

Effects of valproic acid on transcript levels in neurotrophin signaling pathway in mice hippocampus according to the implementation period

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Abstract

Background/Aim: Valproic acid (VPA), is an antiepileptic drug and it has been known for a long time that exposure to VPA in the fetal period causes many behavioral, cognitive, and structural disorders. In this study, we aimed to evaluate the effects of the implementation period of VPA during pregnancy on both behavioral tests and the expression of 45 key genes belonging to the neurotrophin signaling pathway.

Methods: The study was designed to form four different groups of five female mice in each group. The experimental and control groups were divided into mature (n = 5) and immature (n = 5) subgroups, respectively. VPA (500 mg/kg dose) was subcutaneously administered to the experimental groups. All mice were first tested for behavioral changes, and then the hippocampal tissues were used for RNA isolation. The expression levels of 45 transcripts associated with the neurotrophin signaling pathway were determined by real-time RT-PCR in the hippocampus.

Results: No differences were found between the groups in any of the behavioral tests. When gene expression was examined, there was a difference between the groups in the transcript levels of *Ngf* (neurotrophic growth factor), *Ntrk2* (neurotrophic receptor tyrosine kinase 2), *Shc1* (SHC adaptor protein 1), *Chka* (choline kinase alpha), and *Mapk1* (mitogen-activated protein kinase 1) genes.

Conclusions: This study demonstrated the effects of VPA on transcript levels at different maturation stages. We believe that it is important to explain the structural and cognitive teratogenic side effects caused by VPA in human fetuses using genes whose expression changes.

Keywords: Behavioral tests, Neurotrophins, Pregnancy, Valproate.

Introduction

Valproic acid (VPA), a short-chain fatty acid called 2-propylpentanoic acid, is a well-known strong anticonvulsant that has been used for many years in the treatment of epilepsy [1]. In particular, valproic acid is highly effective and provides stronger seizure control than many advanced modern antiepileptics. In addition to epilepsy, VPA can be widely used for the treatment of diseases, such as anxiety disorder, bipolar disorder, and migraine prophylaxis. In recent studies, limitations have been imposed on its prescription to fertile women due to its teratogenic adverse effects [2].

Human studies have shown that exposure to VPA during the prenatal period leads to effects at various stages of the neurodevelopmental process, increases the risk of neural tube defects, and causes abnormal behaviors, resulting in cognitive and developmental retardation and autism-like behavioral phenotypes [3-5]. In many experimental studies, the harmful effects of VPA and behavioral changes observed in rodents exposed to VPA are similar to behavioral disorders in patients with autism spectrum disorder (ASD), and the presence of accompanying stereotypical behaviors

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with a decrease in social interaction in these rodents has also been reported [6, 7].

The changes caused by valproic acid in the fetal brain may vary depending on maturation. The prenatal VPA model was applied as a single high-dose on the 12.5th day of pregnancy in rodents. This period is accepted as the period approximately corresponding to the first trimester of human pregnancy [6, 8]. The postnatal VPA model is applied in the early postpartum period (0-14 days) and corresponds to the third trimester of human pregnancy. As a result of VPA exposure during the prenatal period, clinical pictures similar to those of human autism have been shown, such as decreased cerebellar Purkinje cell counts and symptoms of brainstem damage in the rodent brain [7]. In addition, VPA has been shown to increase brain-derived neurotrophic factor (*BDNF*) mRNA and protein levels 5-6 times in the fetal brain in rodent models [9]. Recent studies suggest that other abnormal neurotrophin levels, especially nerve growth factor (*NGF*) and *BDNF*, may be among the factors involved in the pathogenesis of ASD [10-12].

Neurotrophins play an important role in brain development and neuronal survival in other neuroplastic processes, such as axonal and dendritic growth and the formation of new synapses, and they consist of several different protein families. The most important of these are *BDNF*, *NGF*, and neurotrophins (NT-3, NT-4/5, NT-6, NT-7) [13]. Almeida et al. showed a 2.5-fold increase in *NT-2* transcript levels and a 2-fold increase in *NT-4* and *NT* transcript levels in rodents administered in utero VPA [9]. It is thought that autism, in particular, is associated with the increased expression of *BDNF* [10, 12, 14, 15]. *BDNF* gene plays a role in neurogenesis and synaptic plasticity and allows neural stem cells to differentiate into neurons in the hippocampus. It has important effects on learning and memory [14-17]. Although *BDNF* is necessary for normal brain function, its excessive activation can be pathological. There was a tendency for seizures in transgenic mice overexpressing *Bdnf*. Epilepsy studies have shown increased *Bdnf* and its receptor Tyrosine Kinase B (*TrkB*) in both the temporal and hippocampal regions in humans and animals [18]. In serum studies on *NGF*, *NGF* concentrations were found to be significantly higher in the serum of sick children than in controls [11]. In a study investigating the effect of VPA on *Ngf*, *Ngf* protein levels in the hippocampus were shown to increase in rats treated with VPA [19].

The issue of the correct dose and timing for prenatal or postnatal VPA application in rodents remains unclear. There are controversies in the literature regarding whether the effect is more significant when applied in the early prenatal or postnatal period [6, 8, 20-25]. One study showed that prenatal VPA administration resulted in more salient findings and more effective results, and animal mortality was lower after VPA administration [26].

In this study, we predicted that prenatal or postnatal administration of VPA might produce different results, depending on the maturity of the brain. In the literature, no study has examined prenatal-postnatal VPA models and

compared them with the normal control group in terms of both behavioral tests and transcript analysis involved in the neurotrophin signaling pathway. Based on these considerations, we aimed to examine and evaluate the transcript levels of 45 key genes involved in the neurotrophin signaling pathway in the hippocampus, as well as with the behavioral tests of mice that underwent VPA in the prenatal and postnatal periods, and to compare the groups both among themselves and with normal controls.

Materials and Methods

2.1. Animals:

Balb/c mice were used in our study with the permission of the ethics committee obtained by Erciyes University Animal Experiments Local Ethics Committee (decision number 2017/122). Mice were maintained in Erciyes University, Genome and Stem Cell Center, Transgenic Animals Department, Kayseri, Turkey, under controlled conditions (lights 06:00 to 18:00, temperature 23 °C, and humidity 55%). All experiments were performed on a homogeneous group of animals of the same age, maintained at the same facility. The animals were cared for and treated according to the Principles of Laboratory Animal Care (European rules). OECD guidelines for testing chemicals on mice were followed [27].

2.2. VPA Mouse Model:

Male and female *Balb/c* mice were housed in plastic cages. Females were checked before 10 AM for the presence of a vaginal plug, which was recorded on day 0.5 of embryonic development. In the prenatal group, pregnant females were treated subcutaneously on embryonic day 12 (E12.5) with 500 mg/kg sodium valproic acid [28]. In the prenatal control group, control females were treated subcutaneously with 200 µL of 0.9% saline on embryonic day 12. The mice were divided by sex one month after the injection was completed and only male mice were selected for the experiment.

Late-pregnant *Balb/c* female mice were housed individually in plastic cages. The day of birth was recorded as day 0.5. The female mice were removed from the cage. In the postnatal control group, five male mice were subcutaneously treated with VPA (500 mg/kg) on postnatal day 14. The other five male mice were treated subcutaneously with 200 µL of 0.9% saline on postnatal day 14.

The study was designed in four different groups, and five male mice were selected from each group.

- i) The group which were injected with VPA (500mg/kg) to healthy pregnant mice on gestation day 12th day: **Immature group (prenatal exposure)**
- ii) The group which were injected with VPA (500mg/kg) to non-treatment offspring 14 days after birth: **Mature Group (postnatal exposure)**
- iii) The group which were injected with 0.9% saline to healthy

pregnant mice on gestation day 12th: **Immature Control Group**

iv) The group which were injected with 0.9% saline to non-treatment offspring 14 days after birth: **Mature Control Group**

When all the groups reached adulthood (2 months old), novel object recognition, social interaction, tail suspension, and marble-burying behavior tests were performed, respectively [28].

2.3. Behavior Tests:

Behavioral experiments began when VPA and saline control mice were two months old. Before the start of the experiment, the mice were brought to the room where the experiment was conducted and kept in the environment for 15-30 minutes. Thus, environmental habitation was provided [28].

2.3.1. Novel Object Recognition Test

The setup used for the novel object recognition test was a square-shaped box with a size of 50 cm x 50 cm x 50 cm, with a clear top, surrounded by lines and a wall, and 300 lx light intensity was applied to the central area. Two identical objects no larger than a mouse were used for this test. On the first day, the mice were left in the middle of the setup and recorded for 5 min using an EthoVision system (Noldus Information Technology, Wageningen, Netherlands). The time the mouse stayed near the objects and the number of times it went to them were calculated. On the second day, one of the objects was changed a new object was placed and a 5-minute recording was repeated. The time the mouse stayed with new and familiar objects, the number of times it went with them, and the discrimination index were measured. When the difference between the time spent with the new object in total and the time spent with the familiar generation in total is proportional to the total time and multiplied by 100, the data obtained is called the discrimination index [28, 29]

2.3.2. Social Interaction Test

Crawley's sociability and social innovation test consists of a rectangular, three-compartment box. Each room is 19 x 45 cm and is a system with divided walls, and a middle compartment made of open clear plexiglass that provides free access to each room. Before starting the experiment, the mice were placed in a room where the experiment was carried out for 30 min to familiarize themselves with the experimental setup environment. Then one of the mice was placed in a chamber. While the other compartment was left blank, the original experimental mouse was left in the middle compartment and recorded with a camera system for 5 min. The data were created with the EthoVision (Noldus Information Technology, Wageningen, Netherlands) system in line with the parameters of interest to the mouse in the compartment, and the statistical analysis was performed and compared with the control group [30].

2.3.3. Tail Suspension Test

The experimental design was established in such a way that three mice could be tested simultaneously. In such a way that the mice could not see each other, 25 cm thick cardboard-like plates were cut and placed between them. As the mice were white, a black background was used. The tapes 12 cm in length were cut and the mouse tails were hung on the experimental setup by sticking to the tip of the tail without any damage. The mice were imaged using a video camera for six min. The recordings were monitored in a computer environment and the immobility time of the mouse in six min was calculated [29, 31].

2.3.4. Marble Burying Test

The marble buried test is commonly used to measure neophobia [32], anxiety and obsessive-compulsive, or repetitive behaviors in rodents. It has also been suggested that the test may have predictive validity for screening new antidepressants, anxiolytics, and antipsychotics [33-37]. Performance in the ball burial test is also related to general digging behavior. The basic structure measured in this experiment, in which social curiosity and fear of new objects, in general, are measured, is still unclear. This behavior is probably a typical defensive burial type in rodents [38]. It seems that the mice could not see the balls clearly, so the selection of the more pronounced colored balls increases the burial [39]. The region in which this behavior is related to the neuronal system has not yet been clarified. However, the hippocampus and septum are thought to be highly affected because lesions in these areas reduce digging [31, 33].

The corncocks that we normally used in the care of our mice were placed in an empty cage at a height of 5 cm. Twenty balls were arranged in five rows with four marbles per row. The experimental mouse was released from one corner of the cage and allowed to explore for 30 min. At the end of 30 min, the mouse was taken from the cage and the total number of buried marbles under the corn cob during this period was counted and noted.

2.4. Molecular Analysis:

After the behavioral tests were completed, the mice were sacrificed by cervical dislocation, their brains were dissected, and the hippocampal tissues were placed in 500 µL PureZOL (Biorad, Germany Cat No:7326890) for molecular assays.

2.4.1. RNA Isolation

To determine gene expression levels by real-time PCR, RNA isolation was performed from hippocampal tissues taken into PureZOL following the manufacturer's recommendations. Each sample was placed in PureZOL and homogenized using injectors. 180 µl of chloroform was added to all the homogenized tissues and vortexed for 15 seconds. After incubation at room temperature for 5 min, centrifugation was performed at 12000 g for 20 minutes at +4°C. After centrifugation, the upper phase was transferred to the new reaction tubes, 1:1 ratio of

isopropanol was added to the upper phase, which was then turned over and centrifuged at 12000 g at +4°C for 10 minutes. After centrifugation, the supernatant was poured and 1 ml of 75% ETOH was added to the pellet. The pellet was removed by turning it upside down and centrifuged at 7500 g at +4°C for 5 min. After centrifugation, the supernatant was poured and the alcohol remaining on the pellet was removed using a 10 µL pipette and left to dry. After the drying process was completed, the pellet was resuspended by adding 30 µL of nuclease-free water and the obtained RNAs were placed on ice and kept at +4°C for 10-15 minutes. Then, the RNA concentrations were measured using the Nano-Drop device [36, 40].

2.4.2. cDNA Synthesis and Q-PCR Analysis

cDNAs was obtained using the Evo Script cDNA Synthesis Kit (Roche, Germany Cat No: 07912374001). For the reaction mix, 4ul reaction buffer, 10 µL of dH₂O, and 5 µL of RNA were added to each sample, kept on ice for 5 min, and 2 µL of reverse transcriptase was added. Subsequently, cDNA was obtained by incubating the PCR conditions at 42 °C for 15 min, 85 °C for 5 min, and 65 °C for 15 min.

The Custom Panel (Roche, Germany, Cat No: 04707494001) was used to determine the transcript levels of 45 different genes and 3 reference genes shown in supplemental material **Table S1**. Compatible with Probe assays, a probe master (Roche, Germany, Cat No: 05 532 914 001) was used to study the panels. For each sample, the mix was prepared with 15 µL of probe master mix, 3 µL of dH₂O, and 2 µL of cDNA, then distributed to the panel plate. Expression levels were determined using a Roche LC480 II PCR instrument (Roche, Germany) under the following PCR conditions: 95°C for 5 sec 1 cycle, 95°C 10sec, 55°C for 30 sec, 72 °C for 1 sec 45 cycles, and 40 °C for 10 sec. Then, obtained Ct values were normalized with reference genes

using the $2^{-\Delta\Delta C_t}$ method. Gene expression data were analyzed with GraphPad Prism version 8 [41].

2.5. Statistical Analysis

After the behavioral experiments and gene expression results were obtained, comparisons between the experimental and control groups were performed by selecting the appropriate parameters. The compliance of the data to a normal distribution was evaluated using the histogram, q-q graphs, and Shapiro-Wilk test. Depending on whether the data showed a normal distribution, analyses were performed using one-way ANOVA, Kruskal Wallis, Student's t-test, and Mann-Whitney U tests. Data were analyzed using SPSS version 22 (IBM, USA) and Prism version 8 (GraphPad, USA). Results with p values <0.05 were considered statistically significant.

Ethics: The study was approved by the Local Ethics Committee for Animal Experiments, Erciyes University, and efforts were made to reduce the total number of animals used as well as their potential pain and suffering.

Results

3.1. Behavioral tests

3.1.1. Novel object recognition test

The cognitive status of the mice in the prenatal and postnatal experimental groups was compared to of that the control group and among themselves. As shown in Figure 1, there was no difference between the experimental and control groups in terms of the total duration (cumulative duration), discrimination index, total number of movements, and velocity ($p > 0.05$). Both the experimental and control groups were more interested in the new object than the familiar object (Fig.

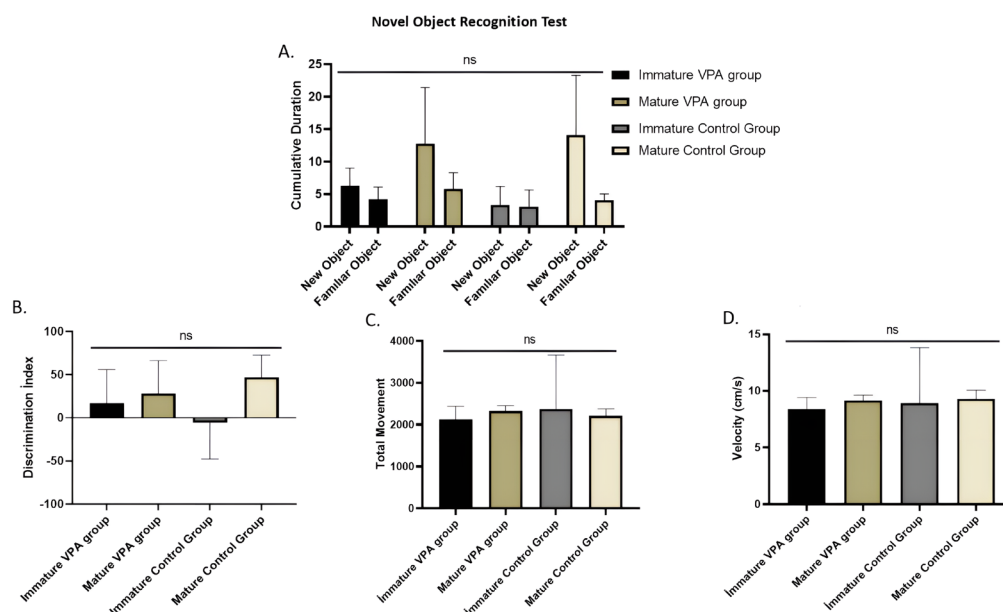


Figure 1. Novel object recognition test with comparisons of cumulative duration, discrimination index, total movement, and velocity.

1).3.1.2. Social interaction test results

In the three-chamber sociality test, the prenatal and postnatal experimental groups and the prenatal control group spent more time with foreign mice than the empty cage group (paired *t*-test; prenatal VPA group $p = 0.002$; postnatal VPA group $p < 0.001$; prenatal control group $p < 0.001$, Fig. 2). The postnatal control group also spent more time with foreign mice, but the difference was not statistically significant.

and 4e). On the other hand, *Chka* gene expression levels were found to be increased in the mature VPA group compared to the immature VPA group (Figure 4d). The effects of valproate on transcript levels in mice are given in Table 1.

Discussion

Neurotrophins not only play a key role in processes such as

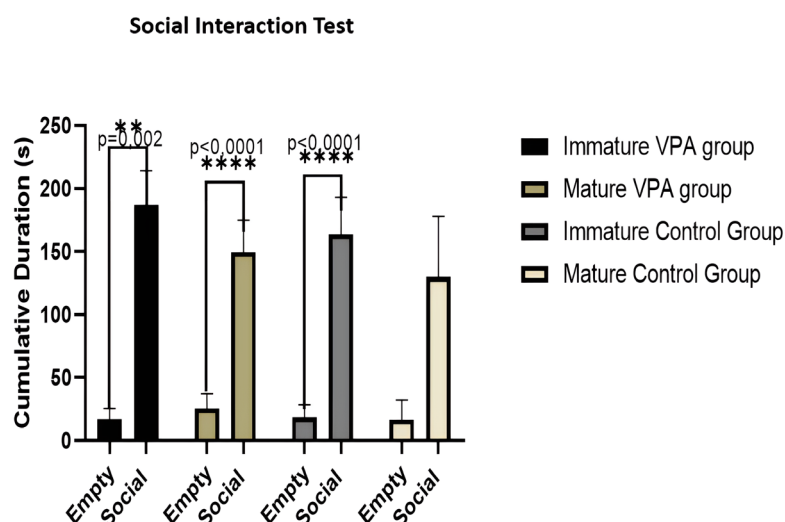


Figure 2. Social interaction test data

3.1.3. Marble test results

In the marble-burying test, there was no difference in the number of buried marbles between the control and experimental groups ($p > 0.05$) (Fig. 3).

3.1.4. Tail suspension test results:

There was no difference between the groups in terms of the inactivity time in the tail suspension test ($p > 0.05$) (Fig. 3).

3.2. Transcript levels of synaptic plasticity and neurogenesis-associated genes:

Forty-five key genes in the *BDNF* neurotrophin signaling pathway were examined. Gene expression of 5 of these genes differed statistically between the groups ($p < 0.005$). The findings are shown in Figure 4.

Ngf gene transcript levels were found to be increased in the immature VPA group compared to both immature and mature control groups (Figure 4a). Likewise, it was observed that the expression levels of the *Ntrk2* gene increased in the immature VPA group compared to the immature period control (Figure 4b). At the same time, it was determined that the expression levels of *Shc1* and *Mapk1* genes were increased in the immature VPA group compared to the mature VPA group (Figure 4c

brain development and neuronal survival but also affect the formation and elimination of neuronal connections [42]. In our study, significant differences were observed between the groups in the expression of some of the genes involved in the neurotrophin signaling pathway depending on the prenatal or postnatal administration of VPA. However, no significant differences were found between the groups with regard to the behavioral tests. This was associated with the low number of animals in the groups when evaluating behavioral tests.

The primary objective of this study was to investigate the effects of prenatal and postnatal VPA administration on the expression of neurotrophins in the brain. The relationship between neurotrophins, especially *Bdnf* and VPA has been emphasized in previous studies [10, 12, 14, 15]. However, we could not find any difference between the groups in terms of *Bdnf* transcript levels in the hippocampus. Nevertheless, *Bdnf* is not the only modulator of neurotrophin in brain development affected by VPA. *Ngf*, another important neurotrophin, plays a role in the growth, differentiation, survival, and regeneration of nerve cells by stimulating *TrkA* (a transmembrane tyrosine kinase) and *p75* receptors [43]. In our study, we found that hippocampal *Ngf* transcript levels were higher in the immature VPA group than in both immature and mature controls. *Ngf*

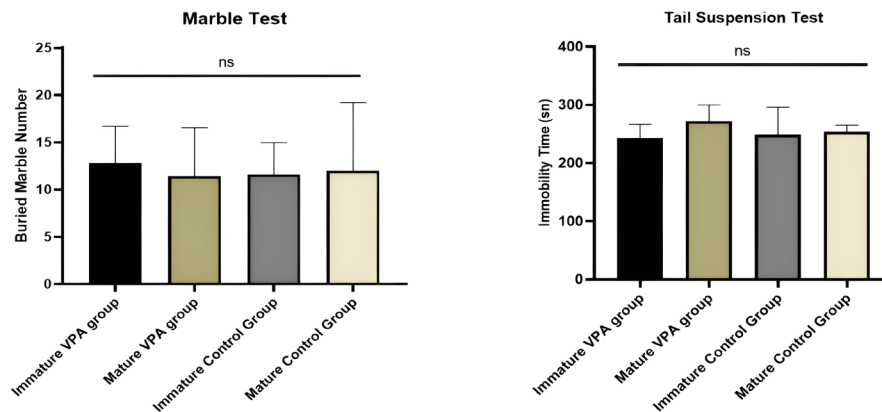


Figure 3. Marble burying (A) and (B) tail suspension test data

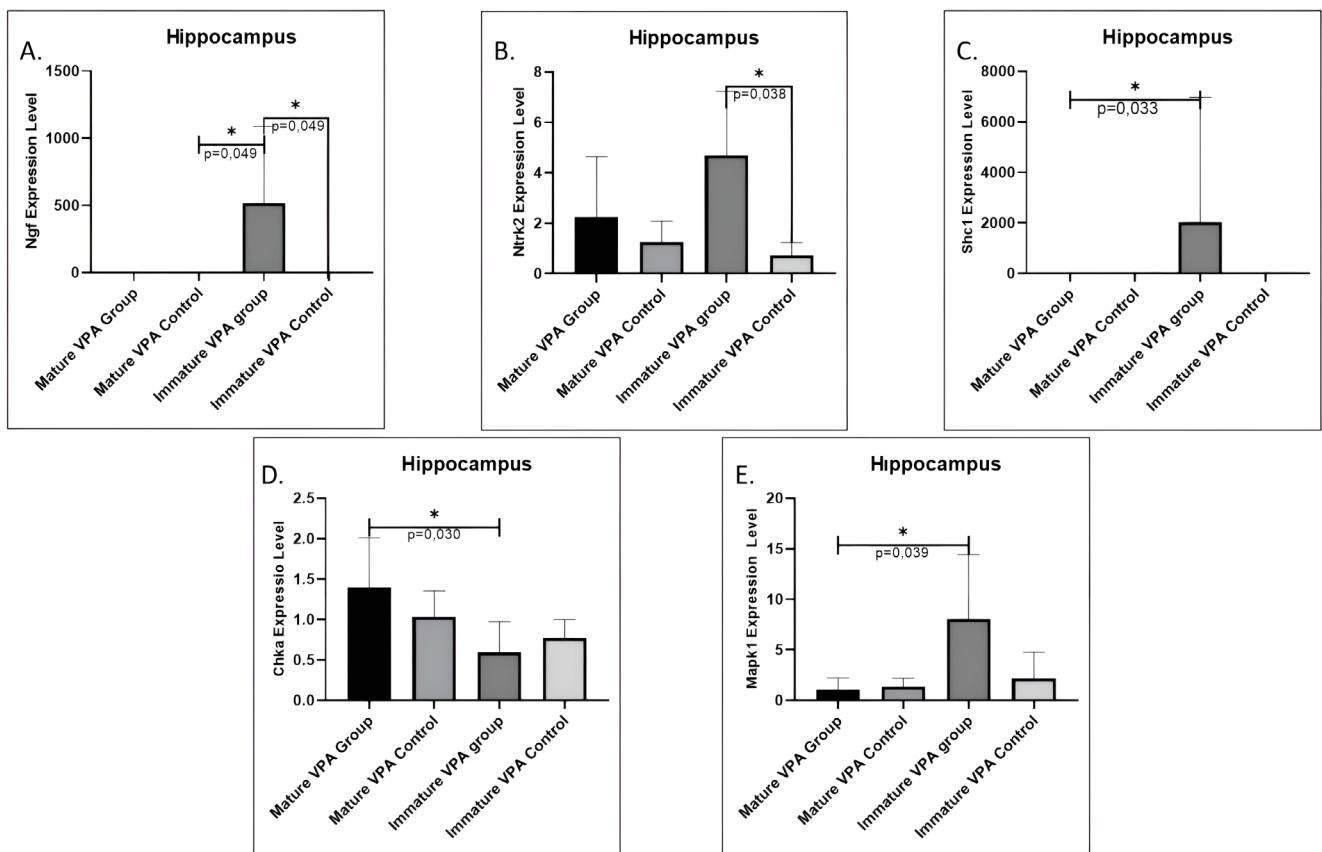


Figure 4. Transcript levels of the neurotrophin signaling pathway-associated genes in the VPA-treated and control groups.

A. Immature VPA group vs immature control group, $p=0.049$, mature VPA control vs immature VPA control, $p=0.049$

B. Immature VPA group vs immature VPA control group, $p=0.038$

C. Immature VPA group vs mature VPA group, $p=0.033$

D. Mature VPA group vs immature VPA group, $p=0.030$

E. Mature VPA group vs immature VPA group, $p=0.039$

Table 1. The effects of valproate on transcript levels in mice groups.

Gene Symbol	Mature VPA Average 2 ^Δ Delta Ct	Mature VPA Control Group Average 2 ^Δ Delta Ct	Immature VPA Average 2 ^Δ Delta Ct	Immature VPA Control Group Average 2 ^Δ Delta Ct	P Value (Mature VPA vs Immature VPA)	p Value (Immature VPA vs Immature VPA Control)	p Value (Mature VPA Control vs Immature VPA Control)
Ngf	2,592822829	1,357616658	347,1304341	2,869827766	>0,05	0,049	0,049
Ntrk2	2,228895133	1,444525795	571,2143328	1,863894632	>0,05	0,038	>0,05
Shc1	0,2202056	1,33083513	2030,717778	5,220802679	0,033	>0,05	>0,05
Chka	1,397573933	1,10326353	0,591355749	1,314456177	0,030	>0,05	>0,05
Mapk1	1,087426934	1,287417029	8,04732717	2,170518531	0,039	>0,05	>0,05

Statistically significant p values are shown in bold.

levels are found at the highest rates in areas with the highest number of cholinergic projections from the basal forebrain, namely, the hippocampus and cortex [44]. The *Ngf* signal is responsible for protecting cholinergic corticospinal projections and prevents them from degenerating after axonal damage to these neurons [45]. *Ngf* modulates cholinergic function by increasing the release of acetylcholine from the basal anterior brain [46]. Interruption of *Ngf* signaling results in cholinergic neuron atrophy. The cholinergic neurons of the basal anterior brain form the only neuronal system in the brain that structurally expresses both the Tropomyosine Receptor Kinase A (*TrkA*) and the P75 Neurotrophic Receptor (p75^{NTR}) [47], which means that they have a constant hypersensitivity to *Ngf*. As a result of the blockade of endogenous *Ngf*, hippocampal long-term potentiation (LTP) decreases, and spatial memory is affected [48]. These findings show that *Ngf* has an important effect on plasticity and memory.

Ngf has been increasingly investigated for the ASD spectrum in recent years. Abnormal *Ngf* protein levels have been shown especially in the serum of autistic children, and it is emphasized that this may be a serological biomarker for cognitive impairment, regression, and epilepsy in individuals with autism. In one study, 41 children with autism were compared with the same number of control groups in terms of serum *Ngf* levels, which were *higher* in children with autism than in the control group [11]. In a rodent study, no increase was detected in terms of *NGF* mRNA expression in the fetal brain cortex in rats administered VPA in utero, while a slight effect was observed in *Bdnf*, *Nt-3*, *Nt-4/5*, and in the neurotrophin receptors *TrkA*, *TrkB*, *TrkC*, but p75^{NTR} doubled [9]. High levels of *Ngf* have also been detected in both animal models and human serum in attention deficit hyperactivity syndrome (ADHD) [49, 50].

The *Ntrk2* gene encodes the high-affinity tyrosine kinase B receptor (TrkB), whose primary ligand is *Bdnf*. However, *Nt-3* can bind to the *TrkB* receptor at *Nt-4/5* [51, 52]. TrkB signaling interacts with various intracellular signaling pathways, including ligand-activated TrkB, mitogen-activated protein kinase (*Mapk*), phosphoinositide 3-kinase (*PI3*), and extracellular signal-regulated kinase (*Erk*), and plays a role in plasticity, neurodifferentiation, and neurotransmission [53]. *TrkB*

signaling has serious consequences on brain development and function. There have been studies on abnormal *Bdnf/TrkB* signaling in ASD. We found that immature VPA administration significantly increased *Ntrk2* expression compared to that in immature controls. However, there was no difference between the controls in mature VPA application. In human studies, six single nucleotide polymorphisms and multiple haplotypes have been detected in the *Ntrk2* gene in platelet-rich plasma (PRP) obtained from the blood of children with autism. This study shows that the *Ntrk2* gene is a factor that can play a role in the development of autism [15]. Our study showed that the effect of VPA exposure on *Ntrk2* is more pronounced in the early pre-natal period.

The mammalian Shc1 homolog p66^{Shc} is the main regulator of mitochondrial reactive oxygen radicals (ROS) production. It has also been shown to play an important role in amyloid β -derived ROS production and in vitro cytotoxicity [54-56]. Under cellular stress conditions, p66 Shc acts as a redox enzyme that strengthens ROS production and initiates apoptosis [52]. Mice knockout for p66 Shc have been shown to have prolonged life spans, lower ROS levels, less atherosclerosis and are protected from physical and cognitive impairment due to aging [54, 57-61]. In our study, we found that the *Shc1* transcript levels were significantly higher in the immature VPA group than in the mature VPA group. It can be thought that the use of VPA in earlier periods of maturation will also have negative effects on oxidative stress. When p66 Shc was knocked down in bovine embryos, the stress resistance of these embryos increased significantly, intracellular ROS levels decreased, and DNA damage and apoptosis decreased [62]. It can be predicted that overexpression of this gene may be a risk factor for cognitive impairment. However, further studies are required on this subject.

It has been shown that *Chk*, a cytoplasmic protein kinase that is a homolog of CSK (control of Src kinase), interacts with tyrosine-phosphorylated *TrkA* receptors by stimulating *Ngf* in PC12 cells (hippocampal neuron model system) [63]. *Chk* expression results in increased activity of the mitogen-activated protein kinase/extracellular-signal-regulated kinase 1/2 (MPaK, ERK 1/2) pathway through neurite growth factor (*Ngf*) and triggers neurite growth in PC12 cells [64]. In our study,

the *Chka* transcript levels were higher in the mature than in the immature VPA group. The *Chka* gene encodes choline kinase alpha. This enzyme plays a role in the phosphorylation of choline via phosphocholine in the Kennedy pathway. Phosphatidylcholine is the major lipid molecule that forms the mammalian cell membrane. This gene has been investigated in many cancer types, and its overexpression has been associated with cancer [65]. There is data on the relationship between this pathway and gene-related pathologies with resistant epilepsy and microcephaly. In this study, it was thought that the decrease in the *Chka* enzymatic activity caused disruption in the first step of the Kennedy pathway, and as a result, existing neurological manifestations were revealed [66]. No studies in the literature have discussed the effects of VPA on this gene. The neurological reflections of the overexpression of this gene are unknown, and further research is also needed on this subject.

The *Mapk1* gene encodes a member of the map kinase family. *Map* kinases, also known as extracellular signal-regulated kinases (ERKs), serve as an integration point for multiple biochemical signals and are involved in a wide range of cellular processes, such as reproduction, differentiation, transcription regulation, and development. Neurotrophins activate many intracellular signaling pathways via Trk receptors, including those controlled by Ras, leading to the activation of mitogen-activated protein kinase (*Mapk*), phospholipase C (PLC)- γ 1, and phosphatidylinositol 3-kinase. Thus, they affect both the development and function of the nervous system [67]. Valproic acid has been reported to inhibit the *Mapk* signaling pathway in *Saccharomyces cerevisiae* [68]. We found an increase in *Mapk1* transcript levels in the immature VPA group compared to those in the mature VPA group. However, we do not have sufficient information to discuss the effects of this increase. We believe that similar studies to be conducted in the following years will shed light on the effects of this increase.

In our study, we examined the transcript levels of genes in the neurotrophin signaling pathway, but could not investigate the proteins synthesized by these genes, the changes developed on them, and the functions of these proteins. Another limitation is that the number of mice was low in the groups, and which negatively affected the comparability of the results, especially in behavioral tests. Owing to the high number of groups and the lack of ethical violations, the number of mice in the groups was kept limited.

Conclusion

As a result, the effects of VPA on the transcript levels of some genes in the neurotrophin signaling pathway in mice may vary according to maturity. Especially in the immature period, it was observed that this period, which corresponds to the initial three months of human pregnancy, had a special importance, and the effect was higher in this period. In the immature period, the increases in *Ngf*, *Ntrk2*, *Shc1*, and *Mapk1* transcript levels, which are among the genes belonging to the neurotrophin signaling pathway, were more pronounced than in the mature

period. It was observed that the *Chka* transcript was more highly expressed in the mature group. Discussion of the effects of increased expression of these genes will shed light on the determination of complications and side effects that may occur as a result of exposure to VPA in human fetuses in the early and late stages of pregnancy and even in the breastfeeding process. However, further studies are required to obtain more definitive results on the subject.

Author Contributions

Müge Gülcihan Önal, Zeynep Yılmaz Şükranlı, Ecma Güvenilir, and Samed Yora performed the animal experiments and assisted with the molecular biology component. Duygu Kurt Gök, Füsun Ferda Erdoğan, Mehmet Fatih Göl, and Serpil Taheri advised on the research design and methodology, problem-solving, and interpretation of results, and they also reviewed the manuscript before submission. Duygu Kurt Gök wrote the manuscript.

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Conflicts of Interest

The authors have no conflict of interest to declare.

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Table S1. Custom Panel Plate Design and Genes

	1	2	3	4	5	6	7	8	9	10	11	12
A	Ngf <i>M. musculus</i>	Bdnf <i>M. musculus</i>	Ntf5 <i>M. musculus</i>	Ntf3 <i>M. musculus</i>	Ntrk1 <i>M. musculus</i>	Ntrk2 <i>M. musculus</i>	Ngf <i>M. musculus</i>	Bdnf <i>M. musculus</i>	Ntf5 <i>M. musculus</i>	Ntf3 <i>M. musculus</i>	Ntrk1 <i>M. musculus</i>	Ntrk2 <i>M. musculus</i>
B	Ntrk3 <i>M. musculus</i>	Rapsn <i>M. musculus</i>	Sh2b1 <i>M. musculus</i>	Shc1 <i>M. musculus</i>	Irs1 <i>M. musculus</i>	Chka <i>M. musculus</i>	Ntrk3 <i>M. musculus</i>	Rapsn <i>M. musculus</i>	Sh2b1 <i>M. musculus</i>	Shc1 <i>M. musculus</i>	Irs1 <i>M. musculus</i>	Chka <i>M. musculus</i>
C	P60k3r1 <i>M. musculus</i>	Akt1 <i>M. musculus</i>	Prkca <i>M. musculus</i>	Rhoa <i>M. musculus</i>	Alcam <i>M. musculus</i>	Maged1 <i>M. musculus</i>	P60k3r1 <i>M. musculus</i>	Akt1 <i>M. musculus</i>	Prkca <i>M. musculus</i>	Rhoa <i>M. musculus</i>	Alcam <i>M. musculus</i>	Maged1 <i>M. musculus</i>
D	Trp73 <i>M. musculus</i>	R60pk2 <i>M. musculus</i>	Mapk7 <i>M. musculus</i>	Mapk14 <i>M. musculus</i>	Map2k1 <i>M. musculus</i>	Map2k2 <i>M. musculus</i>	Trp73 <i>M. musculus</i>	R60pk2 <i>M. musculus</i>	Mapk7 <i>M. musculus</i>	Mapk14 <i>M. musculus</i>	Map2k1 <i>M. musculus</i>	Map2k2 <i>M. musculus</i>
E	Mapk3 <i>M. musculus</i>	Mapk1 <i>M. musculus</i>	Bad <i>M. musculus</i>	Gsk3b <i>M. musculus</i>	Trp53 <i>M. musculus</i>	Bax <i>M. musculus</i>	Mapk3 <i>M. musculus</i>	Mapk1 <i>M. musculus</i>	Bad <i>M. musculus</i>	Gsk3b <i>M. musculus</i>	Trp53 <i>M. musculus</i>	Bax <i>M. musculus</i>
F	Jun <i>M. musculus</i>	Creb1 <i>M. musculus</i>	Bcl2 <i>M. musculus</i>	Fas1 <i>M. musculus</i>	H2-Ab1 <i>M. musculus</i>	Actb <i>M. musculus</i> Reference Gene	Jun <i>M. musculus</i>	Creb1 <i>M. musculus</i>	Bcl2 <i>M. musculus</i>	Fas1 <i>M. musculus</i>	H2-Ab1 <i>M. musculus</i>	Actb <i>M. musculus</i> Reference Gene
G	Frs2 <i>M. musculus</i>	Grb2 <i>M. musculus</i>	Gab1 <i>M. musculus</i>	Map2k7 <i>M. musculus</i>	Rapgef1 <i>M. musculus</i>	Gapdh <i>M. musculus</i> Reference Gene	Frs2 <i>M. musculus</i>	Grb2 <i>M. musculus</i>	Gab1 <i>M. musculus</i>	Map2k7 <i>M. musculus</i>	Rapgef1 <i>M. musculus</i>	Gapdh <i>M. musculus</i> Reference Gene
H	Map2k5 <i>M. musculus</i>	Rap1a <i>M. musculus</i>	Traf6 <i>M. musculus</i>	Rps6ka5 <i>M. musculus</i>	Zfp110 <i>M. musculus</i>	Hprt1 <i>M. musculus</i> Reference Gene	Map2k5 <i>M. musculus</i>	Rap1a <i>M. musculus</i>	Traf6 <i>M. musculus</i>	Rps6ka5 <i>M. musculus</i>	Zfp110 <i>M. musculus</i>	Hprt1 <i>M. musculus</i> Reference Gene