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A Study on the Behavioral and Molecular Changes in the Cerebral Cortex and Hippocampus of a Valproic Acid-Induced Autism Mouse Model

발프로산에 의한 자폐증 동물 모델의 행동학적 이상 및 대뇌 피질과 해마에서의 분자적 변화에 관한 연구

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A thesis of the Degree of Masters of Science

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by
Usman Mahmood

A thesis submitted to the Department of Natural Sciences in partial fulfillment of the requirements for the Degree of Master of Science in Interdisciplinary Neuroscience at Seoul National University College of Medicine

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ABSTRACT

Introduction: Autism spectrum disorder (ASD) is a developmental disorder with manifestations of obscure neural development patterns, deficient dendritic arborization, molecular discrepancies and behavior impairments. Currently, 523 genes are linked with ASD and only 5% of all autistic patients have a genetic component. The remaining portion of ASD is thought to be associated with environmental factors. Given the vast amount of genetic discrepancy, there are currently over 540 different genetic autism mouse models and 20 environmentally induced models. Currently the valproic acid (VPA)-induced animal model is yet to be considered a valid autism animal model. VPA, an antiepileptic drug, is known to induce ASD if administered during pregnancy. The goal of this study is to elucidate behavioral and molecular changes that occur in an environmental mouse model as well as finding potential molecular targets that may explain autistic phenotypes.

Methods: In order to investigate molecular pathways in VPA-induced autism mice, western blotting, immunohistochemistry, Golgi-Cox staining, Nissl staining, dendritic spine analysis were used. In addition, to examine behavioral alterations in the mouse model, self-righting, eye opening, mother scent choice, thermal nociception, self-grooming, home cage interaction, three chamber social interaction, T-maze, open field and elevated plus-maze tests were performed.

Results: VPA mice showed impairments in developmental milestones, social interaction, repetitive behavior and spatial memory. VPA mice also exhibited difference in dendritic spine density and had an increase in the amount of mushroom and thin spine types shown in the cerebral cortex. VPA mice also showed deregulation of molecular pathways, i.e.; PTEN and P-AKT in the cerebral cortex and hippocampus throughout development and post-weaning period.

Conclusion: VPA mice show severe impairments in development, and mimic several different autistic molecular patterns reported previously. I showed that VPA mice show consistent synaptic abnormalities in early as well as post-weaning period coinciding with deregulations of PTEN expression in the hippocampus and cortex. Taken together, this study suggests that PTEN may be an important causative gene which has implications in synaptic disruption and behavioral changes observed in ASD with an environmental etiology.

Keywords: Autism, Autism Spectrum Disorder (ASD), Valproic acid, Behavioral Studies, PTEN
Student number: 2012-20407

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LIST OF ABBREVIATIONS

AKT: protein kinase B

ASD: autism spectrum disorder

BSA: bovine serum albumin

CA1: cornu ammonis region 1

CA3: cornu ammonis region 3

CTX: cerebral cortex

DAPI: 4',6-diamidino-2-phenylindole

DG: dentate gyrus

DIV: day in vitro

DMEM: Dulbecco's modified Eagle's medium

DNA: deoxyribonucleic acid

E13: embryonic day 13

E18: embryonic day 18

FMRP: fragile X mental retardation protein

GFP: green fluorescent protein

H&E: hematoxylin and eosin

HBSS: HEPES buffered salt solution

HDAC: histone deacetylase

HIP: hippocampus

HRP: horseradish peroxidase

IHC: immunohistochemistry

MAP2: microtubule associated protein 2

mTOR: mammalian target of rapamycin

p-AKT: phosphorylated protein kinase B

PBS-T: phosphate buffered saline with Triton-X 100

PBS: phosphate buffered saline

PI3K: phosphoinositide 3-kinase

PND: postnatal day

PTEN: phosphate and tensin homolog

PVDF: poly vinylidene fluoride

RT: room temperature

SAL: saline-treated group

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

SHANK2: SH3 and multiple ankyrin repeat domains 2

SHANK3: SH3 and multiple ankyrin repeat domains 3

TSC1/2: tuberous sclerosis complex 1/2

VPA: valproic acid, valproic acid-treated group

GENERAL INTRODUCTION

History of Autism

Autism is a developmental disorder commonly diagnosed in toddlers and early childhood ranging from the ages of 1-3, which was initially described by the psychiatrist Leo Kanner in 1943. Autism has a neurological origin primarily affecting social cognition and disturbances in interaction. Childhood autism is broadly defined by the presence of abnormal and impaired development, which manifests into a series of clinically relevant areas (or symptoms). As first described by Dr. Kanner,

> *"The outstanding, "pathognomonic", fundamental disorder is the **children's inability to relate themselves** in ordinary way to people and situations from the beginning of life. Their parents referred to them as always been "self-sufficient "; "like in a shell";" happiest when left alone"; "acting as if people weren't there": "perfectly oblivious to everything about him: "giving the impression of silent wisdom";" failing to develop the usual amount of social awareness"; "acting almost as if hypnotized." That is not, as in schizophrenic children or adults, a departure from an initially present relationship; it is not "withdrawal" from formerly existing participation. There is from the start of extreme **autistic loneliness** that, whenever possible, disregards, ignores, shouts out anything that comes to the child from the outside. Direct physical contact or such motion or noises as threatens to disrupt the aloneness is either treated "as if it weren't there" or, if this is no longer sufficient, resented painfully as distressing interference."(Kanner, 1946)*

Thus began Kanner's epoch-making first account of the condition now known as infantile autism in the mid 1900's. After a brilliant clinical

description, and stating that "*we must, then assume that these children have come into the world with innate inability to from the usual, biologically provided affective contact with people, just as other children come into the world with innate physical or intellectual handicaps*" started controversy and different school of thoughts regarding autism. Some believed that children of infantile autism differed from children with psychiatric disorder, and others questioned whether autistic children differed qualitatively or merely quantitatively from the normal. For several years after Kanner's first description of autism, it was believed that autism was a consequence of bad parenting. However, in 1967 Dr. Michael Rutter suggested that autism has a neurological and genetic background.

> *"Genetic studies have been disappointingly limited and inconclusive so far, and there is a need for a well conducted twin study on adequate numbers. The rate of autism sibling (less than 2%) is low, which is rather against a decisive hereditary element. On the other hand the rate is considerably above the general population 4 to 5 per 10,000) and it may be that there is a genetically determined type of autism that constitutes a small subgroup of autistic disorders. Several investigations have shown that autistic children have a normal chromosome complement (Bisele, Shmid, & Lawlis, 1968; BööK, Nichtern, & Gruenberg, 1963; Judd & Mandell, 1968) but Judd and Mandell reported that some autistic children as well as some fathers had an unusually long arm to the Y chromosome. This significance of this, if any, is quite unknown at present."(Rutter, 1967)*

Dr. Rutter's study was the first literature review that started the investigation into the biological and genetic influence of the pathogenesis of autism. Dr. Rutter's findings started an era of research into autism.

Diagnosis

Diagnosis of autism is based on behavior, not cause or mechanism (London, 2007, Brentani, 2013). Autism is defined in the DSM-IV-TR as exhibiting at least six symptoms total, including at least two symptoms of qualitative impairment in social interaction, at least one symptom of qualitative impairment in communication, and at least one symptom of restricted and repetitive behavior. Sample symptoms include lack of social or emotional reciprocity, stereotyped and repetitive use of language or idiosyncratic language, and persistent preoccupation with parts of objects. Several different disorders fall under the criteria of autism, and thus autism is now referred to as autism Spectrum disorder (ASD). Onset must be prior to age three years, with delays or abnormal functioning in either social interaction, language as used in social communication, or symbolic or imaginative play (American Psychiatric Association. & American Psychiatric Association. Task Force on DSM-IV., 2000).

As of 2014, there are currently no known universal biomarkers for ASD. Elevated blood serotonin (5-HT) levels have been consistently recorded in 25-41% of individuals with ASD and have been the best up-to-date biomarker found thus far in autism research (Ruggeri, Sarkans, Schumann, & Persico, 2014). However, due to the vast complexities and several different types of ASD, not all ASD individuals show signs of hyperserotonemia. Recently, several morphological biomarkers have been identified in autistic patients. 23 studies regarding head circumference show that autistic children follow a peculiar developmental trajectory (a) it is within normal limits or slightly below average at birth; (b) it starts accelerating during the first year of life, peaking sometime between 6 months and 4 years of age and (c) it then decelerates so that at puberty the head size typically does not significantly differ between autistic individuals and control (Bartholomeusz, Courchesne, & Karns, 2002). Observation of macrocephaly has also become a morphological biomarker since 45% of macrocephalic autistic patients have at least one macrocephalic parent (Sacco et al., 2010).

However there are several autism-related proteins such as Tuberous sclerosis 1 and 2 complex, Fragile X mental retardation protein, Phoshatase and tensin homolog, SH3 and multiple ankyrin repeat

domains 2 and 3 (TSC1/2, FMRP, PTEN, SHANK2 and 3) provide insight into the genetics and molecular pathways disrupted in autism.

Causes

It has long been presumed that there is a common cause at the genetic, cognitive and neural levels for autism's characteristic triad of symptoms (Happe & Ronald, 2008). However, there is increasing speculation that ASD is instead a complex disorder whose core aspects have distinct causes that often co-occur (Happe, Ronald, & Plomin, 2006).

ASD has a strong genetic basis, however even though the genetics of ASD are often complex and unclear how genetic variations and mutations cause major effects. Intricacy arise due to interactions among multiple genes, the environment, and epigenetic factors which do not change DNA but are heritable and influence gene expression in several different pathways (Rapin & Tuchman, 2008). The large portion of ASD individuals with unaffected family may result from copy number variations — spontaneous deletions or duplications in genetic material during meiosis (Cook & Scherer, 2008). Hence, a substantial fraction of ASD cases may be traceable to genetic causes that are highly heritable but not inherited: that is, the mutation that causes the autism is not present in the parental genome (Rapin & Tuchman, 2008).

Empirical evidence provides insight and suggests that synaptic dysfunction may be a causative pathological hallmark of ASD.

Some rare mutations, like fragile X syndrome (FXS), may lead to autism by disrupting synaptic pathways such as synapse development, maintenance, or synaptic elimination.

Mechanism

ASD does not have a clear unifying mechanism at either the molecular, cellular, or systems level; it is not known whether ASD is a few disorders caused by mutations converging on a few common molecular pathways, or such as intellectual disability, a large set of disorders with diverse mechanisms (Geschwind, 2008), thus creating a difficulty in determining the molecular mechanisms that may result in autistic like behaviors.

VPA

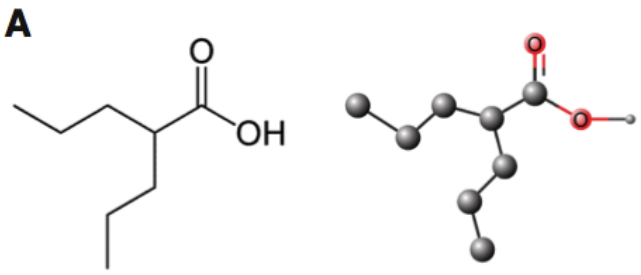


Figure 1. The molecular structure of VPA

VPA, an acidic chemical compound, is primarily used in medicine for the treatment of epilepsy, bipolar mania and migraine prophylaxis. VPA has a broad spectrum of anticonvulsant activity, although it is primarily used as a first-line treatment for tonic-clonic seizures, absence seizures and myoclonic seizures and as a second-line treatment for partial seizures and infantile spasms (Loscher, 2002).

US Food and Drug Administration (FDA) first approved VPA in 1978 for the treatment of seizure disorders. In 1980, adverse fetus exposure to VPA was reported (Dalens, Raynaud, & Gaulme, 1980). Since then, an increasing amount of interest has been invested in determining the effects of VPA *in utero*. The critical period for exposure to any teratogen has been shown to increase the risk of autism in the first trimester (Arndt, Stodgell, & Rodier, 2005) Several studies have paved the way in validating VPA mice as an environmentally induced model of autism. The main aspects to take into account in developing animal models, is (i) to reproduce a circumstance that would lead to a certain condition, for example, inducing a genetic disease by manipulating a specific gene; (ii) to induce similar patterns found in the studied condition, for example, observing the same behavioral alterations found in a particular impairment; (iii) to observe if the model has similarities to a human features when exposed to certain treatment (Crawley,

2007). Table 1 shows a systematic literature review of several studies regarding VPA mice and their similarities to human autism patients.

Catergory		Autism Patients	VPA mice	Reference
Neuroanatomical	Abnormalities of the cranial nerve motor nuclei, hypoplasia of brain stem structures, reduced volume of posterior parts of cerebellar vermis and hemisphere, loss of purkinje cells, injruy to deep nuclei of crebelleum.	✓	✓	Ingram et al, 2000; Rodier et al, 1996; Rodier et al, 1997b
	Purkinje cells numbers in posterior loubes VI-VII and X of the vermis were reduced normal in anterior lobes	✓	✓	Ingram et al, 2000;
	Decreased size of posterior cerbellar vermis	✓	✓	Courcheesne et al, 1994a; Courchesne et al, 1994b; hashimoto et al, 1995
	Dentate nucleus was significantly reduced	✓	✓	Roider et al 1997b
	Globose and emoliform nuclei are much more severely affected than dentate nuclei	✓	✓	Kemper & bauman, 1998
Behavioral	Lower sensitivity to pain and higher sensitivity to non painful stmuli	✓	✓	Markram et al 2008a; Pierce & Courchesne, 2001
	Diminished acoustic prepulse inhibition	✓	✓	Narita et al, 2010, Kaufmann et al 2004
	Locamotor and repetitive/ stereotypic-like hyperactiity combined with lower explactory activity	✓	✓	Schneider et al, 2001; Militerni et al, 2002
	Decreased number of social behavior and increased latency to social behaviors	✓	✓	Schneider et al, 2007;
	Decreased sizures threshold	✓	✓	Schnider et al 2008; Volkmar & Nelson, 1990
	Higher anxiety	✓	✓	Schnider & Przewlocki, 2005; Evans et al, 2005; Gillott et al, 2001
	Animals did not display a higher rate of acquistion of conditioned responses.	✓	0	Bolbecker et al, 2009, Hogg et al, 1979
Biochemical	Altered sterotonergic neuronal differentiation and migration in the dorsal raphe nucleus	✓	✓	Miyazaki et al, 2005; Tsujino et al, 2007; Anderson et al, 1990; Lam et al, 2006
	Dendritic arborization in apical dendrites of pyramidal cells in motor cortex	✓	✓	Snow et al 2008,
	Altered function of opioidergic, glutamatergic systems	✓	✓	Schneider et al, 2007; Rinaldi et al, 2007
	Decreased NLGN3 mRNA	✓	✓	Kolozsi et al, 2009; Roullet et al, 2010
Immunological	Decreased weight of thymus, decreased spenocytes proliferating response to concanavalineA	✓	✓	Cohly & Panja, 2005
	Lower IFN-γ/IL-10 ratio, increased production of NO by peritoneal marcrophases	✓	✓	Schneider et al, 2008, Katsiki et al 1995
	Inadequate response to enviormental stressors	✓	✓	Merlot et al, 2008; Petrovosky, 2001
	Increased susceptibility to stress and increased levels of corticosterones	✓	✓	Gorski et al, 1998, Elenkov & Chrousos, 1999
Cellular	Over expression of NR2A and NR2B and CAMKI1	✓	✓	Rinaldi et al, 2007
	Dramatic increase reactivity to electrical stimulation and deficit in inhibition in amygdala	✓	✓	Markram et al, 2008b, Rinaldi et al 2007, Silva et al, 2009
	Pyramindal neurons target more neurons even at the expense of using less synapses per connection	✓	✓	Rinaldi et al, 2007; Casanova et al 2002
Response to therapy	Enviromental enrichment reverese almost all autisticlike behavioral aberrations	✓	✓	Schneider et al 2006, Bruel-Jungerman et al 2005; Rampon et al 2000

Table 1. Detailed literature review of the neuroanatomical, behavioral, biochemical, immunological, and cellular impairments in VPA mice and Human patients

More recently, VPA has been described as a histone deacetylase (HDAC) inhibitor, and it has been established that histone acetylation leads to relaxation of the nucleosome structure, releasing DNA and allowing transcription. Inhibition of HDAC promotes decondensed chromatin formation, thereby promoting the expression of genes (Chateauvieux, Morceau, Dicato, & Diederich, 2010). In cancer research, VPA prevented LPS-induced degradation of phosphatase and tensin homologue deleted on chromosome ten (PTEN) and up-regulated the PTEN expression (Jambalganiin et al., 2014). VPA specifically targets 2 of the 4 classes of HDACs, and more interesting to mention that class I and II have been reported to be strongly implicated in neuronal function, which may partially explain the action of VPA in neural pathologies (Bhattacharya, Ramchandani, Cervoni, & Szyf, 1999).

In Chapter 1, I investigated whether exposure to VPA causes early behavioral impairments as well as morphological and biological changes during developmental stage. In Chapter 2, I examined behavioral and molecular impairments in post-weaning stage of mice.

Neuroanatomical and molecular alterations that occur in the hippocampus and cortex of VPA mice were also examined.

CHAPTER 1

Early Developmental Behaviors and Molecular Complexities in VPA-Induced Mouse Model of Autism

INTRODUCTION

ASD is a neurological disorder affecting cognition and social interaction. Abnormal development is often observed in ASD patients in the early stages of life, weight fluctuation, abnormal brain development, disruption in synaptic connection and hyperactive neuronal connections resulting in behavioral complexities. Animal models have shown inconsistencies in providing selective empirical evidence in replicating autistic traits. Currently there are approximately 540 autism animal models. Genetic manipulation of autism-related genes provides evidence into possible molecular pathophysiology, however they fail to provide the correlation between genetic manipulation and all behavioral symptoms. (Bruining et al., 2014)

Several studies regarding VPA mice show only behavioral abnormalities on the post-weaning period of mice and fail to show early developmental impairments. In this Chapter, I provide evidence of early developmental milestone delays, a novel form of social recognition in early development in autistic animals and first report to show pathological changes in primary neurons from an environmental autism model. Figure 1 displays scheme of experimental study on a VPA autism mouse model.

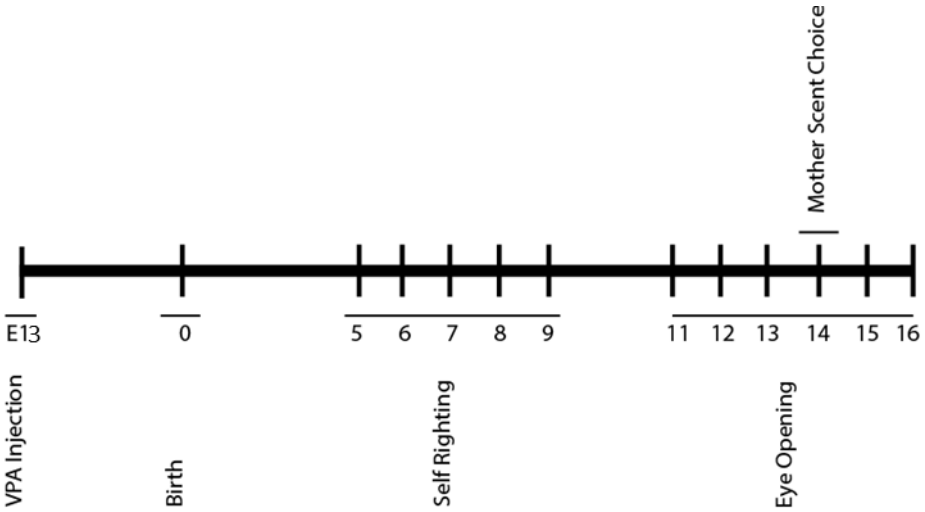


Figure 1-1. Experimental timeline

The scheme of experimental study on a VPA autism mouse model, after VPA injection on embryonic day 13 (E13), behavior studies were conducted as shown after birth.

MATERIALS AND METHODS

1. Animals

All animal procedures were performed following the National Institutes of Health Guidelines for the Humane Treatment of Animals, with approval from the Institutional Animal Care and Use Committee of Seoul National University (IACUC No. SNU-130319-01). Animals of only male were used in this study. BALB/c mice were obtained at E12 from Central Lab Animal Inc. Korea. C57Bl/6N mice were obtained at E12 from Koatech Korea for *in vitro* studies. On E13 dams received a single subcutaneous injection of 600mg/kg VPA. Control females received an equal amount of saline (SAL) only.

2. Behavioral Studies

Self-righting test (Surface righting test)

On PND 5-9, each mouse was placed on its back and gently held with all four limbs extended outwards at which time it was released. Time to right such that all four paws were touching the surface was recorded. A maximum score of 30 s was recorded when the mouse failed to right in that period.

Eye opening test

On PND 12-16, pups were inspected daily if the eyes were opened. 1 point was scored for each eye, resulting with a score from 0 to 2 for each pup.

Mother scent choice (Maternal scent preference) test

Maternal scent preference was conducted on PND 14 pups in a transparent polycarbonate cage (20x30x15 cm). The left third of the test cage was filled to a depth of 3 cm with litter from the mother's cage, the center third contained clean litter, and the right third contained litter from the cage of a stranger dam. The position of the test litters (mother and stranger) was alternated across subjects to control for any side preferences. Three 1 min trials, with inter-trial intervals of 10 sec, were administered for each pup. For the first trial, pups were placed in the center of the fresh litter facing the back wall of the test cage. For the second trial, pups were placed in the center of the fresh litter facing the section containing its mother's cage litter. For the third trial, pups faced the section containing the litter of the stranger dam. Time spent in each section of the cage was recorded and averaged across the three trials. The pup was considered to be inside a section when all four paws were touching the litter within the specified region.

3. Tissue preparation

To obtain tissues for experiments, all animals were anaesthetized and immediately cardiac-perfused with PBS containing heparin. For morphological analyses, one hemisphere was fixed in 4% paraformaldehyde solution for 24 hr, incubated in 30% sucrose solution for 72 hr at 4℃ and then sequential 25 μm coronal sections were taken on a cryostat (Shandon Cryotome FE Thermo Scientific, Waltham, Massachusetts, USA) and stored at 4℃. For biochemical analyses, including western blotting, the other half was quickly frozen on dry ice and stored at -70℃. Tissues were lysed in RIPA buffer with a cocktail of protease inhibitors (Roche, Basel, Switzerland).

4. Primary hippocampal neuron culture

Mouse primary hippocampal neuron cultures were prepared from the hippocampi of E18-19 pregnant C57BL/6 mice by dissociation with 0.25 % trypsin and plating onto 18 mmΦ coverslips or 6-well coated with 1 mg/ml poly-L-lysine. The neurons were grown in Neurobasal medium (Invitrogen, CA, USA) supplemented with B27 (Invitrogen, CA, USA), 2 mM GlutaMAX-I supplement (Invitrogen, CA, USA) and 100 μg/ml penicillin/streptomycin (Invitrogen, CA, USA) at 37℃ in a humidified environment of 95 % Air/5 % CO_2.

5. Dendritic spine density analysis

Primary hippocampal neuron cultures (DIV 10-12) from SAL and VPA E18 mice were transfected with IRES-mGFP vector. The number of dendritic spines was evaluated at DIV 18-20. The fluorescent images were acquired with an LSM 510 confocal microscope (Carl Zeiss, Jena, Germany), using the same settings for all of the samples. The dendritic spines were counted within the 50-100 μm segments on the secondary dendrites that extended at least 40-80 μm beyond the cell body (soma).

6. Immunohistochemistry

Sections (20 μm) containing cortex and hippocampus were obtained using a cryostat (Shandon Cryotome FE Thermo Scientific, Waltham, Massachusetts, USA), and mounted on slides. Sections were mounted on slides and boiled in pH 8.5 citric acid for 1 hr, and then blocked in 5% horse serum, 5% BSA and 0.03% of triton X-100. Sections were then incubated overnight with the following antibody and ratio, PTEN 1:200, MAP2 1:200 (Santa Cruz biotechnology, CA, USA). and DAPI 1:1000. After overnight incubation, samples were washed 3 times with 1x PBS and incubated in secondary antibody for 1 hr. The fluorescent images were acquired with an LSM 510 confocal microscope (Carl Zeiss, Jena, Germany), using the same settings for all of the samples.

7. Histological staining

For Nissl staining sections (20 μm) containing cortex and hippocampus were obtained using a cryostat (Shandon Cryotome FE Thermo Scientific, Waltham, Massachusetts, USA), and mounted on slides. The sections were air-dried for 3 h and placed in 1:1 ratio of absolute ethanol and chloroform. Sections were then rehydrated through 100% and 95% alcohol to distilled water stained with 0.1% Cresyl Violet solution for 5–10 min, then rinsed quickly in distilled water, Differentiated in 95% ethyl alcohol for 2-30 min and checked microscopically for best result. Dehydrate in 100% alcohol 2 x 5 min and the slides were placed clear in xylene 2 x 5 min and mounted with permanent mounting medium and protected with a coverslip. Digitized images of the Nissl-stained sections were obtained with an LSM 510 confocal microscope (Carl Zeiss, Jena, Germany), using the same settings for all of the samples.

For hematoxylin and eosin (H&E) staining, sections (20 μm) containing cortex and hippocampus were obtained using a cryostat (Shandon Cryotome FE Thermo Scientific, Waltham, Massachusetts, USA), and mounted on slides. The sections were air-dried 24h followed by a 1x PBS wash for 10 min and placed in xylene twice for 10 min. Slides were then placed in 100, 90, 80, 70% alcohol for 1 min, and washed in tap water for 30s and placed in hematoxylin for 10 min. Slides were

washed in tap water for 30s and placed into 1% HCL solution in 80% alcohol. Washing in tap water for 10 min followed by slides placed in Eosin for 1 min. After exposure to eosin washing for 10 min under tap water and placed back into 70, 80, 90, 100% alcohol. Slides were then placed in xylene twice for 5 min mounted with permanent mounting medium and protected with a coverslip. Digitized images of the H&E-stained sections were obtained with an LSM 510 confocal microscope (Carl Zeiss, Jena, Germany), using the same settings for all of the samples.

8. Western blotting

For the western blotting, 50-100 μg of protein lysed by RIPA buffer from whole cells or hippocampi was loaded onto denaturing 10-15 % SDS-PAGE gels and transferred to PVDF membranes (Millipore, MA, USA). Each membrane was then incubated in 5 % skim milk or 5 % BSA for 1 hr at room temperature followed by overnight incubation with appropriate primary antibodies PTEN, p-AKT, β-actin at 1:2,000 (Santa Cruz biotechnology, CA, USA). The membrane was then incubated for 1 hr at RT with anti-rabbit or anti-mouse secondary antibodies conjugated with HRP (1:5,000, Invitrogen, CA, USA). The HRP signals were visualized by WestSave chemiluminescent detection kit (AbFrontier, Seoul, Korea).

9. Statistical analysis

Data were expressed as mean ±SEM value. Significant differences between two groups were tested with unpaired Student's *t*-test using SPSS software (Chicago, IL, USA). A p-value < 0.05 was considered significant.

RESULTS

VPA mice showed developmental delays

To investigate whether VPA mice showed body deformities commonly associated with teratogen administered during pregnancy, I checked for tail kinks and measured body weight. I found no physical differences between the VPA and SAL mice. However, I found that VPA mice showed a decrease in weight from PND 5 to PND 11(SAL PND 5-11, 4.78±0.17, 5.52±0.18, 6.36±0.21, 7.08±0.24, 8.08±0.24 ,8.70±0.29, 9.00±0.34, n=10 ; VPA PND 5-11 4.08±0.13, 4.60±0.14, 5.33±0.18, 5.91±0.21, 6.52±0.25, 6.79±0.30, 7.38±0.32; PND 5-11, n=11; $p<0.01$, $p<0.01$, $p<0.01$, $p<0.01$, $p<0.01$, $p<0.01$, respectively), however by PND 12, VPA showed no difference in contrast to SAL mice (Figure 1-2A). These results are similar to a previous report with infant screening which showed a higher correlation between autism and low body weight in infants (Dudova et al., 2014).

Self-righting reflex is a well-documented developmental milestone that is recorded and examined from PND 5 to PND 9. Mice are placed on to a flat surface with all four paws extended away from the surface. Mice are expected to self-right and place all four paws flat on the surface. Duration of reflex decreases as days increase. I found that there was no difference between self-righting on PND 5 (SAL 18.51±1.69).

However, from PND 6 to 9 VPA mice were unable to match the duration of self-righting compared to SAL (Figure 1-2B) (SAL PND 6-9 11.76±1.38, 5.19±1.38, 2.33±1.24, 1.31±0.78, n=10 and VPA PND 6-9 17.99±0.90, 15.71±0.51, 8.40±0.19, 4.45±1.53, n=11; $p<0.01$, $p<0.001$, $p<0.001$, $p<0.001$, respectively). Similar results were found in infants who could not roll over or self-right as a diagnostic tool in determining developmental delays with autistic children (Teitelbaum, Teitelbaum, Nye, Fryman, & Maurer, 1998)

Eye opening has been documented to occur from PND 12 to PND 16 (Williams & Scott, 1953). Eye opening is a developmental milestone that is well studied and has epigenetic time points that determine the rate of eye opening which still needs to be elucidated. VPA mice showed no significant difference in PND 12 (SAL 0.083±0.057, VPA 0±0.077), however VPA mice are significantly delayed in the rate of eye opening compared to SAL (Figure 1-2C). There is statistical difference between VPA and SAL from PND 13 to PND 15 (SAL PND 13-15 0.625±0132, 1.565±0.105, 2±0, n=10, VPA PND 13-15 0.194±0.077, 1.08±0.140, 1.76±0.087, n=11; $p<0.01$, $p<0.01$, $p<0.05$). Nonetheless, VPA mice show significant delay in eye opening from PND 13 to 15.

These results provide evidence that VPA mice not only exhibit physical changes early in development yet also continue to have developmental impairment throughout the early stages of life.

VPA mice showed social recognition impairment

Impairment in social interaction is one of the core symptoms in ASD (American Psychiatric Association. & American Psychiatric Association. Task Force on DSM-IV., 2000). There are currently no tests that provide empirical evidence that show social impairment in early development (Silverman, Yang, Lord, & Crawley, 2010). It was shown that mother scent choice may be a useful behavioral test that may provide insight into social recognition in early postnatal development. Pups were placed in the middle cage on neutral bedding. One third of the cage is filled with nesting from home cage while the other one third is filled with stranger cage bedding. Pups were measured on duration spent in bedding. SAL pups crawled towards familiar bedding and significantly spent more time in familiar bedding (familiar 28.56±3.94, stranger 4.92±2.26, n=9; $p<0.001$) (Figure 1-3). VPA mice showed no statistical difference between preferences from stranger to familiar bedding (familiar 21.05±6.80, stranger 21.53±5.59, n=9; p=0.96) (Figure 1-3). Since social interaction in mice is mainly dependent on odor and urine deposits, bedding from stranger and

familiar mice provide a surplus of material. Taken all together, mother scent choice can be used as a novel behavioral assay in determining social recognition or social interaction in early postnatal development in autism animal models.

Brain weight and synaptic impairment was observed in VPA mice

Accurate brain development is crucial for intelligence, and injection of VPA may cause a disruption in this normal pathological occurrence. VPA-injected pups had a significantly reduced brain volume compared to SAL at E18 (SAL 60.66±2.34, n=10; VPA 71.81±1.44, n=9; $p<0.001$) as well as body weight (VPA 886.68±34.08, n=9, SAL 126.71±19.85, n=9; $p<0.001$) (Figure 1-4A-B). Interestingly, this phenomenon continued to occur at PND13 and VPA pups showed a significant reduction in brain weight (SAL 330.62±4.98, n=10; VPA 383.32±5.75, n=9; $p<0.001$). However VPA mice showed no difference in body weight (Figure 1-4C-D).

Synaptic regulation is a key modulator of memory and intelligence. There are several synaptic irregularities in ASD, such as synaptic formation, synaptic connection, synaptic maintenance and synaptic elimination. In human studies, cerebral cortex of FXS patients, excessive synaptic pruning was observed. Recently, it was shown that FXS mice lacking the FMRP1 protein failed to regulate synapse by

synaptic elimination (Tsai et al., 2012).

To investigate whether dendritic spines were altered in the cortical neuron cultures from E18 VPA and SAL mice, neurons were transfected with IRES-mGFP vector at DIV10-12 and the dendritic spines were evaluated on DIV 18-20. In cortical neurons transfected with mGFP, an increase in spine density in VPA was observed (10.54±0.2064, N=4, n=16,) compared to SAL neurons (7.33±0.0962, N=4, n=16; $p<0.001$). These neuroanatomical and morphological results taken together provide evidence that a single injection of VPA induces severe brain volume reduction during the developmental period and synaptic irregularities that may be the underlying cause of autism.

VPA mice showed anatomical changes in the hippocampus

The limbic system is known to mediate memory and social functions which are typically disturbed in ASD. (DeLong, 1992). Additionally, imaging studies were able to show abnormalities in the limbic region of young subjects (Saitoh, Karns, & Courchesne, 2001). I then checked for gross anatomical changes of the hippocampus of PND 13 brains with H&E staining and Nissl staining. Apart from the fact that VPA mice had generally smaller brain size, I was able to visually observe reduction in the CA1-subiculum area in VPA mice (n=4) (Figure 1-5 A).

The protein level of PTEN was decreased in VPA mice

PTEN is a tumor suppressor gene that is frequently mutated in human cancers and plays an important role in brain development (Endersby & Baker, 2008; Li et al., 1997). Neurological features of inherited PTEN mutations include macrocephaly, seizures, and mental retardation (Endersby & Baker, 2008; Waite & Eng, 2003). PTEN is a negative regulator of phosphatidylinositol-3-kinase (PI3K) signaling. Changes in the activity of PI3K/AKT/mTOR/GSK3β pathway components have been associated with diverse brain disorders such as brain tumors, schizophrenia, and ASD. PTEN mutations have been identified in ASD patients with macrocephaly (Yue et al., 2005). Deletion of PTEN in mature neuronal populations in the cerebral cortex and hippocampus resulted in macrocephaly, abnormal dendritic and axonal growth and synapse number. These mice also showed behavioral abnormalities resembling certain features of human ASDs (Kwon et al., 2006).

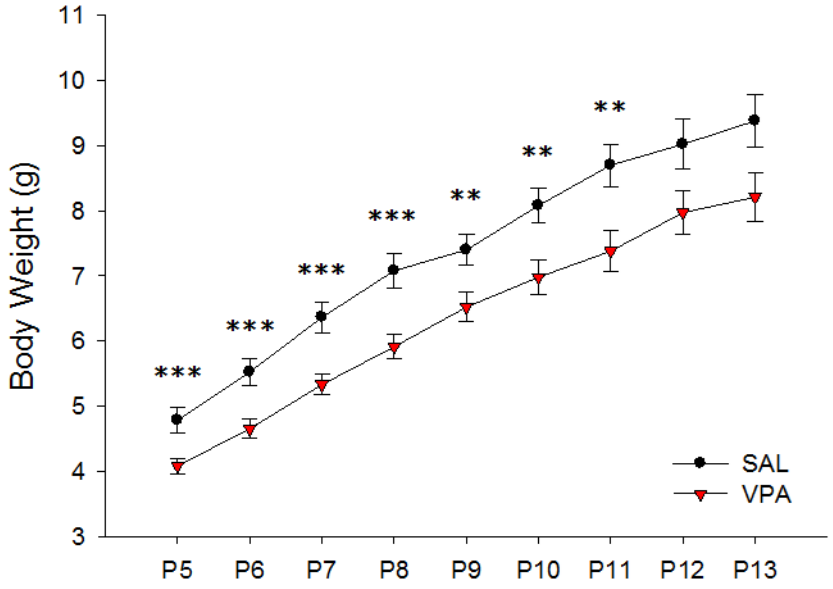
In E18 hippocampus and cortex, I found that PTEN expression was significantly reduced in VPA mice compared to SAL mice (HIP VPA 0.41±.028, n=5; SAL 0.81±0.10, n=4; $p<0.01$; CTX VPA 0.63 ±0.03, n=5; SAL 0.769±0.02, n=4; $p<0.05$) (Figure 1-6A,C). PTEN expression was also reduced at PND 13 of both VPA hippocampus and cortex (HIP VPA 0.57±0.07, n=5, SAL 1.73 ±0.09, n=4; $p<0.001$; CTX

VPA 0.407 ±0.04, n=5, SAL 0.633 ±0.06, n=4; $p<0.05$) (Figure 1-6 B, C)

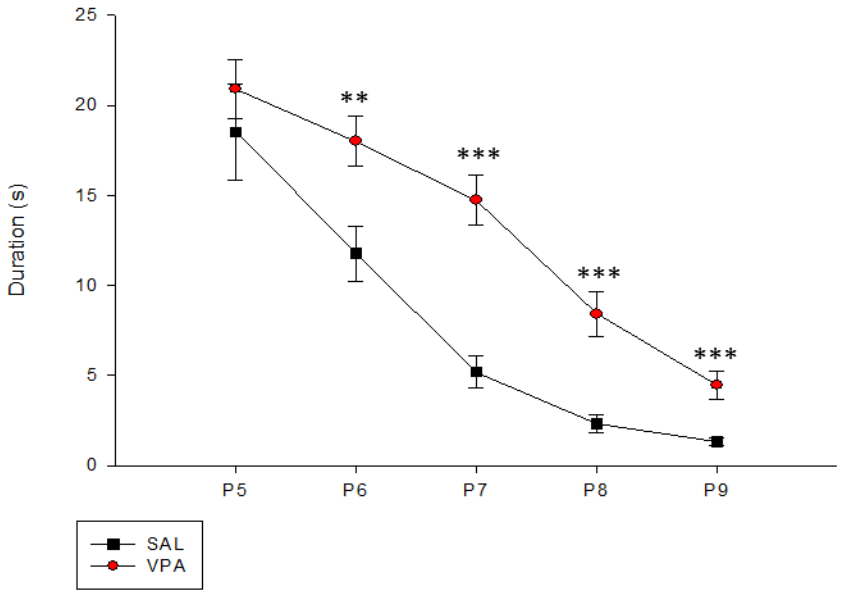
PTEN is a major regulator in the PI3K/AKT/mTOR pathway, I also measured the changes in phosphorylated AKT (p-AKT) and found that in E18 hippocampus, p-AKT was significantly increased (VPA 1.57± 0.07, n=5, SAL 1.05±0.10, n=4; $p<0.05$) (Figure 1-6 A, D). There was no significant change in p-AKT among groups in the cortex (VPA 0.77±0.04, n=5, SAL 0.72±0.04, n=4). p-AKT was significantly increased in both PND 13 hippocampus and cortex (Hip VPA 0.73±0.16, n=5, SAL 0.08±0.03, n=4; $p<0.05$; CTX VPA 0.65±0.07, n=5, SAL 0.344±0.03, n=4; $p<0.05$) (Figure 1-6-B,D). I also preformed immunohistochemistry on PND 13 SAL and VPA mice and found that PTEN expression was reduced in CA1, CA3, DG as well as the cortex in VPA mice (Figure 1-6 E)

Pten-deficient mice displayed deficiencies in classic social interaction paradigms designed to test autism-like behavior in mice (Crawley, 2004). Taken together with previous behavior data may suggest that PTEN may play an important role in VPA social interaction impairments.

(A)


Body Weight (g)
11
10
9
8
7
6
5
4
3
P5
P6
P7
P8
P9
P10
P11
P12
P13
***
***
***
***
**
**
**
SAL
VPA

(B)


Duration (s)
25
20
15
10
5
0
P5
P6
P7
P8
P9
**
***
***
***
SAL
VPA

(C)

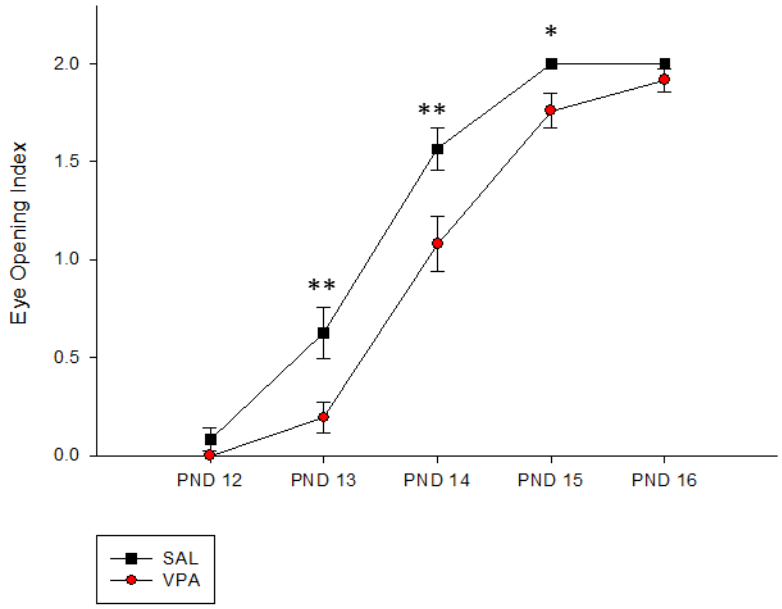

Eye Opening Index
2.0
1.5
1.0
0.5
0.0
**
**
*
PND 12
PND 13
PND 14
PND 15
PND 16
SAL
VPA

Figure 1-2. Developmental profile of SAL and VPA mice

(**A**) Body weight was measured daily from P5-13 consecutively, VPA mice showed a decrease in weight from PND 5 to PND 11, SAL 5-11, 4.78±0.17, 5.52±0.18, 6.36±0.21, 7.08±0.24, 8.08±0.24, 8.7±0.29, 9.0±0.34, n=10, VPA 4.08±0.13, 4.6±0.14, 5.33±0.18, 5.91±0.21, 6.52±0.25, 6.798±0.3, 7.38±0.32, , n=11; PND 5-11; $p<0.01$, $p<0.01$, $p<0.01$, $p<0.01$, $p<0.01$, $p<0.01$, $p<0.01$, p,=0.08 p=0.072, however by PND 12 VPA showed no difference in contrast to SAL mice.

(**B**) Self-righting reflex was conducted on PND 5-9. no difference between self-righting on PND 5, PND 6 to 9 VPA mice were unable to match the duration of self-righting compared to SAL, SAL PND 6-9 11.76±1.38, 5.19±1.38, 2.33±1.24, 1.31±0.78, n=10 and VPA PND 6-9 17.99±0.90, 1.71±0.51, 8.40±0.19, 4.45±1.53, n=11; $p<0.01$, $p<0.001$, $p<0.001$, $p<0.001$

(**C**) Eye opening was observed from PND 12-16. VPA mice show no significant difference on PND 12, SAL 0.0833±0.0576, VPA 0±0.0779. There is a statistical difference between VPA and SAL from PND 13 to PND 15, SAL PND 13-15 0.625±0132, 1.5652±0.1057, 2±0, n=10, VPA PND 13-15 0.1944±0.0779, 1.08±0.1405, 1.76±0.0872, n=11; $p<0.01$, $p<0.01$, $p<0.05$

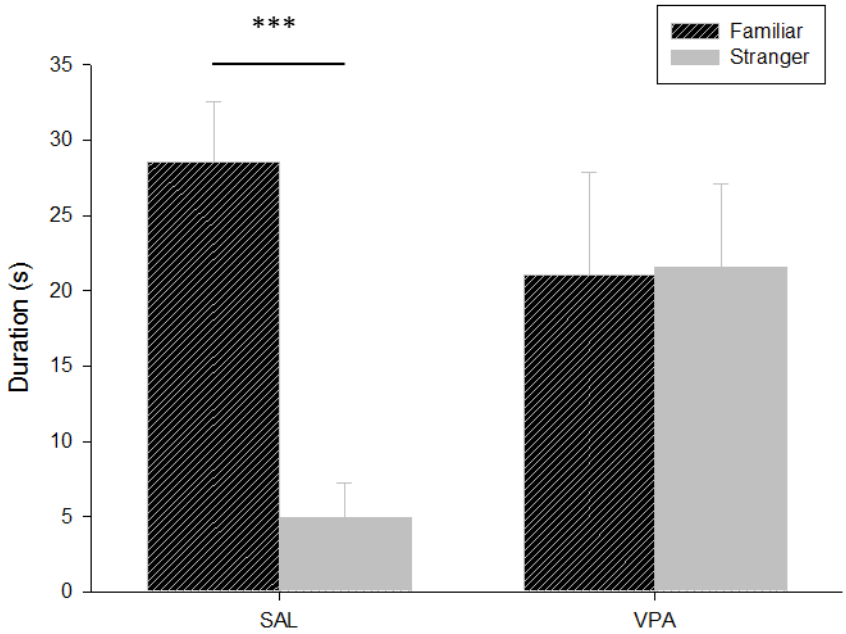
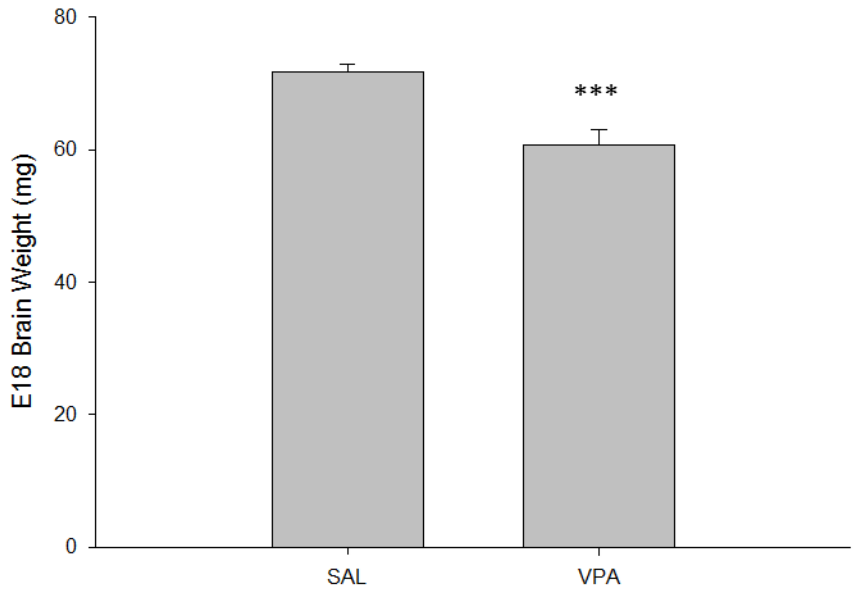


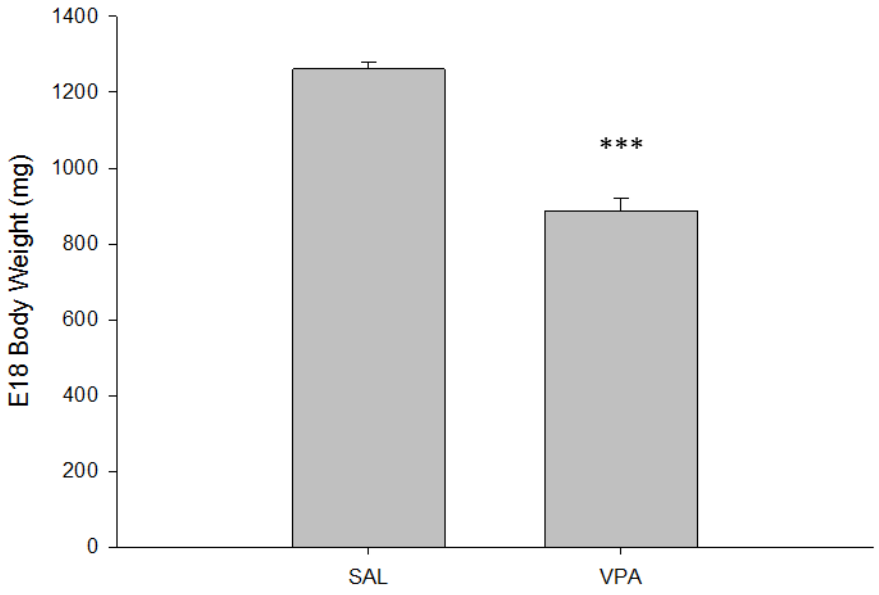
Figure 1-3. VPA mice showed social recognition and social impairment

SAL pups crawled towards familiar bedding and significantly spent more time in familiar bedding, Familiar 28.5685±3.94, Stranger 4.9233±2.2621, n=9; $p<0.001$, VPA mice showed no statistical difference between preferences from stranger to familiar bedding, Familiar 21.05±6.805, Stranger 21.539±5.5992, n=9; p=0.96

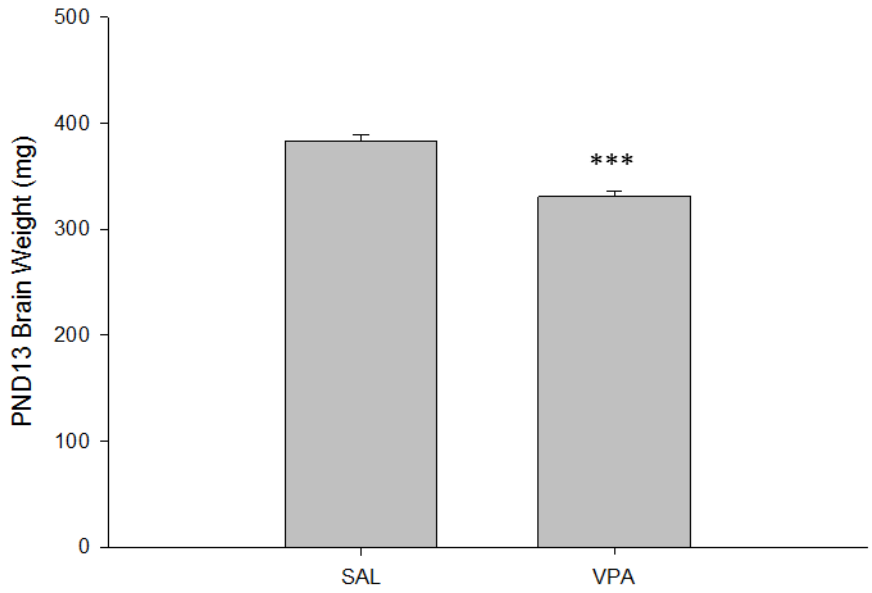
(A)


80
60
40
20
0
E18 Brain Weight (mg)
***
SAL
VPA

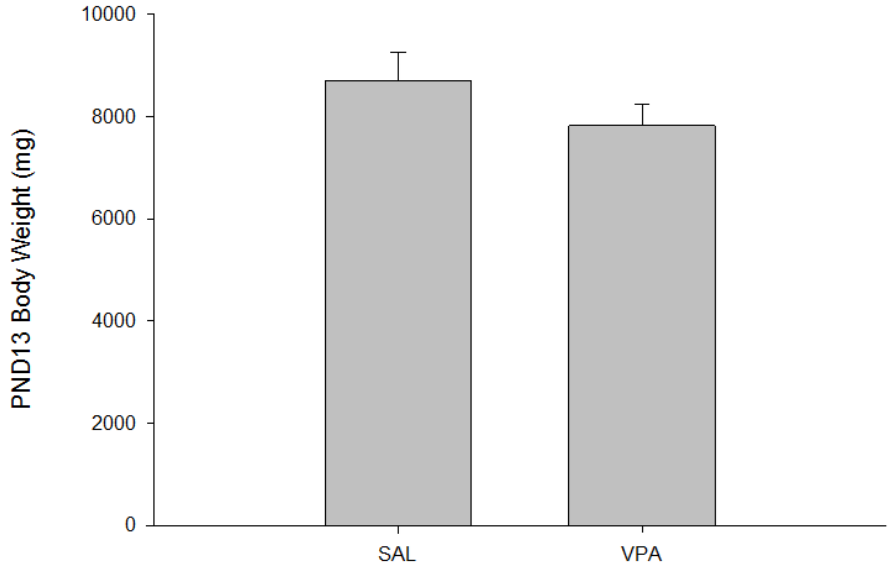
(B)


1400
1200
1000
800
600
400
200
0
E18 Body Weight (mg)
***
SAL
VPA

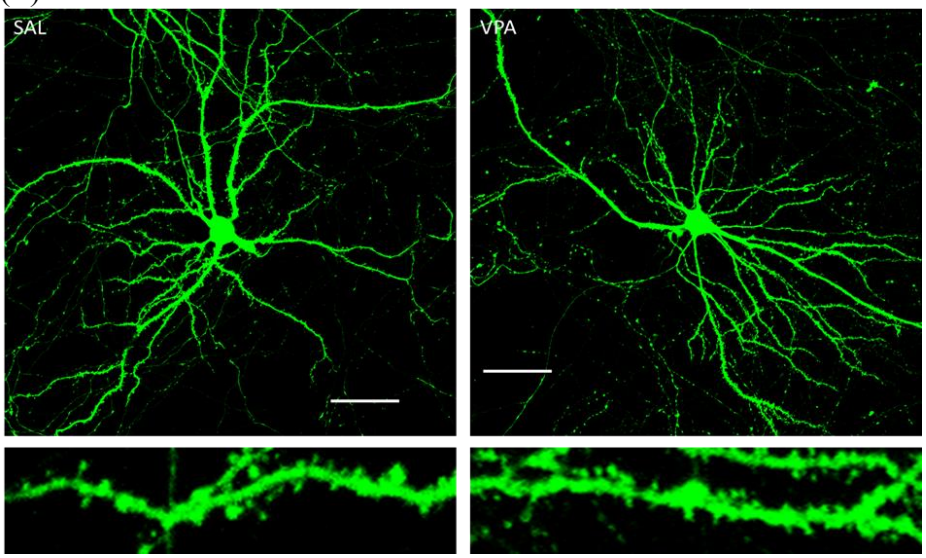
(C)


500
400
300
200
100
0
PND13 Brain Weight (mg)
***
SAL
VPA

(D)


10000
8000
6000
4000
2000
0
PND13 Body Weight (mg)
SAL
VPA

(E)



(F)

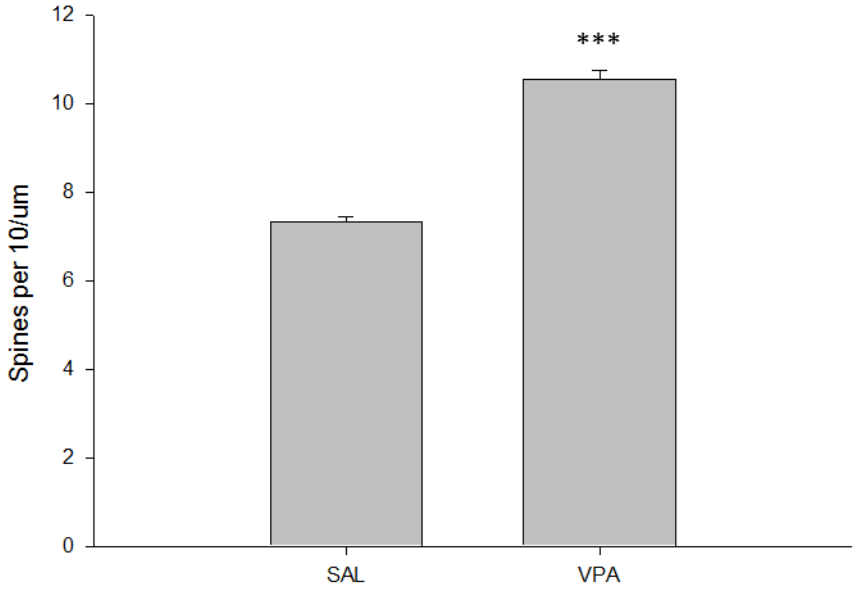


Figure 1-4. VPA mice showed decreased brain weight and increase in spine density in cortical primary neuron cultures

(A&C) From early development to early adulthood VPA mice show a significant reduction in brain weight at E18 and PND 13, SAL 60.66±2.34, n=10, VPA 330.62±4.98, n=9; $p<0.001$, compared to SAL, SAL 71.81±1.44, n=9; VPA 383.32±5.75, n=9; $p<0.001$, respectively.

(B&D) Low birth weight is commonly associated with autism, on E18 VPA, 886.68±34.08 n=9, mice show a reduction of weight compared to SAL, 126.71±19.85, n=10, $p<0.001$. On PND 13 there was no statistical difference between body weight with both VPA and SAL.

(**E**) Representative images of dendritic spines in primary cortical neuron cultures of E18 SAL and VPA (VPA treatment of 600mg/kg on E13) at DIV 18-20 after transfection with IRES-mGFP vector at DIV10-12. The dendritic segment outlined with a white box is magnified to delineate the spine morphology with a 3x optic zoom. The scale bars indicate 20-10um in the low- and high-magnification images, respectively.

(**F**) Quantification of the spine densities (secondary dendrites spines 50-100um from the soma) of E18 cultured cortical neurons at DIV 18-20 after transfection at DIV 10-12 of the SAL and VPA mice. The neurons of the VPA mice exhibited a significant increase in the number of dendritic spines per 10um of dendrites, 10.54±0.20, compared to SAL neurons, 7.33 ± 0.09, N=4, n=16; $p<0.001$

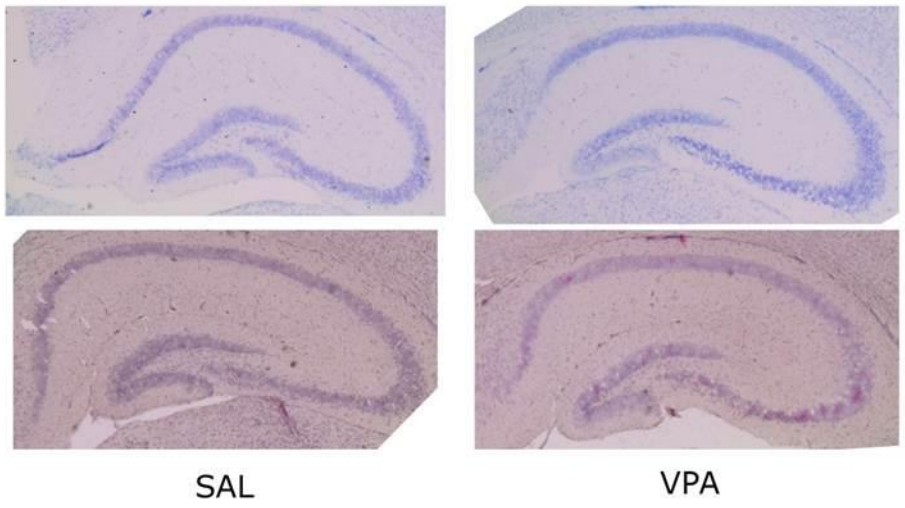
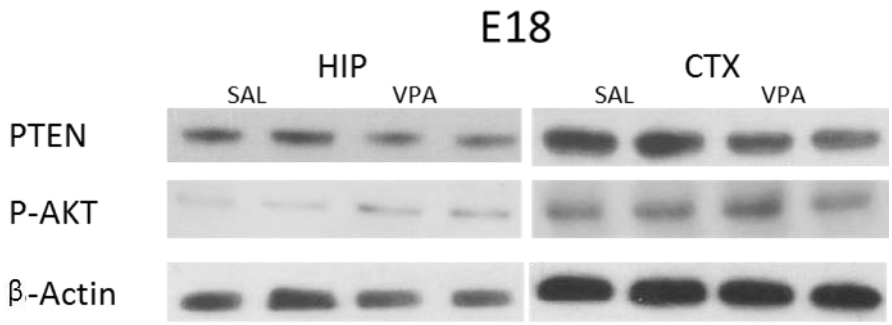


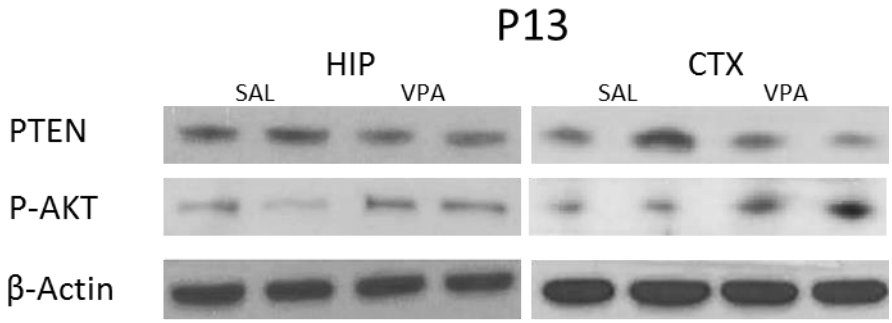
Figure 1-5. VPA mice showed neuroanatomical deformities in the hippocampus

Nissl staining and hematoxylin and eosin staining of the hippocampus. VPA hippocampus shows hypocellularity in the CA1-subiculum transition area, SAL, n=4, VPA, n=4

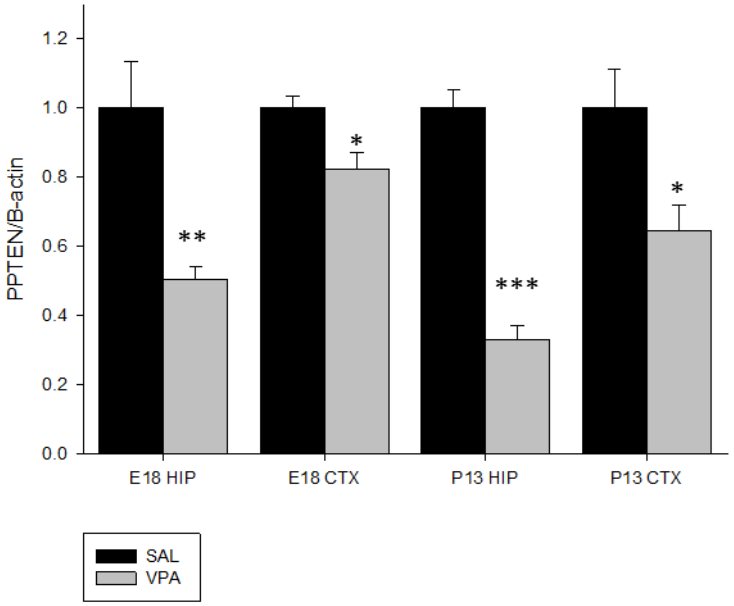
(A)


E18
HIP
CTX
SAL
VPA
SAL
VPA
PTEN
P-AKT
β-Actin

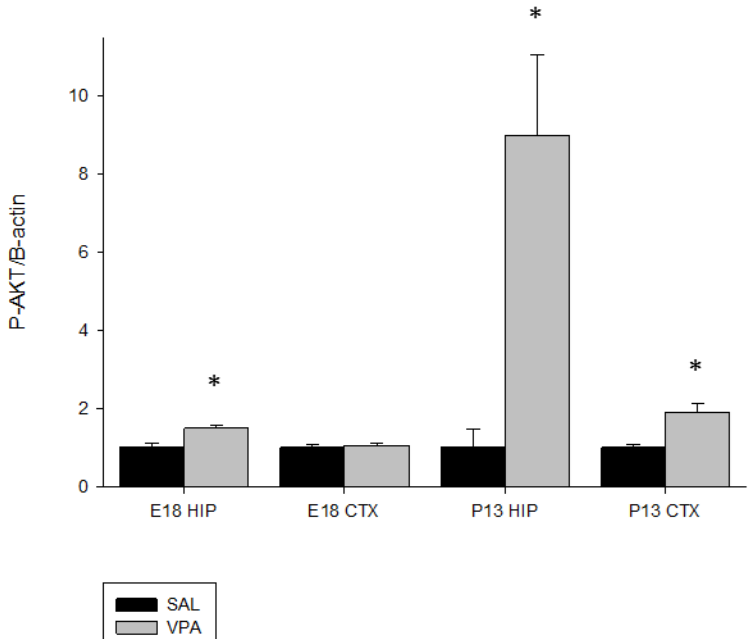
(B)


P13
HIP
CTX
SAL
VPA
SAL
VPA
PTEN
P-AKT
β-Actin

(C)


PPTEN/B-actin
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
*
***
*
E18 HIP
E18 CTX
P13 HIP
P13 CTX
SAL
VPA

(D)



(E)

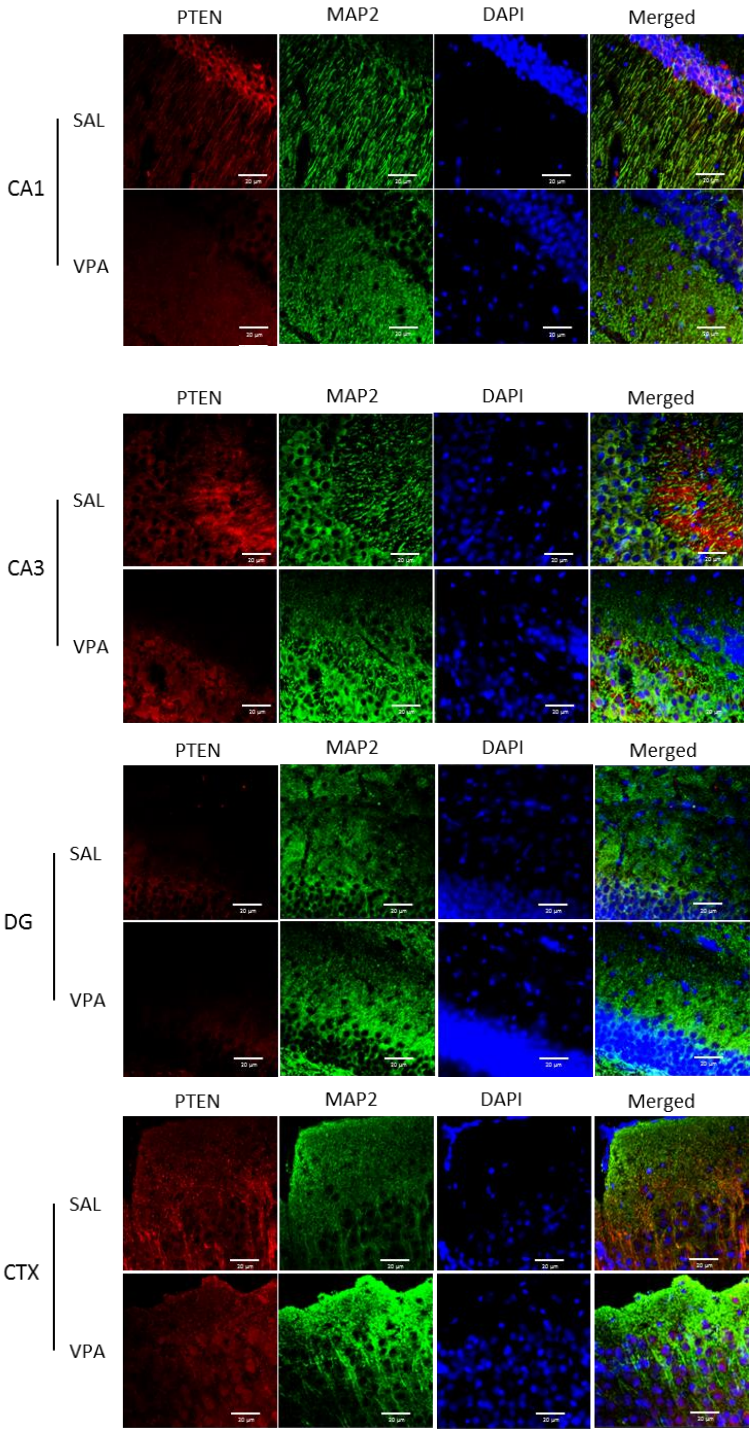

PTEN
MAP2
DAPI
Merged
CA1
SAL
VPA
CA3
SAL
VPA
DG
SAL
VPA
CTX
SAL
VPA

Figure 1-6. Reduction of PTEN expression in hippocampus and cortex of E18 and PND 13 VPA mice

(**A**&**B**) PTEN and P-AKT representative immunoblotting images

(**C**) Quantification of PTEN expression in hippocampus and cortex of E18 and PND 13 of VPA exposed mice. PTEN expression was significantly reduced in VPA treated mice compared to SAL mice, HIP VPA 0.411±0.028, n=5; SAL 0.814±0.108, n=4; $p<0.05$; CTX VPA 0.63±0.036, n=5; SAL 0.769±0.025, n=4; $p<0.001$. PTEN expression was also reduced on PND 13 of both VPA hippocampus and cortex, HIP VPA 0.57±0.071, n=5, SAL 1.73±0.093, n=4; $p<0.001$; CTX VPA 0.407±0.047, n=5, SAL 0.633 ±0.069, n=4; $p<0.05$

(**D**) Quantification of phosphorylated AKT expression in hippocampus and cortex of E18 and PND 13 of VPA exposed mice. P-AKT in E18 hippocampus, p-AKT was significantly increased, VPA 1.574± 0.074, n=5, SAL 1.05±0.103, n=4; $p<0.05$. There was no significant change in p-AKT among groups in the cortex, VPA 0.7705±0.048, n=5, SAL 0.729±0.042, n=4. P-AKT was significantly increased in both PND 13 hippocampus and Cortex, Hip VPA 0.73±0.168, n=5, SAL 0.08±0.030, n=4; CTX VPA 0.657±0.079, n=5, SAL 0.344±0.031, n=4; $p<0.05$, $p<0.05$

(**E)** PTEN expression (Red), MAP2 (Green) DAPI in CA1, CA3, DG and Cortex of PND 13 mice SAL, n=4, VPA, n-4. scale bar represents 20 μm

DISCUSSION

The present study demonstrates that prenatal VPA exposure in mice leads to early-life neurodevelopmental and molecular changes, which accord with previous studies about ASD subjects and other animal studies. VPA-exposed pups showed delayed developmental milestones and impaired social recognition. These changes were accompanied by neuroanatomical changes and PTEN, PI3K/AKT pathway perturbations. Maternal use of VPA during pregnancy shows growing evidence that it is a risk factor of autism and other neurodevelopmental disorders. A recent prospective cohort study demonstrated that prenatal exposure of VPA increased the likelihood of neurodevelopmental disorders, such as ASDs, attention deficit hyperactivity disorder and dyspraxia (Dawson et al., 2010). Since early intervention in young children with ASD leads to improvements (Dawson et al., 2010), I focused on the early dates of VPA mice to better understand neuroanatomical and molecular changes. VPA-exposed pups showed a decrease in brain, E18 and PND 13, and body weight on PND 5-11 as well as impairments in early neurodevelopmental assessments such as self-righting (on PND 5-9) and eye-opening (on PND 11-16).

Dendritic spines are small protrusions along the dendrites where most glutamatergic excitatory synapses occur. ASD, schizophrenia and Alzheimer's disease are the representative neuropsychiatric disorders that can be characterized by impairments in neuronal connectivity and plasticity (Penzes, Cahill, Jones, VanLeeuwen, & Woolfrey, 2011). Culturing primary neurons is an important experimental tool that enables transfection of exogenous genes and live imaging. By injecting pregnant mice on E13 and obtaining neuronal cells on E18, I was able to demonstrate increased spine density in primary neuronal cultures. Furthermore the use of golgi-cox staining was not applicable it enables clear staining and visualization of only mature brain neurons and not embryonic neurons (Koyama & Tohyama, 2012). Genetic model mice of ASD including SHANK3 (Durand et al., 2012) and FMR1 (Levenga et al., 2009) mutation have been used to obtain primary cultured neurons, but to my knowledge, this is the first report to show pathological changes in primary neurons from an environmental autism model. I expect that primary cultured neurons from VPA mice can be further used to explore the molecular mechanisms in dendritic spine pathology.

PTEN mutation is a recently recognized causative factor and its conditional knockout studies are corroborating the link between autism

and PTEN (Clipperton-Allen & Page, 2014; Kwon et al., 2006; Lugo et al., 2014; Zhou et al., 2009). A typical pathology observed in the hippocampus of PTEN knockout model is the hypertrophy of the dentate gyrus and compression of CA1 region. I observed a similar histological change in the hippocampus of PND 13 VPA mice. Additionally, dendritic spines were increased in VPA-exposed primary neuronal cultures which correspond with the changes that were shown by Golgi staining in PTEN mice (Fraser, Bayazitov, Zakharenko, & Baker, 2008). These findings suggest that the decrease of PTEN in VPA mice brains may lead to similar neuroanatomical changes.

My results show early changes in a VPA-induced mouse model. Autism-related behavioral changes can be detected in early life of VPA mice. Taking that PTEN is decreased in the cerebral cortex and hippocampus of VPA mice and that PTEN conditional knockout shows specific effects on behavioral and anatomical changes relevant to ASD, PTEN may be a significant molecular target in the VPA model.

CHAPTER 2

Behavioral and Molecular Findings in the Post-Weaning Period of VPA mice

INTRODUCTION

Considering human evidence of autism followed by early *in utreo* exposure to VPA, VPA induced mouse model reproduces similar human ASD features when treated. Several behavioral abnormalities have been observed in mice exposed to VPA during embryonic development that meet the behavioral symptom criteria for ASD, increased repetitive behavior (Mehta, Gandal, & Siegel, 2011), social interaction and spatial memory impairment (Bambini-Junior et al., 2011). As well as morphological changes, reduction in the number of putative synaptic contrasts in connection between layer 5 pyramidal neurons (Gogolla et al., 2009), decreased number of purkinje cells (Sandhya, Sowjanya, & Veeresh, 2012), Nissl positive cell loss in the middle and lower layers of prefrontal cortex (Hara et al., 2012).

In this Chapter, I report the consequences of early embryonic exposure to VPA and its effect on autistic-like behavioral patterns as well as anatomical and molecular changes in the post-weaning phase.

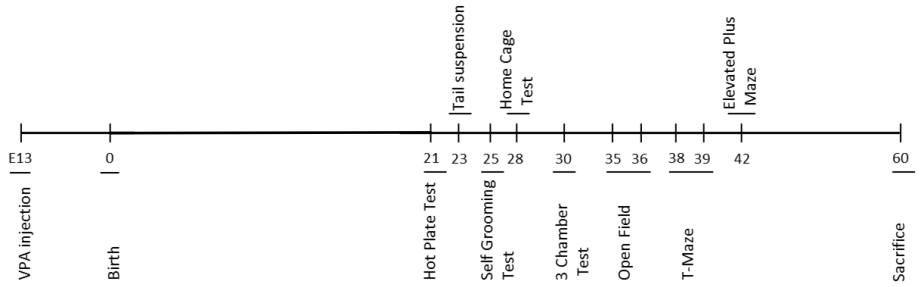


Figure 2-1. Experimental timeline

The scheme of experimental study on a VPA autism mouse model. After VPA injection on E13 behavior studies were conducted as shown after birth till mice were sacrificed and used for molecular studies.

MATERIALS AND METHODS

1. Animal

All animal procedures were performed following the National Institutes of Health Guidelines for the Humane Treatment of Animals, with approval from the Institutional Animal Care and Use Committee of Seoul National University (IACUC No. SNU-130319-01). BALB/c mice were obtained at E12 from Central Lab Animal Inc. Korea for behavioral studies. On E13 seven dams received a single subcutaneous injection of 600 mg/kg VPA. Control females received an equal amount of saline only.

Pups were used for nociception, self-grooming, home cage interaction test, three chamber interaction, open field, elevated plus maze and T-maze tests. In addition, the brains from SAL and VPA mice were used for Golgi-Cox staining.

2. Behavior Studies

Thermal nociception test (Hot plate test)

On PND 20, pups were placed on a 50℃ hot plate and the time to lick or flick its hind paw was recorded and removed into its home cage immediately. Pups which did not show response until 1 min was recorded as 1 min and removed from the hot plate to prevent tissue

damage. 2 trials were conducted on each pup with an interval more than 10 min.

Self-grooming test

On PND 25, pups were placed in a clean empty plastic cage for 20 min. Bedding was not used in order to prevent digging behavior. The habituation period, the first 10 min, was not assessed and the next 10 min was recorded with a digital video camera. Videos were later analyzed for the frequency and total duration of self-grooming behavior.

Home cage interaction test

Age-matched stranger mice were placed in a transparent plastic apparatus (13.5x6.2x15.6 cm). In the beginning, the empty apparatus without a stranger mouse was placed in the home cage of a test mouse (SAL or VPA mouse). After 10 min of free exploration of the container by the test mouse, a stranger mouse was placed in the apparatus, and the test mouse was allowed to explore the stranger mouse for 10 min. Exploration was defined as each instance in which SAL or VPA mouse tries to sniff the stranger mouse, or orients its nose towards and come close to the stranger. Experiments and analyses were performed by independent researchers in a blind manner.

Three-chamber social interaction assay

Three-chamber social interaction assay consists of three phases. In the first phase, a SAL or VPA mouse was placed in the three-chambered apparatus with two small containers in the left or right (not center) chamber, and was allowed to explore the environment freely for 10 min for habituation. After 10 min, the SAL or VPA mouse was gently guided to the center chamber, and the two entrances to the center chamber were blocked while an inanimate object (Object) and a stranger mouse (Stranger 1) were placed in the two containers. Then, the two entrances were opened to allow the mouse in the center to explore the new environment freely for 10 min. In the third phase, the test mouse was gently guided to the center chamber again, with the blockade of the entrances. The Object was replaced with Stranger 2, followed by exploration of the Stranger 1 or 2 by the test mouse for 10 min. Time spent in each chamber was measured by Ethovision 8.5 program (Noldus, Netherlands). Individual movement tracks were analyzed by Ethovision and modified by ImageJ to generate heat maps.

T-maze test

The dimension of each arm 30x15x7 cm and 7x7 cm center piece was constructed using acrylic plastic. On PND 36, mice were moved to testing area, after 10 min, the first trial started by placing the mouse

into start arm facing the goal arms, once mouse has chosen a goal arm, the mouse was confined by placing an acrylic piece (6.5x6.5x15 cm) into the center after choosing goal arm. After 30 sec, the mouse was removed and placed back into its home cage for 1 min. After 1 min, the mouse was returned to the start arm and was allowed to choose between the two open goal arms. After 30 sec, the mouse was removed and the first trial ended. After the second trial was repeated after a 1 hr interval. On day 2, two more trials were performed, resulting in a total of 4 trials over 2 days. Analysis of behavior was done by scoring, if the same goal arm was repeatedly chosen in the same trial the score was 0, if different goal arms were chosen in the same trail a score of 1 was given.

Open field test

The size of the open field box was 40x40x40 cm, and the center zone line was 10 cm apart from the edge. Mice were placed in the center of chamber in the beginning of the test, and mouse movements were recorded with a video camera for 5 min, and analyzed by Ethovision 8.5 program (Noldus, Netherlands).

Elevated plus-maze test

The elevated plus-maze consists of two open arms, two closed arms, and a center area, elevated to a height of 50 cm above the floor. Mice were placed in the center area and allowed to explore the space for 5 min, and analyzed by Ethovision 8.5 program (Noldus, Netherlands).

3. Tissue preparation

To obtain tissues for experiments, all animals were anaesthetized and immediately cardiac-perfused with PBS containing heparin. For morphological analyses, one hemisphere was fixed in 4% paraformaldehyde solution for 24 hr, incubated in 30% sucrose solution for 72 hr at 4℃ and then sequential 25 μm coronal sections were taken on a cryostat (Cryotome, Thermo electron cooperation) and stored at 4℃. For biochemical analyses, including western blotting, the other half was quickly frozen on dry ice and stored at -70℃. Tissues were lysed in RIPA buffer with a cocktail of protease inhibitors (Roche, Basel, Switzerland).

4. Golgi-Cox Staining

Golgi staining was performed using the FD Rapid Golgi Stain Kit (FD Neuro Technologies, MD, USA) following the manufacturer's instructions. Images were acquired with an LSM 510 confocal microscope (Carl Zeiss, Jena, Germany), using the same settings for all

of the samples. The dendritic spines were counted within the 50-100 μm segments on the secondary dendrites that extended at least 40-80 μm beyond the cell body (soma).

5. Histological staining and Immunochemistry

For Nissl staining, sections (20 μm) containing cortex and hippocampus were obtained using a cryostat (Shandon Cryotome FE Thermo Scientific), and mounted on slides. The sections were air-dried for 3 hours and placed in 1:1 ratio of absolute ethanol and chloroform. Sections were then rehydrated through 100% and 95% alcohol to distilled water stained with 0.1% Cresyl Violet solution for 5–10 min, Slides where then rinsed quickly in distilled water, Differentiated in 95% ethyl alcohol for 2-30 min and check microscopically for best result. Sections were dehydrated in 100% alcohol two times for 5 min and were placed clear in xylene twice for 5 min and mounted with permanent mounting medium and protected with a coverslip. Digitized images of the Nissl-stained sections were obtained with an LSM 510 confocal microscope (Carl Zeiss, Jena, Germany), using the same settings for all of the samples. Nissl-positive neuronal cell numbers were manually and rigidly counted within the hippocampus of the scanned digital images. The total cell counts were averaged from at least three sections per animal.

For IHC sections (20 μm) containing cortex and hippocampus were obtained using a cryostat (Shandon Cryotome FE Thermo Scientific), and mounted on slides. Sections were mounted on slides and boiled in pH 8.5 citric acid for 1 hr, and then blocked in 5% horse serum, 5% BSA and 0.03% of triton X-100. Sections were then incubated overnight with the following antibody and ratio, PTEN 1:200, MAP2 1:200 (Santa Cruz biotechnology, CA, USA) and DAPI 1:1000. After overnight incubation, samples were washed 3 times with 1x PBS and incubated in secondary antibody for 1 hr. The fluorescent images were acquired with an LSM 510 confocal microscope (Carl Zeiss, Jena, Germany), using the same settings for all of the samples.

6. Western Blot

For the western blotting, 50-100 μg of protein lysed by RIPA buffer from cortex or hippocampi was loaded onto denaturing 10-15 % SDS-PAGE gels and transferred to PVDF membranes (Millipore, MA, USA). Each membrane was then incubated in 5 % skim milk or 5 % bovine serum albumin for 1 hr at RT followed by overnight incubation with appropriate primary antibodies. The membrane was then incubated for 1 hr at RT with anti-rabbit or anti-mouse secondary antibodies conjugated with HRP (1:5,000, Invitrogen, CA, USA). The HRP

signals were visualized by WestSave chemiluminescent detection kit (AbFrontier, Seoul, Korea).

7. Statistical analysis

Data were expressed as mean ±SEM value. Significant differences between groups were tested with unpaired Student's *t*-test, 1-way ANOVA, 2-way ANOVA using SPSS software (Chicago, IL, USA). A p-value < 0.05 was considered significant.

RESULTS

Noncore autistic behavioral symptoms were observed in VPA mice

To investigate thermal nociception differences between SAL and VPA, I performed a hotplate test and found that VPA males and female mice had a significant increase in duration of latency for paw licking, similar to previously reported results (VPA Males 42.34±3.03, n=8, Females 42.25±2.65, n=10; SAL Males 28.59±1.93, n=9, Females 28.58±1.69, n=11; $p<0.001$, $p<0.001$) (Markram, Rinaldi, La Mendola, Sandi, & Markram, 2008) (Figure 2-2). This result provides evidence that VPA exposure in utero causes increased pain threshold, similar to human autistic patients.

Core autistic behavioral symptoms observed in VPA mice

In order to investigate the core symptoms I performed self-grooming assay, home cage interaction test and three-chamber interaction assay.

To test repetitive and restrictive behavior, I tested self-grooming on PND 25. Two-way ANOVA indicated that there were no statistical differences in duration of self-grooming among groups and gender (Groups: $F_{1,1}=2.164$, $p=0.150$; Gender: $F_{1,1}=0.690$, $p=0.412$) (Figure 2-3A). However, in the case of grooming frequency, there was a significant effect in both groups and gender (Groups: $F_{1,1}=43.243$, $p<0.001$; Gender: $F_{1,1}=26.074$, $p<0.001$). Since there was a significant

interaction between groups and gender ($F_{1,1}$=12.287, p=0.0013), I additionally performed 1-way ANOVA on all 4 groups. There was a significant difference among groups ($F_{1,3}$ = 26.397, $p < 0.001$) and the post-hoc test indicated that only VPA males had a significant change in grooming frequency compared to all other groups ($p < 0.001$ compared to SAL Males 5.55±0.85, n=9; SAL Females 4.09±0.68, n=11; VPA Females 6.9±1.02, n=10) (Figure 2-3B). The sexual dimorphism in grooming frequency is consistent as previously reported (Mehta et al., 2011). Since autism is more prevalent in males and VPA female pups failed to meet the core symptom of repetitive behavior, I focused primarily on male mice.

In order to determine social interaction impairment I performed a home cage interaction test on PND 28 and found that VPA mice significantly reduced time spent near the stranger mouse (VPA 338.62±26.35, n=8, SAL 486.24±12.24, n=9; $p<0.001$) (Figure 2-3C). Since duration spent in Zone A compared to Zone B does not correlate to real interaction, I measured the duration and frequency of nose pokes. VPA mice showed a significant decrease in both duration (VPA 90.25±10.49, n=8, SAL 138.04±12.60, n=9; $p<0.05$), and frequency (VPA 36±3.39, n=8, SAL 64.71±6.68, n=9; $p<0.01$) of nose pokes (Figure 2-3D, E).

I also performed three chamber social interaction assay on PND 30 and found that VPA mice could not distinguish the difference between a familiar mice compared to empty cage (Stranger 203.08±29.57, Object 227.34±39.08, n=8) in comparison to the SAL mice (Stranger 262.13±20.99, Object 155.16±13.66, n=9; $p<0.01$) (Figure 2-3F). Furthermore VPA mice showed no discrimination between familiar and stranger (Familiar 182.76±57.34, Stranger 168.37±10.93, n=8) mice when compared to SAL (Familiar 154.88±14.76, Stranger 203.47±16.54, n=9; $p<0.05$) (Figure 2-3G). I observed no difference in locomotor activity in open field (VPA 2538.73±296.29, n=8, SAL 1916.62±221.07, n=9) (Figure 2-3H). These results suggest that exposure to VPA *in utreo* causes core autistic behavioral symptoms of social impairment.

VPA mice show spatial memory impairment and normal anxiety

To investigate if VPA exposure *in utreo* alters spatial memory and anxiety, I performed T-maze and elevated plus maze tests on PND 38-39 and PND 42 respectively. I observed that VPA mice showed significant impairments in spatial memory compared to SAL mice (VPA 0.333±0.11, n=8, SAL 0.722±0.0, n=9, $p<0.001$)(Figure 2-4A). However VPA mice did not show any significant difference in anxiety (Figure 2-4B).

Exposure to VPA in utero causes neuroanatomical changes

I first measured brain weight to determine if there were any physical changes in the brain. VPA male brains were slightly lighter (425.66±4.33, n=8) compared to SAL mice (445.667±5.23, n=9) however it did not reach statistical significances (p=0.064) (Figure 2-5A).

I then checked for gross anatomical changes of the hippocampus by using Nissl staining. I measured and counted the Nissl positive stained cells per each section of hippocampus (Figure 2-5 B). I found no difference between SAL and VPA in the total number of Nissl positive cells (SAL 4130.05±226.00, N=4, n=16, VPA 4027.83±171.13, N=4, n=16) (Figure 2-5 C). Furthermore, I could not find difference between the DG (SAL 1805.83±90.90, N=4, n=16, VPA 1971.50±112.73, N=4, n=16) and CA3 (SAL 891.44±64.71,N=4, n=16, VPA 878.61±46.44, N=4, n=16) regions (Figure 2-5D, E). Interestingly enough, I found that much like the CA1 region in PND 13, in PND 56 VPA mice, the CA1 region was abnormal. I found that CA1 region of the VPA mice had statistically fewer Nissl positive cells compared SAL (SAL 1432.78±87.38, N=4, n=16, VPA 1177.72±62.34, N=4, n=16, $p<0.05$) (Figure 2-5 F).

Dendritic spines are reported to be altered in several different types of autistic patients (Irwin et al., 2001; Weiler et al., 1997). To explore the effects of VPA on neuroanatomical changes I performed Golgi-Cox staining (Figure 2-6 A). I found pyramidal neurons in the 2nd and 3th layer of the cortex had an increased number of dendritic spines (SAL 94.82±1.51, N=6, n=18, VPA 105.00±5.00 N=5, n=15; $p<0.05$) (figure 2-6B) and a reduction in filopodum (SAL 2.21±0.28, N=6, n=18, VPA 0.15±0.09, N=5, n=15; $p<0.001$) and stubby (SAL 51.41±1.02, N=6, n=18, VPA 39.11±2.37, N=5, n=15; $p<0.001$) type spines and an increase in thin (SAL 31.10±0.94, N=6, n=18, VPA 49.44±4.78, N=5, n=15; $p<0.001$) and mushroom-like spines (SAL 10.10±0.40, N=6, n=18, VPA 16.30±1.13, N=5, n=15; $p<0.001$) (Figure 2-6C, D, E). I also found that VPA mushroom spine heads were significantly reduced in size in comparison to Sal mushroom heads (SAL 1.46±0.02, N=6, n=18, VPA 0.86±0.02, N=5, n=15; $p<0.001$ (Figure 2-6F).

These results are in parallel with findings in other autism models, such as FMRP, and PTEN KO mice (Fraser et al., 2008; He & Portera-Cailliau, 2013), suggesting that cortical synaptic plasticity may be compromised in VPA mice and may help determine a molecular target in finding potential therapeutics.

Autism- associated protein pathways are disrupted in VPA mice

Mutations in Phosphate and Tensin homolog has been linked to the phenotype of autism associated with macrocephaly (Varga, Pastore, Prior, Herman, & McBride, 2009). PTEN is considered a susceptibility gene for autism similar to FXS and TSC1/2 complex, PTEN mutations may account as much as 5% of autism associated macrocephaly and 1% of autism in general (Buxbaum et al., 2007). Conditional PTEN knockout mice show several behavioral similarities to VPA mice. PTEN cKO mice also show an increase in spine density in hippocampal and cortical pyramidal neurons (Zhou et al., 2009). Given that VPA mice showed increased spine density in cortical neurons I checked PTEN levels in the hippocampus and cortex of VPA mice (Figure 2-7A). I found that PTEN was significantly reduced in the hippocampus (SAL 1.01±0.15, n=6, VPA 0.36±0.10, n=6; $p<0.01$) as well as the cortex (SAL 0.51±0.05, n=6, VPA 0.15±0.025, n=6; $p<0.001$)(Figure 2-7B). I also found that phosphorylated protein kinase B (p-AKT) which is downstream of PTEN inhibition of PIP3 were significantly increased in the hippocampus (SAL 0.66±0.13, n=6, VPA 1.49±0.22, n=6; $p<0.05$) (Figure 2-7C). I also performed IHC and checked PTEN expression in the CA1, CA3, DG of the hippocampus as well as the cerebral cortex. Figure 2-7D gives a representative image of PTEN expression in different locations of VPA mice in comparison to SAL.

(A)

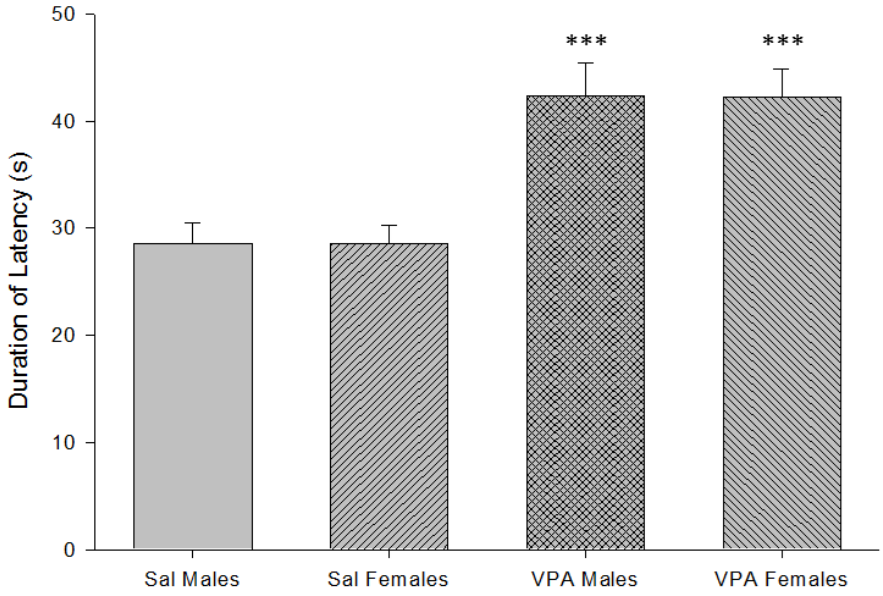
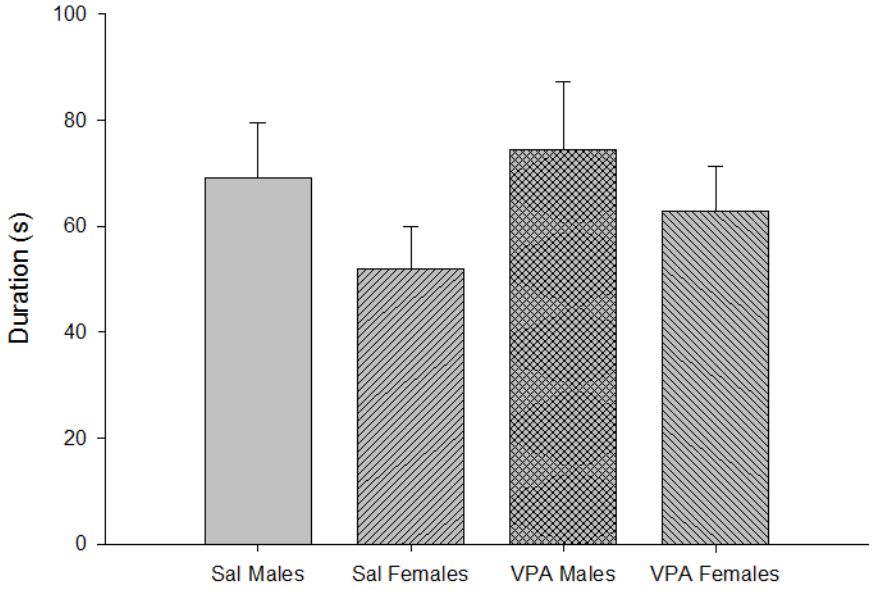


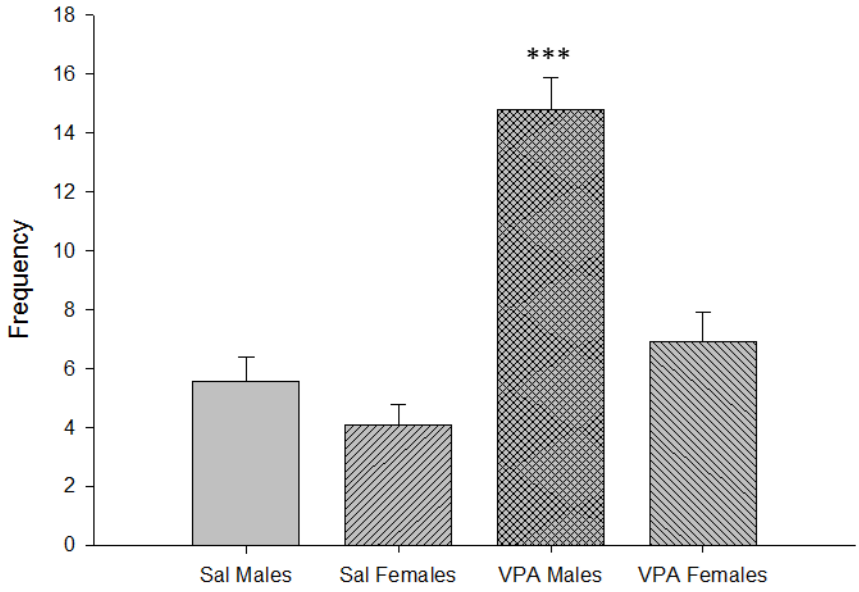
Figure 2-2. VPA mice showed noncore autistic behavioral symptoms

(A) VPA males and female mice had a significant increase in duration of latency for paw licking, VPA Males 42.34±3.03, n=8, Females 42.25±2.65, n=10; SAL Males 28.59±1.93, n=9, Females 28.58±1.69, n=11; $p<0.001$, $p<0.001$

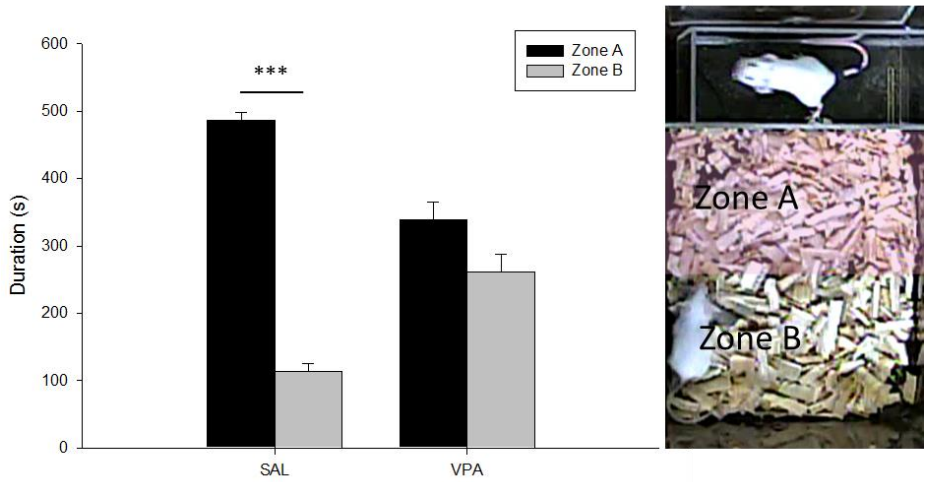
(A)


100
80
60
40
20
0
Duration (s)
Sal Males
Sal Females
VPA Males
VPA Females

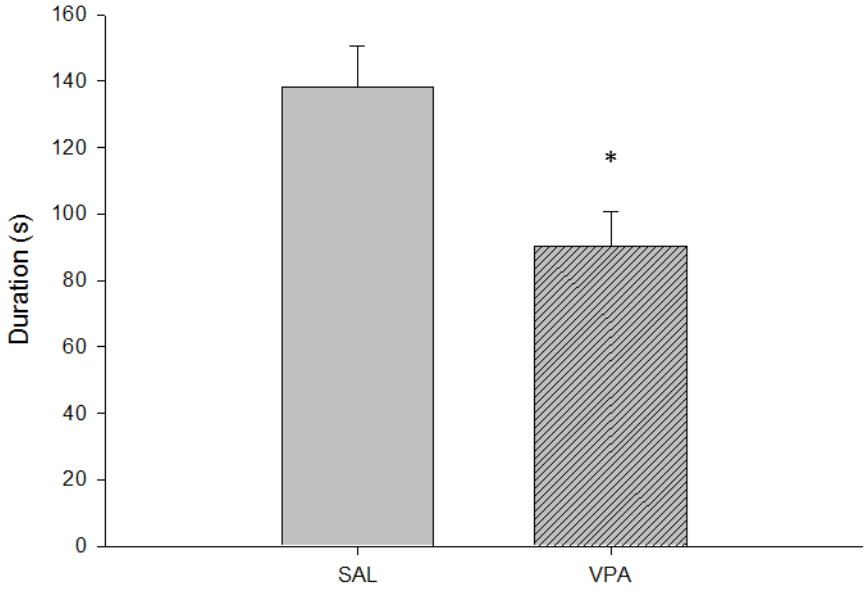
(B)


18
16
14
12
10
8
6
4
2
0
Frequency
***
Sal Males
Sal Females
VPA Males
VPA Females

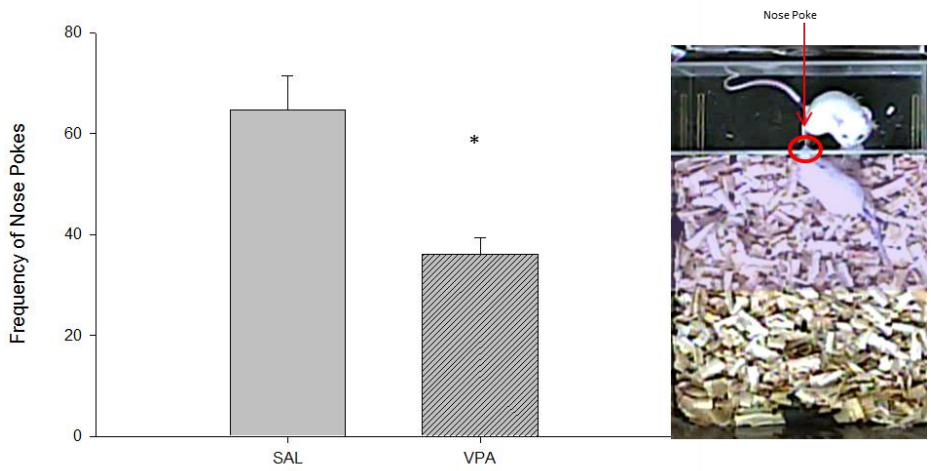
(C)


600
500
400
300
200
100
0
Duration (s)
***
Zone A
Zone B
SAL
VPA
Zone A
Zone B

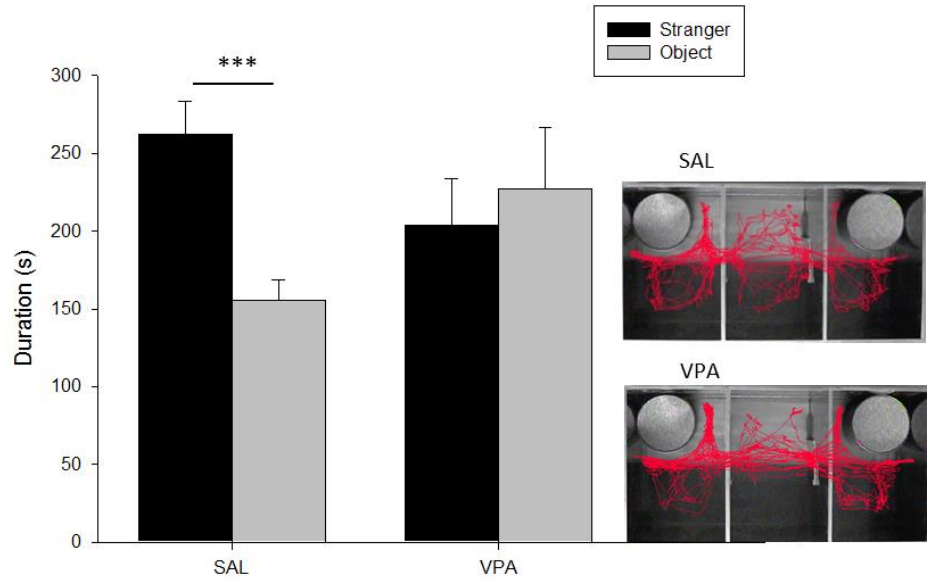
(D)


160
140
120
100
80
60
40
20
0
Duration (s)
*
SAL
VPA

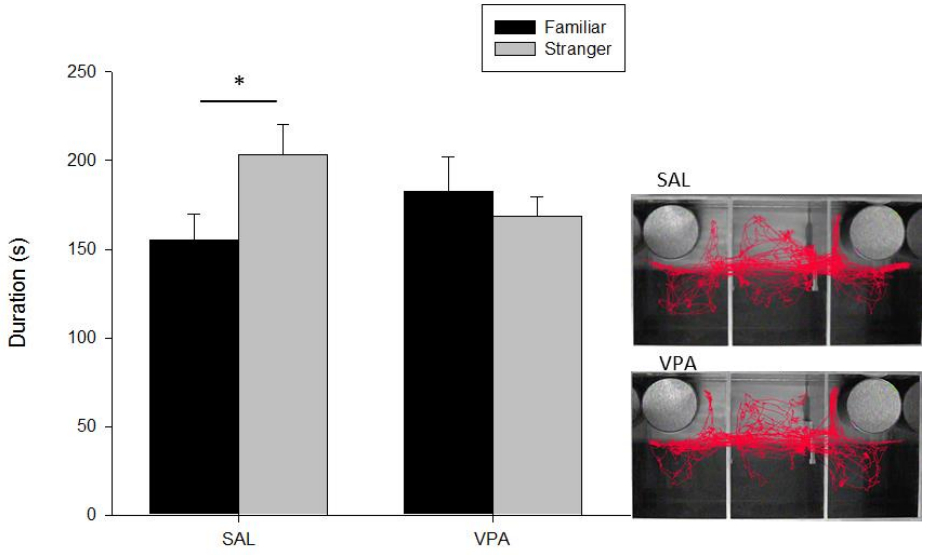
(E)


Nose Poke
Frequency of Nose Pokes
80
60
40
20
0
*
SAL
VPA

(F)


Stranger
Object
***
Duration (s)
300
250
200
150
100
50
0
SAL
VPA
SAL
VPA

(G)


Familiar
Stranger
*
250
200
150
100
50
0
Duration (s)
SAL
VPA
SAL
VPA

(H)

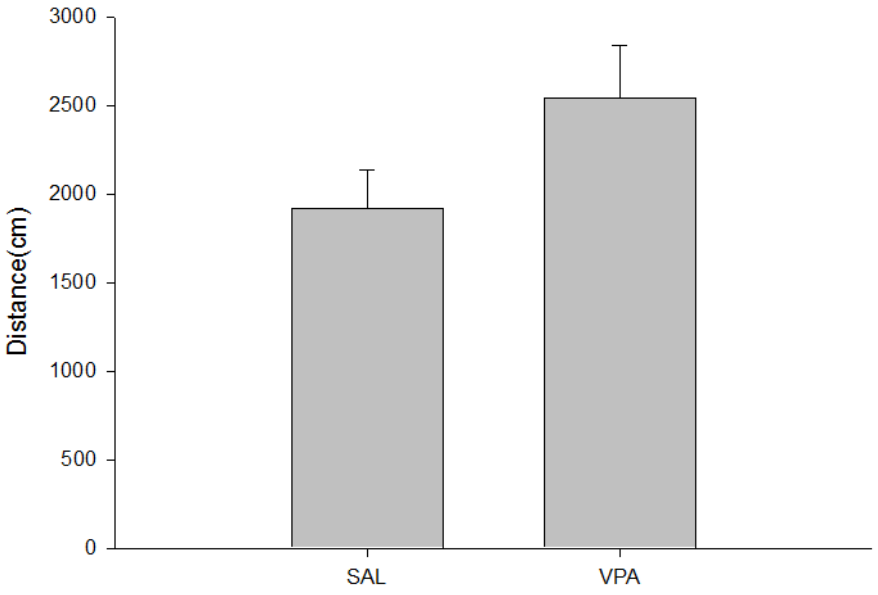

3000
2500
2000
1500
1000
500
0
Distance(cm)
SAL
VPA

Figure 2-3. VPA mice exhibited repetitive behavior and social interaction impairment

(A) In order to investigate receptive behavior, self-grooming test was conducted. No statistical difference in duration of self-grooming among groups and genders, SAL Males 69.12±10.40, n=9, Females 51.95±8.08, n=11; VPA Males 74.56±12.60, n=8, Females 62.83±8.34, n=9

(B) VPA male pups show significantly increased frequency of self-grooming compared VPA females, VPA males 14.77±01.09, n=8, Females 6.9±1.02, n=10; $p<0.001$ and SAL males 5.55±0.85, n=9, SAL females 4.09±0.68, n=11

(C) In home-cage interaction test VPA mice significantly reduced time spent near the stranger mouse, VPA 338.62±26.35, n=8, SAL 486.24±12.24, n=9; $p<0.001$

(D) In the home-cage test duration of nose pokes was measured, VPA mice showed a significant decrease in both duration, VPA 90.25±10.49, n=8, SAL 138.04±12.60, n=9; $p<0.05$

(E) In the home-cage test frequency of nose pokes was measured, VPA 36±3.39, n=8, SAL 64.71±6.68, n=9; $p<0.01$

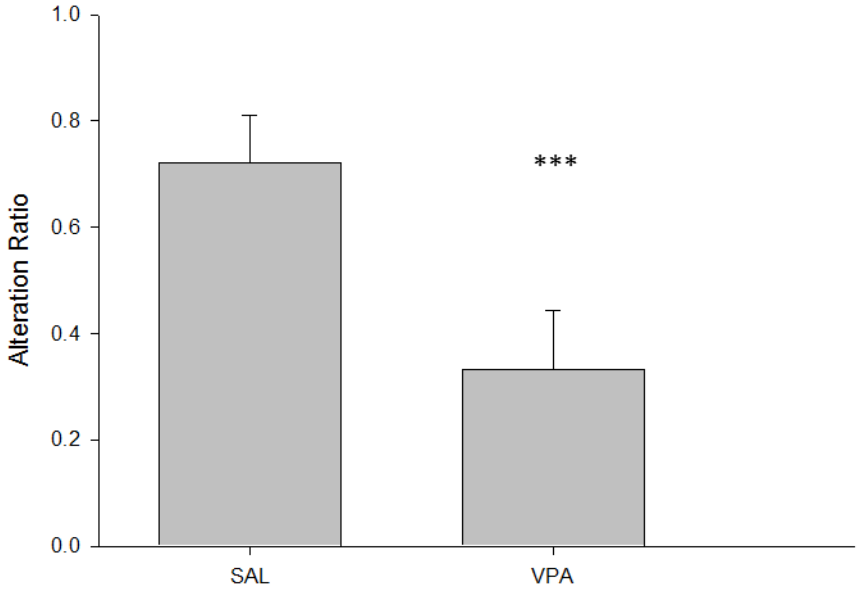
(F) Three chamber social interaction assay was conducted and VPA mice could not distinguish the difference between a familiar mice compared to empty cage, Stranger 203.08±29.5, Object 227.34±39.08,

n=8, in comparison to the SAL mice Stranger 262.13±20.99, Object 155.16±13.66; , n=9 $p<0.01$

(G) VPA mice also showed no discrimination between familiar and stranger, Familiar 182.76±57.34, Stranger 168.379±10.93, n=8, mice when compared to SAL, Familiar 154.88±14.76, Stranger 203.47±16.54, n=9; $p<0.05$

(H) No difference was observed in amount of distance moved in open-field.

(A)


1.0
0.8
0.6
0.4
0.2
0.0
Alteration Ratio
***
SAL
VPA

(B)

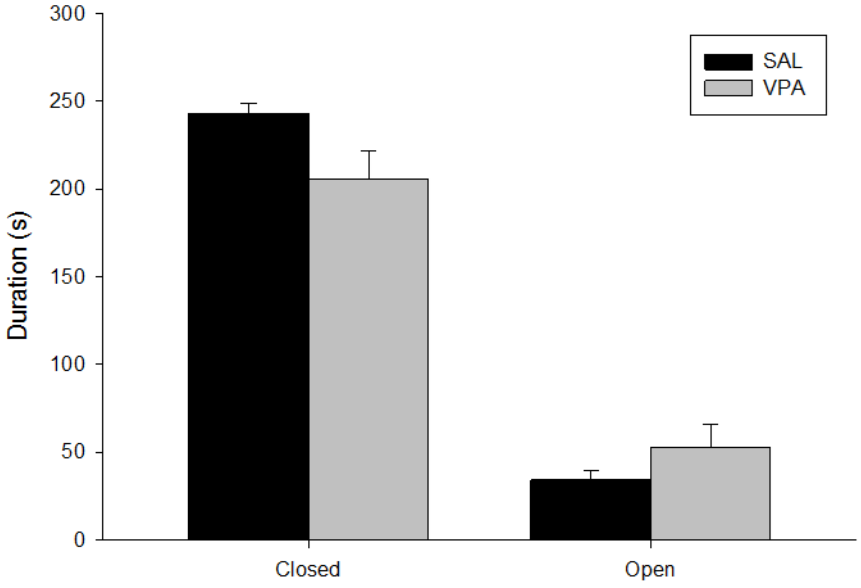
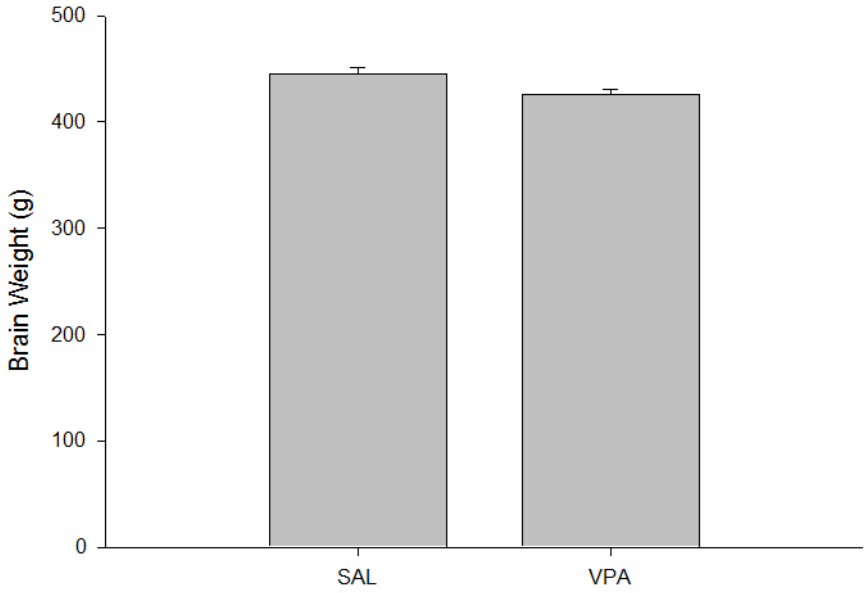

300
250
200
150
100
50
0
Duration (s)
SAL
VPA
Closed
Open

Figure 2-4. VPA mice showed spatial memory impairment and normal anxiety

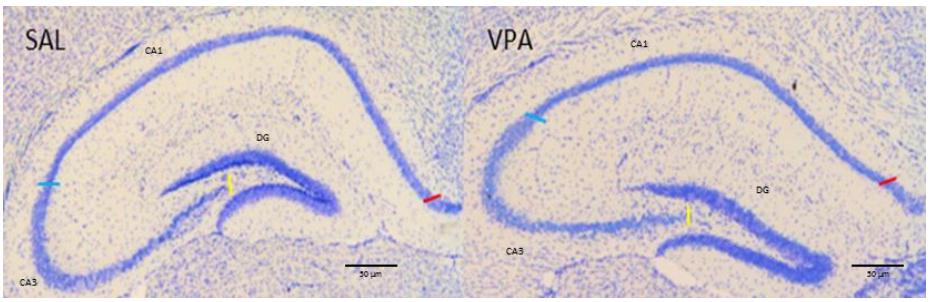
(A) VPA mice showed significant impairment in spatial memory compared to SAL mice VPA 0.33±0.11, n=8, SAL 0.72±0.08, n=9, $p<0.001$

(B) VPA mice did not show any significant difference in anxiety

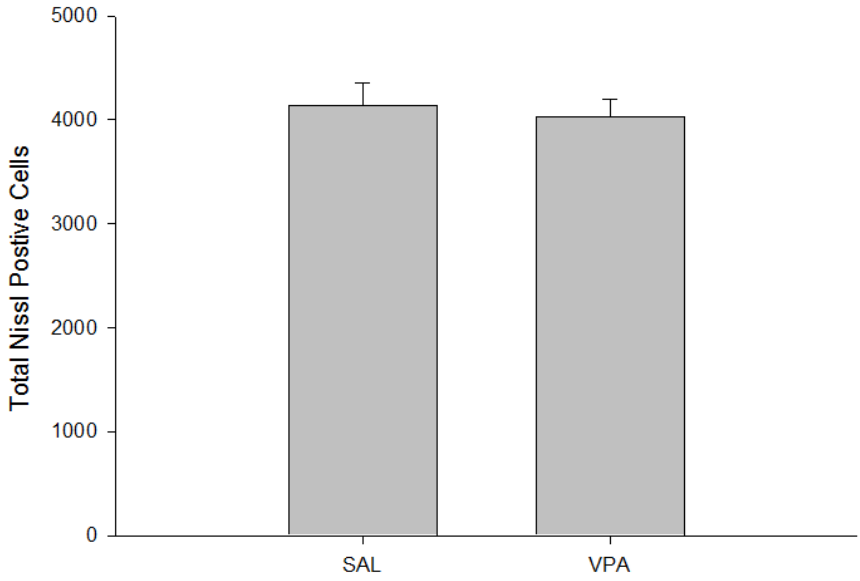
(A)


500
400
300
200
100
0
Brain Weight (g)
SAL
VPA

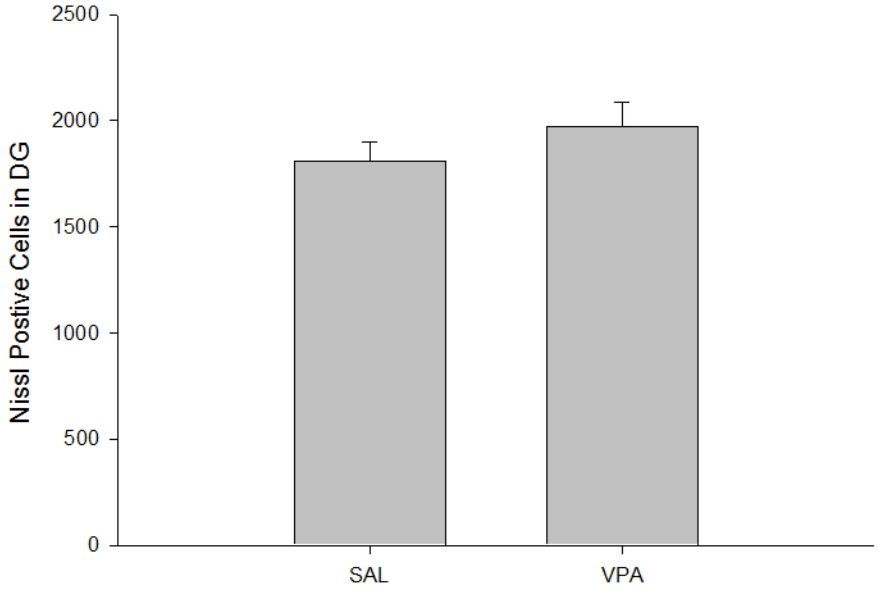
(B)


SAL
CA1
DG
CA3
50 μm
VPA
CA1
DG
CA3
50 μm

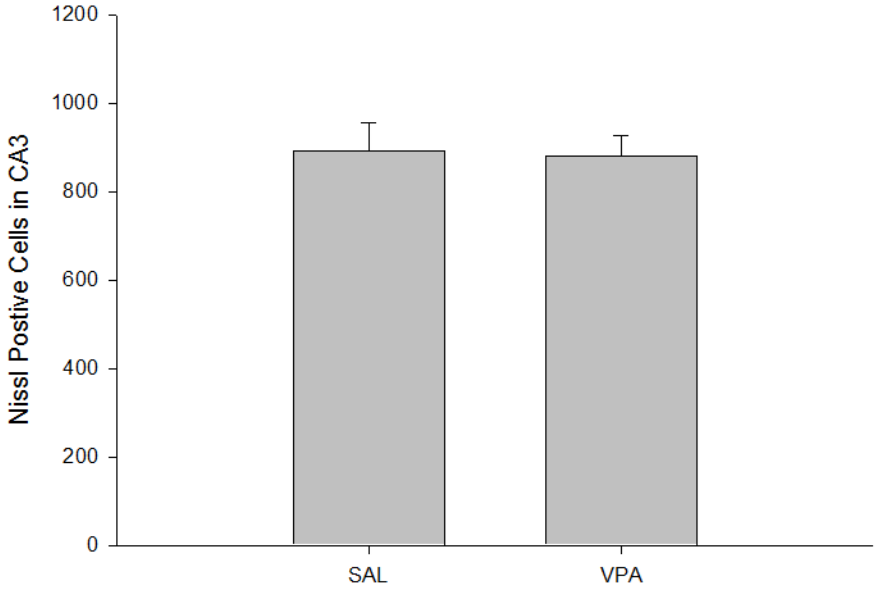
(C)


Total Nissl Postive Cells
5000
4000
3000
2000
1000
0
SAL
VPA

(D)


Nissl Postive Cells in DG
2500
2000
1500
1000
500
0
SAL
VPA

(E)


Nissl Postive Cells in CA3
1200
1000
800
600
400
200
0
SAL
VPA

(F)

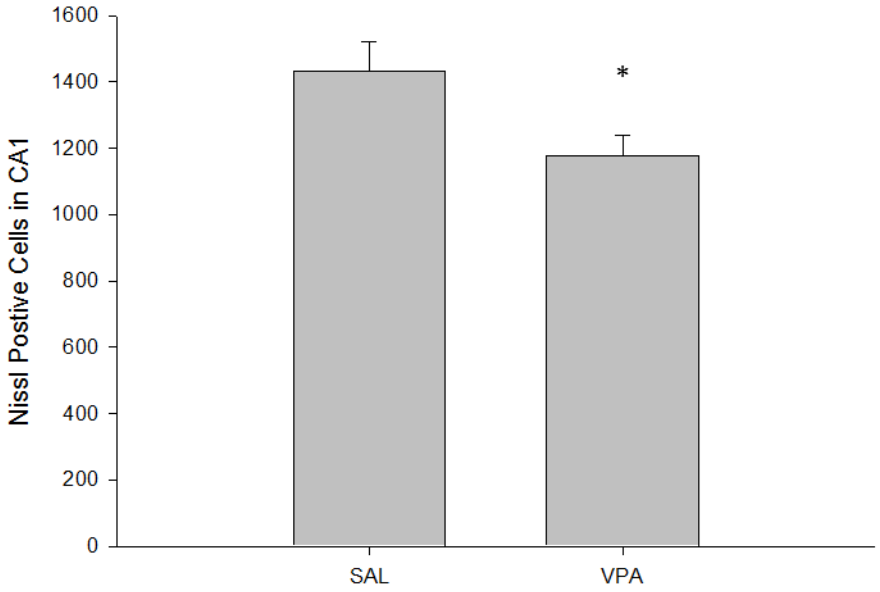

Nissl Postive Cells in CA1
1600
1400
1200
1000
800
600
400
200
0
*
SAL
VPA

Figure 2-5. VPA exposure caused neuroanatomical changes in the hippocampus

(A) Brain weight was measured and showed VPA male brains were slightly lighter, 425.66±4.33, n=8, compared to Sal mice, 445.66±5.23, n=9, however it did not reach statistical significances, p=0.064.

(B) Representative picture of Nissl staining in SAL and VPA sectioned tissue**,** Scale bar represents 50 µm.

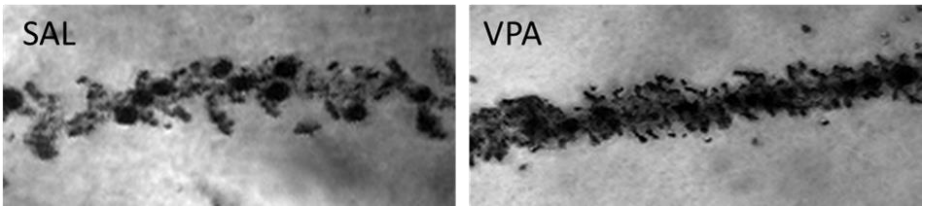
(C) No difference was found between SAL and VPA in the total number of Nissl-positive cells, SAL 4130.05±226.0, N=4, n=16, VPA 4027.83±171.138, N=4, n=16

(D) Statistical significance could not be found between SAL 1805.83±90.90, N=4, n=16, VPA 1971.50±112.73, N=4, n=16 in the DG region.

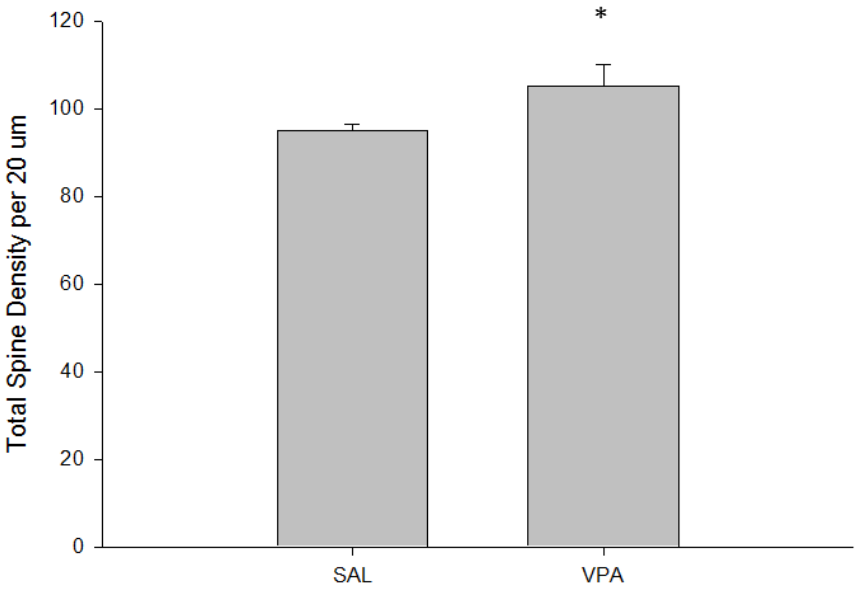
(E) Statistical significance could not be found between SAL 891.44±64.71, N=4, n=16, VPA 878.61±46.44, N=4, n=16 in the CA3 region.

(F) CA1 region of the VPA mice had statistically fewer nissl positive cells compared to SAL 1432.78±87.38, N=4, n=16, VPA 1177.72±62.34, N=4, n=16, $p<0.05$

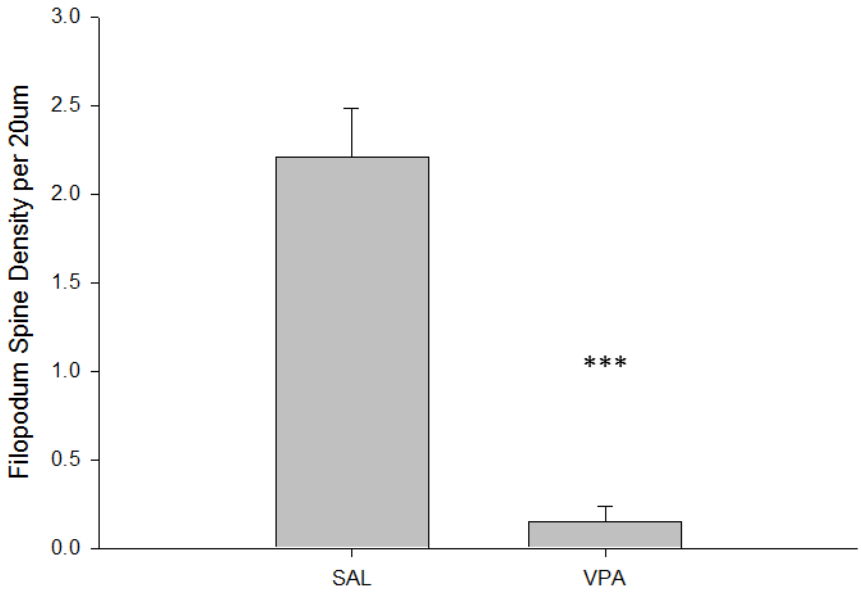
(A)


SAL
VPA

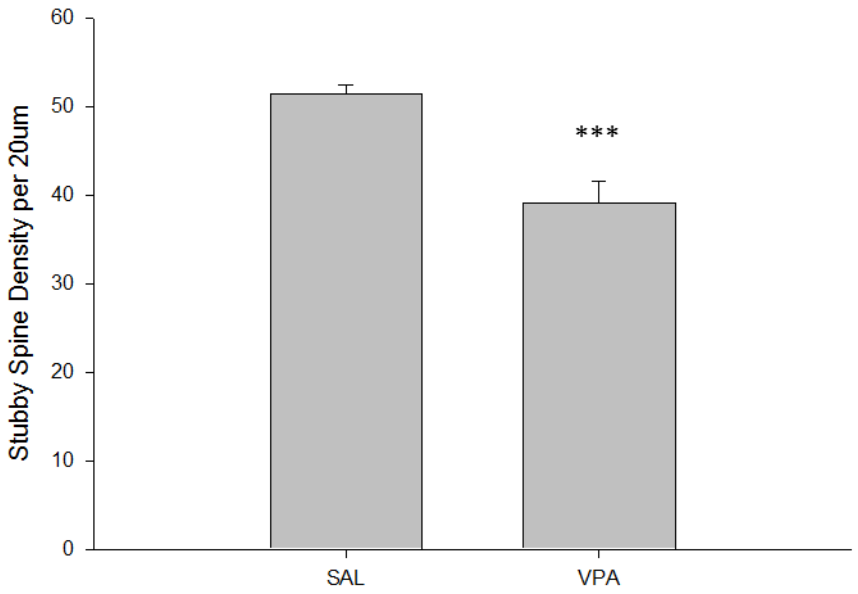
(B)


Total Spine Density per 20 um
120
100
80
60
40
20
0
*
SAL
VPA

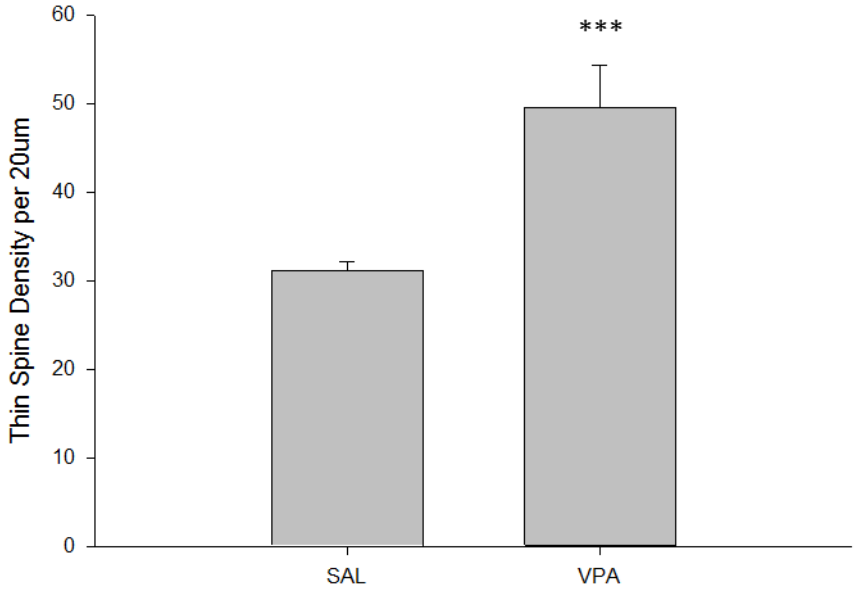
(C)


Filopodum Spine Density per 20um
3.0
2.5
2.0
1.5
1.0
0.5
0.0
***
SAL
VPA

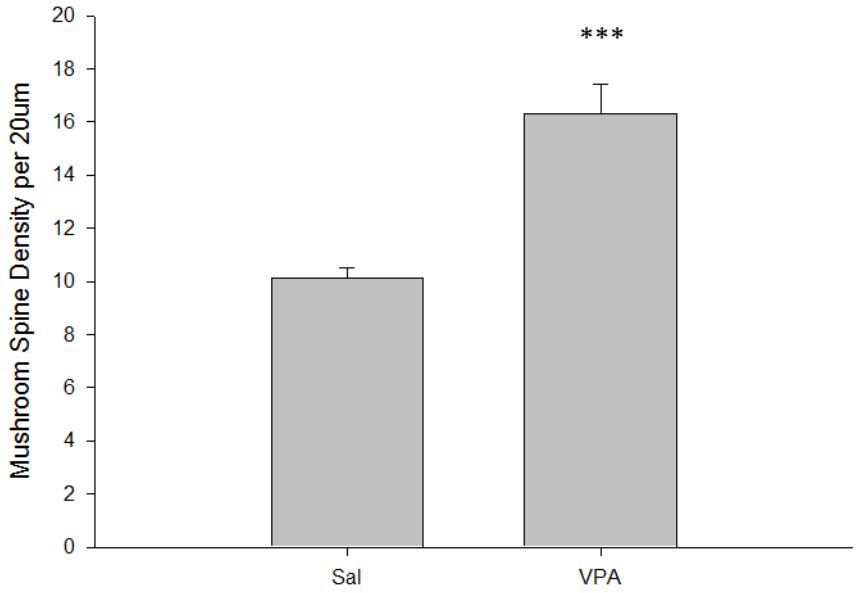
(D)


Stubby Spine Density per 20um
60
50
40
30
20
10
0
***
SAL
VPA

(E)


Thin Spine Density per 20um
60
50
40
30
20
10
0
***
SAL
VPA

(F)


Mushroom Spine Density per 20um
20
18
16
14
12
10
8
6
4
2
0
***
Sal
VPA

(G)

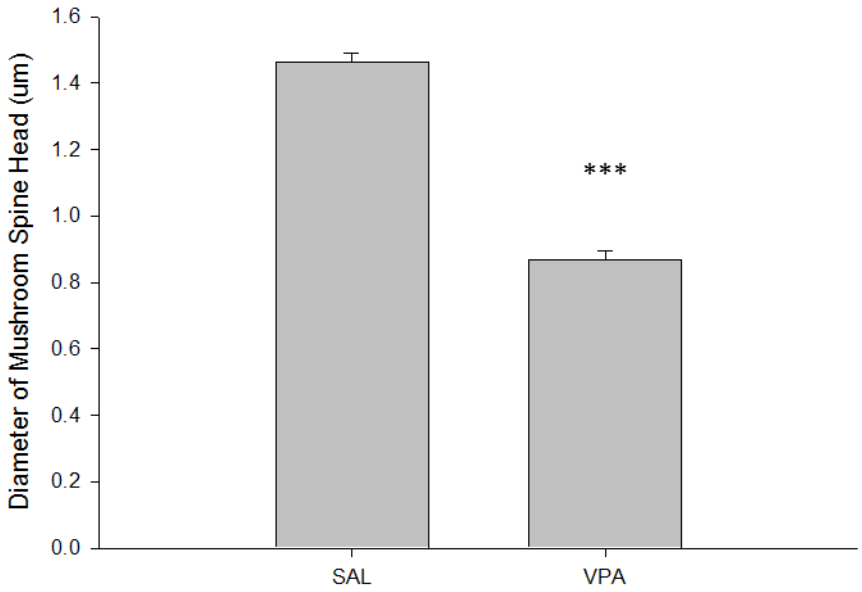


Figure 2-6. Synaptic abnormalities were observed in VPA cortical neurons

(A) A representative image of Golgi-Cox staining of the 2nd and 3rd layer neurons in SAL and VPA mice, a visual difference can be determined.

(B) Pyramidal neurons in the 2nd and 3th layer of the cortex had increased number of dendritic spines, SAL 94.82±1.51, N=6, n=18, VPA 105.00±5.00, N=5, n=15; $p<0.05$

(C) Further analysis shows reduction in filopodum type spines, SAL 2.21±0.28, N=6, n=18, VPA 0.15±0.09, N=5, n=15; $p<0.001$

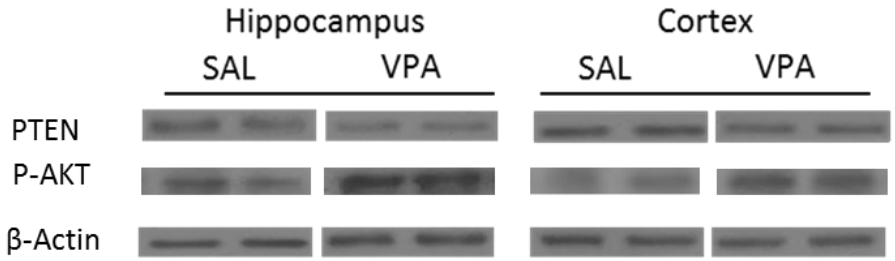
(D) VPA cortical neurons also showed a decrease in stubby type spines, SAL 51.41±1.02, N=6, n=18, VPA 39.11±2.37, N=5, n=15; $p<0.001$

(E) VPA mice showed an increase in thin type spines, SAL 31.10±0.94, N=6, n=18, VPA 49.44±4.78, N=5, n=15

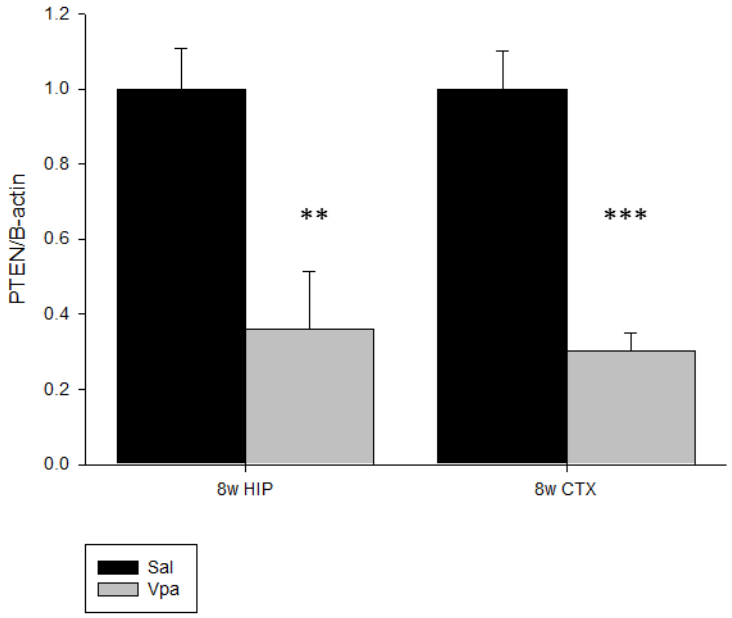
(F) VPA mice showed an increase in mushroom-like spines, SAL 10.10±0.40, N=6, n=18, VPA 16.30±1.13, N=5, n=15; $p<0.001$

(G) VPA mushroom spine heads were significantly reduced in size in comparison to SAL mushroom heads, SAL 1.46±0.0269, N=6, n=18, VPA 0.8697±0.023, N=5, n=15; $p<0.001$

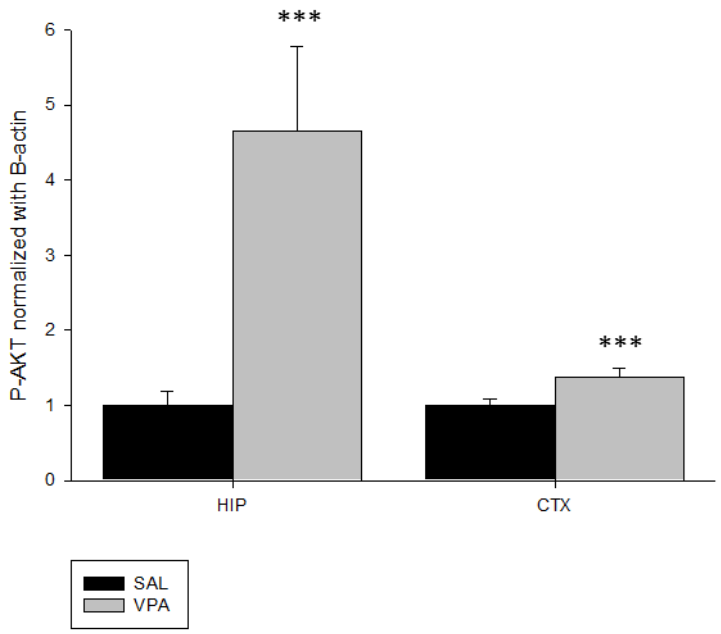
(A)


Hippocampus
Cortex
SAL
VPA
SAL
VPA
PTEN
P-AKT
β-Actin

(B)


PTEN/B-actin
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
***
8w HIP
8w CTX
Sal
Vpa

(C)



(D)

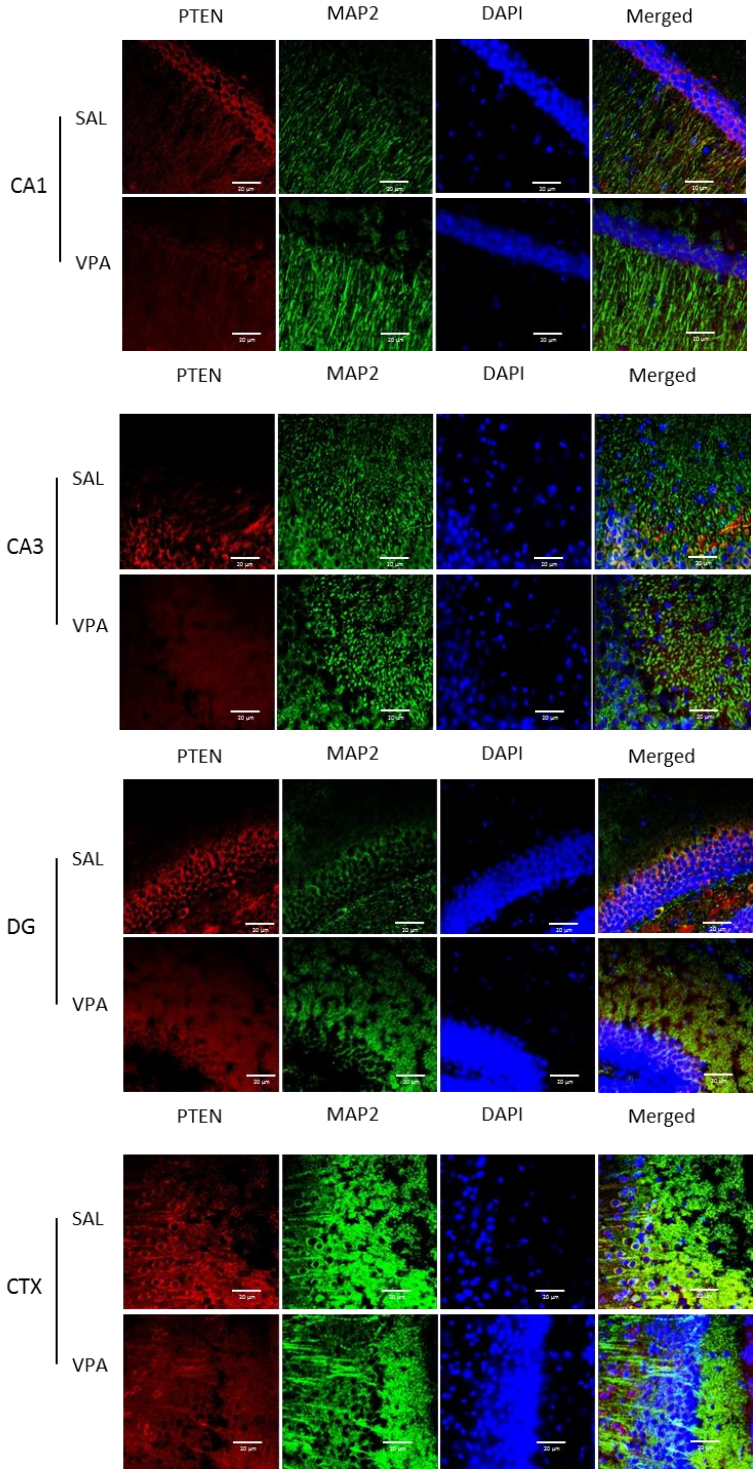

PTEN
MAP2
DAPI
Merged
CA1
SAL
VPA
PTEN
MAP2
DAPI
Merged
CA3
SAL
VPA
PTEN
MAP2
DAPI
Merged
DG
SAL
VPA
PTEN
MAP2
DAPI
Merged
CTX
SAL
VPA

Figure 2-7. Reduction of PTEN expression in hippocampus and cortex of post-weaned mice

(A) Representative image of PTEN levels in the hippocampus and cortex of VPA mice shown in western blots

(B) PTEN was significantly reduced in the hippocampus, SAL 1.01±0.15, n=6, VPA 0.36±0.10, n=6; $p<0.01$, as well as the cortex SAL 0.51±0.05, n=6, VPA 0.15±0.02, n=6; $p<0.001$

(C) Phosphorylated protein kinase B (P-AKT) was significantly increased in the hippocampus, SAL 0.66±0.13, n=6, VPA 1.49±0.22, n=6; $p<0.05$

(D) Representative image of PTEN expression in different locations of VPA mice in comparison to SAL, scale bar represents 20 µm

DISCUSSION

This chapter demonstrates that prenatal VPA exposure in mice leads to behavioral and molecular changes in the post-weaning period, which accord with previous studies about autism subjects and other animal studies. VPA-exposed pups showed noncore autistic behaviors such as increase threshold to pain, and core autistic behaviors such as repetitive behavior as seen by self-grooming test, as well as social impairment observed in home cage interaction test and three chambers social interaction assay. VPA mice also showed spatial memory impairment; however unlike previous reports (Mychasiuk, Richards, Nakahashi, Kolb, & Gibb, 2012), VPA mice did not show increased anxiety.

As Chapter 1 indicated, the synaptic abnormality may be one of the root causes in behavioral changes, mature mice exhibited similar synaptic abnormalities as seen in the primary cortical neurons. In chapter 2, I show that VPA mice showed an increase in overall spine density and an increase in thin and mushroom type spines. Although, the mushroom spine head size was statistically smaller compared to SAL, it may be beneficial to further investigate the significance of increased mushroom spines in regards to behavior. Behaviorally, the possibility of local over-connectivity has been linked to hyper-arousal and reduced selectivity (Hutsler & Zhang, 2010). Multiple types of indirect evidence have been used to support theories of altered cortical

connectivity in ASD (Durand et al., 2012; Lugo et al., 2014; Tsai et al., 2012). In other developmental disorders reduced spine expression is commonly found, while conditions such as fragile X syndrome show increased spine densities. Despite its relevance to theories of altered cortical connectivity, synaptic spine expression has not been systematically explored in ASD (Hutsler & Zhang, 2010). Despite these various predictions of connectional changes and the importance of such responses to behavioral symptoms that characterize autism, a direct examination of the structural microcircuitry within the cerebral cortex has not been elucidated.

PTEN specifically catalyses the dephosporylation of the 3` phosphate of the inositol ring in PIP_3, resulting in the biphosphate product PIP_2 ($PtdIns(4,5)P_2$). This dephosphorylation is important because it results in inhibition of the AKT signaling pathway. Alterations in the PI3K/AKT/mTOR pathway results in many behavioral abnormalities and is expected to play a significant role in ASD (Lugo et al., 2014).

Taken together, VPA mice show several similarities to PTEN cKO mice, including autistic behavioral symptoms, anatomical changes in the CA1 region of the hippocampus and increased dendritic spines (Clipperton-Allen & Page, 2014; Lugo et al., 2014). VPA exposed mice may have similar changes in the PTEN/PI3K/AKT pathway which may lead to similar perturbations. These results provide insight into the

molecular impairments found in an environmental induced factor of autism.

Further research is particularly needed to elucidate the molecular mechanism by which the expression of PTEN is downregulated by VPA, and its effect on neural connectivity. The successful elucidation of this mechanism may expand the possibility to adapt PTEN as a major causal gene in the pathogenesis and also utilize it in diagnostics and therapeutics for the clinical treatment of autism spectrum disorders.

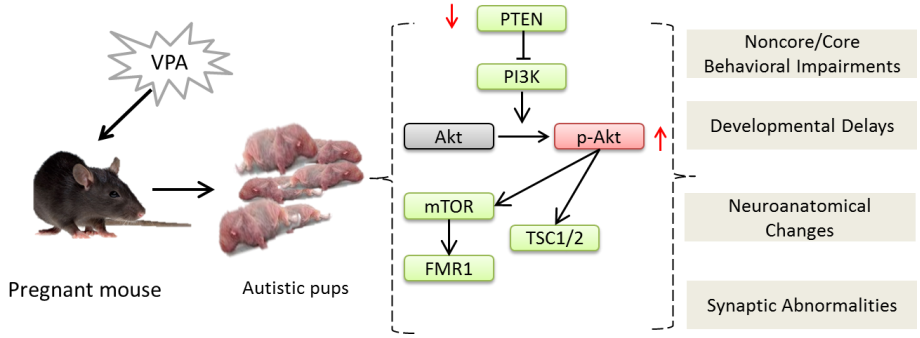


Fig. 2-8. An Illustrated Summary of changes in PTEN/PI3K/AKT pathway in VPA treated mice.
Pups exposed to VPA have a decrease expression of PTEN in the hippocampus as well as the cortex. Conditional knockout of PTEN has been shown to increase autistic behaviors, induce synaptic abnormalities and neuroanatomical changes which correspond to clinical ASD patients. Reduction of PTEN in the PTEN/PI3K/AKT pathway may be a key change that explains pathophysiological changes observed in ASD.

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국문 초록

서론: 자폐증 스펙트럼 장애(Autism spectrum disorder)는 주로 뇌 발생 단계의 이상으로 인한 질환으로 생각되고 있으며 뇌 발달 이상, 신경세포의 수상돌기의 분지 결손, 분자적 이상 및 행동 이상 등의 특징이 나타난다. 현재 523개의 유전자 이상이 자폐증 스펙트럼 장애와 관련되어 보고되고 있으나 모든 자폐환자의 5%만이 유전자 이상을 보인다. 나머지는 환경적인 요인으로 인하여 질환이 발생하는 것으로 생각되고 있다. 자폐증 연구에 사용되는 동물 모델에 현재 540여 종류 유전자 변형 동물과 20여 종류의 환경적 요인에 의한 모델이 있다는 것은 단일 유전자에 의한 질환이 아니라 다양한 유전자와 환경적 요인이 복합적으로 영향을 미쳐 발병한다는 것을 반영한다. 그 중 발프로산(valproic acid, VPA)에 의한 자폐증 동물모델이 가장 널리 사용되고 있다. 항간질약물로 사용되는 발프로산은 임신 중에 복용하면 자폐증 스펙트럼 장애를 유발하는 것으로 보고되어 있다. 본 연구에서는 발프로산에 의한 자폐증 동물모델의 행동 변화와 분자적 변화를 조사함에 의하여 자폐적 행동 특성을 설명할 수 있는 분자적 타겟을 발굴하고자 하였다.

방법: 발프로산에 의한 자폐증 마우스 모델에서 분자적 변화를 조사하기 위하여 웨스턴블롯팅, 면역조직화학염색방법, 골지-콕스 염색, 니슬염색, 수상돌기 가시 분석방법을 사용하였다. 또한 이들 마우스모델의 행동적 변화를 관찰하기 위하여 self-righting test, eye opening test, mother scent choice, thermal nociception test, self-grooming test, home cage interaction test, three chamber social interaction assay, T-maze test, open field test 및 elevated plus-maze test를 사용하였다.

결과: 발프로산 자폐증 모델 마우스는 발달 지표의 결손을 보였으며 사회적 상호작용, 반복적 행동 및 공간기억의 손상을 나타내었다. 또한 대뇌피질 신경세포의 수상돌기 가시 밀도의 변화를 보였으며 수상돌기 가시의 형태도 대조군 마우스에 비하여 유의한 변화를 나타내었다. 대뇌피질 및 해마에서 PTEN과 인산화된 AKT의 변화가 관찰되었다.

결론: 이 연구를 통해 발프로산에 의한 자폐증 마우스모델에서 발달 단계 및 성체 시기에 심각한 결손과 분자적 변화를 관찰할 수 있었다. 발프로산에 노출된 마우스에서 시냅스상 이상이 일관되게 나타나는 점과, 같은 시기에 PTEN의 발현도 해마와 대뇌피질에서 감소해있음을 밝힐 수 있었다. 이러한 결과들은 PTEN이 환경적 요인에 의한 자폐증에서 나타나는 시냅스의 결함과 행동학적

변화에 영향을 미치는 원인 유전자임을 시사한다.

주요어: 자폐증, 자폐증 스펙트럼 장애, 발프로산, 행동장애, PTEN, P-AKT
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