수를 복강 내에 주사하였다. 새끼 백서에서 자폐 행동을 평가하기 위하여 행동 검사(three-chamber social approach test)를 생후 4-6주에 시행하였다. 기저 상태 ^{18}F-FDG 양전자방출단층촬영(^{18}F-fluorodeoxyglucose positron emission tomography)을 생후 6주 또는 62주에 시행하였고, 뇌 대사에 변화가 있는지 알아보기 위하여 SPM(Statistical Parametric Mapping)을 시행하였다. 또한 대사 연결성 변화가 동반되는지 확인하였다. 대사 연결성은 전통적인 상관 분석, 그리고 부분 상관 분석 기법인 SICE(sparse inverse covariance estimation)를

이용하여 분석하였다. 발프론산에 노출된 백서는 사회적 행동이 저하되었으며, 이 차이는 암컷 백서보다 수컷 백서에서 두드러지게 나타났다. 수컷 백서에서는 사회적 새로움 선호(preference for social novelty)가 감소하였고, 암컷 백서에서는 사회성(sociability)이 감소하였다. 발프론산에 노출된 백서에서 대사 활성도 및 대사 연결성에 변화가 나타나는 것을 확인하였다. 대사 연결성 변화는 전통적인 상관 분석으로는 확인할 수 없었고, SICE로 확인할 수 있었다. 발프론산에 노출된 수컷 백서에서 후각망울(olfactory bulb)에서 대사가 유의하게 감소하였고, 현저성 망(salience network)을 구성하는 좌도피질(left insular cortex)과 좌편도(amygdala) 사이에 대사 연결성이 감소하였다. 발프론산에 노출된 암컷 백서에서는 대사 활성도가 감소한 부분이 없었으나 시상(thalamus)과 중뇌(midbrain), 그리고 우내측전전두엽피질(right medial prefrontal cortex)과 꼬리조가비핵(caudoputamen) 사이의 대사적 연결성이 감소하였다. 이러한 대사 활성도 및 대사 연결성 변화는 특히 남성에서 자폐 유사 행동에 대한 신경생물학적인 기질을 대변할 수 있을 것으로 보이며, 발프론산 노출 자폐 모델에서 질병 특유 징후로서 가능성이 있음을 시사한다. 따라서 본 연구는 비침습적인 뇌 영상이 자폐스펙트럼장애를 진단하고, 중증도를 분류하고, 자폐 유사 행동에 대한 신경생물학적인 이해를 돕는 영상 내적표현형으로 역할이 가능할 수 있음을 나타낸다.

핵심되는 말 : 자폐스펙트럼장애, 백서, 발프론산, 대사적 연결성, fluorodeoxyglucose, 양전자방출단층촬영, sparse inverse covariance estimation

PUBLICATION LIST

1. Seo J, **Cho H**, Kim GT, Kim CH, Kim DG (2016). Glutamatergic stimulation of the left dentate gyrus abolishes depressive-like behaviors in a rat learned helplessness paradigm. *Neuroimage*. Advance online publication. doi:10.1016/j.neuroimage.2016.12.047