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RESEARCH ARTICLE

Effects of Prenatal Treatment with Valproic Acid (VPA) on Offspring of Epileptic Adult Rats: MRI Investigation

Anna Petrenko[1,*], Mikhail Gulyaev[2], Dmytriy Tischenko[2], Valery Petuchov[2], and Kenul Abbasova[1]

[1]*Faculty of Biology, Human and Animal Physiology Department, Lomonosov Moscow State University, Vorobjovy Gory, Moscow, 119899, Russia*
[2]*Centre for Magnetic Tomography and Spectroscopy, Lomonosov Moscow State University, Moscow, Russia*

The problem of treating epilepsy in pregnant women is being actively researched now as avoiding side effects of anti-convulsant drugs on children remains unsolved. In order to do so it important to understand why do structural effects appear in the first place and why there is observed a problem of a slower development as a result of using wide spread treatment methods. However, there is insufficient data about the influence of prenatal treatment with Valproic Acid (VPA) on changes in the brain structure of offspring of epileptics in adulthood. The aim of this study was to assess long-term brain damage after prenatal exposure to VPA. The offspring ($n = 70$) of 26 Wistar rats were divided into four groups and treated as follows: valproic acid (VPA—300 mg/kg), pentylenetetrazole (PTZ—75 mg/kg), VPA + PTZ and controls. MRI volumetric and signal analysis of changes in the cortex, hippocampus, thalamus, amygdale and striatum was conducted when the rats reached adulthood. The study provided clear evidence that VPA treatment in pregnancy significantly decreased hippocampal volume in all treatment groups and altered T2 signal intensity in all analysed structures.

KEYWORDS: Epilepsy, Pregnancy, Valproic Acid, Pentylenetetrazole, Magnetic Resonance Tomography.

INTRODUCTION

The difficulty of treating epilepsy during pregnancy has long attracted the attention of physiologists and clinicians. From 25 to 40% of patients suffering from epilepsy are women of childbearing age, and antiepileptic drugs (AEDs) can lead to metabolic changes, menstrual cycle disorders, polycystic ovary syndrome and violation of oral contraceptive pharmacokinetics and, as a result, unplanned pregnancy [1]; on the other hand pregnancy itself causes physiological changes leading to increased cardiac output and renal blood flow [2] and increased plasma volume and fat storage, the consequences of which may be the disturbance of drug pharmacokinetics including that of AEDs [3]. This also means that epilepsy and the different drugs taken by women before and during pregnancy may affect not only their own body but also future children, their physical development, brain functioning and even behaviour in adult life.

The incidence of seizures increases by 17–37% during pregnancy [2] and most patients require chronic administration of AEDs [4]. The offspring of women who took AEDs while pregnant had morphological defects. The situation is even more dangerous for those mothers who take valproic acid in large doses [5, 6]. Exposure to stressors that can be either a drug (a chemical stressor) or a non prevented epilepsy seizure in utero could have long-term effects on behaviour, cognitive function, processes of growth, metabolism and reproduction, and the inflammatory/immune response [7, 8].

Valproic acid is one of the major drugs used to treat epilepsy [9] and is also widely used in the treatment of migraine headaches and schizophrenia [3]. Meanwhile, fetal valproate syndrome (FVS) was described in 1984 [10]. This means that VPA exposure in utero can produce a wide range of defects from urogenital and neural tube defects (spina bifida) to congenital heart disease and intellectual disability [11, 12]. The link between prenatal VPA exposure and autism spectrum disorders is currently being actively examined both in animal models [13] and in humans. Rats treated with VPA in utero demonstrated delayed maturation, a lower body weight, delayed motor development and attenuated integration of a coordinated series of reflexes, lower sensitivity to pain, higher sensitivity to nonpainful stimuli, diminished acoustic prepulse inhibition, locomotor and repetitive, stereotypic-like

*Author to whom correspondence should be addressed.
Email: neshapetrenko@gmail.com
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hyperactivity combined with lower exploratory activity, a reduced number of social behaviours and increased latency to social behaviours [14], all of these being features of autism. Indeed, a study of the use of VPA during pregnancy showed a higher risk of autism-like disorder in the offspring of VPA-treated women risk is higher compared with when other anticonvulsants are used [15–17]. However, despite all the downsides of a valproic acid, there is no way of not using the drug at all at the moment, as it is hard to predict side effects of new drugs that have been invented in last fifteen years using classical pre-clinic models on animals (for example—using pentylenetetrazole), secondly—some of them can lead to a development of a dependency, and, finally, 20 to 30 percent of patients don't feel any effect from them at all [18].

MATERIALS AND METHODS

The experiment was carried out on white Wistar females weighing 180–230 g ($n = 26$) and 10 white Wistar males and their offspring of both sexes ($n = 70$). In total the experiment involved 106 animals.

A minimal VPA dose was chosen according to the VPA dose that completely prevents PTZ-induced seizures within 45 minutes after i.p. injection of PTZ (total dose—75 mg/kg–300 mg/kg), this part of study was conducted on 6 rats.

20 females were placed with the males in ratio 2:1 overnight between 5.00 pm and 8.00 am (total of 15 hours). Vaginal smears were examined for the phase of the oestrous cycle and for a seminal plug as evidence of mating. The day of plug detection was designated gestational day 0. In the absence of a seminal plug mating was repeated.

Females found to be pregnant were randomly divided into four groups. Control group animals were injected i.p. with 1 ml of NaCl 0.9% from the 1st to the 7th day of gestation and the "VPA" group with 300 mg/kg of valproic acid in a solution of NaCl 0.9% 100 mg/ml. In animals in the "PTZ" group PTZ-induced seizures were provoked on the 3rd and 6th day (all animals had generalized tonic-clonic seizures after the second or third injection, corresponding to the 5th stage of seizure). Racine score [19] was used to classify the intensity of behavioral seizures. Generalized seizures were defined by the intensity at Stage III (unilateral myoclonus or tonic myoclonus), IV (bilateral myoclonus or tonic-myoclonic behavior), and V (rearing and falling). The "VPA + PTZ" group was injected with VPA on the 1st to the 7th day following the injection of PTZ on the 3rd and 6th day of gestation (i.e., the seizures were arrested by VPA, the maximum stage of seizures was the 1st stage after the third injection of PTZ). Rats were maintained on a 12-h light/dark cycle. The temperature was kept at a constant 22 ± 2 °C. On the 19th day of pregnancy animals were individually housed. For those females who did not give birth after the 23rd day, or gave birth to significantly fewer rats than in the control group, or did not show maternal behaviour, the pregnancy was considered as a failure. There were 13 liters from all females altogether, giving 70 animals in total. Infant rats were weaned from the mother on the 28–29th day of postnatal development.

Image Analysis

Once the offspring of all four groups had reached the age of 2 months, 9 animals were chosen ($n = 5$ males, $n = 4$ females) from each group (in total $N = (5+4)*4 = 36$) for MRI tomography [20] using a Bruker BioSpec 70/30 (Bruker, Germany) with the software ParaVision 5.0, in the Faculty of Fundamental Medicine. Two-month-old animals were analysed in the T2-RARE mode for T2-weighted images (T2-WI) with high resolution and T2-map (T2) card for quantitative MRI studies.

To evaluate hippocampal volumes, regions of interest (ROIs) on 6 consecutive tomographic slices of rat brain in the frontal projection was thus obtained, starting from the

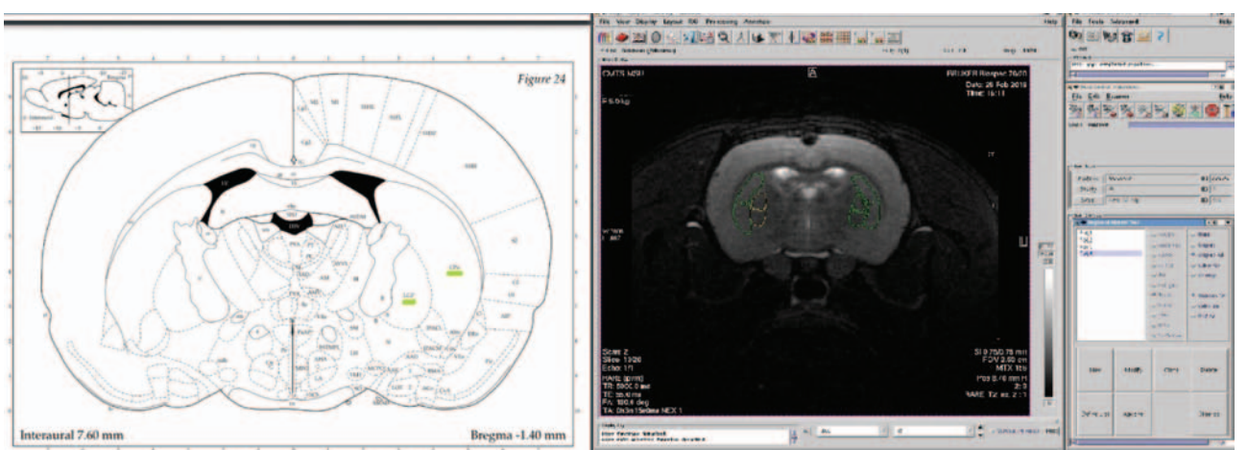

Fig. 1. Left—a scheme of brain structure [21], right—interface of ParaVision 5.0 with highlighted structures: striatum and lateral globus pallidus. The slice is on a level of −1.40 mm from bregma.

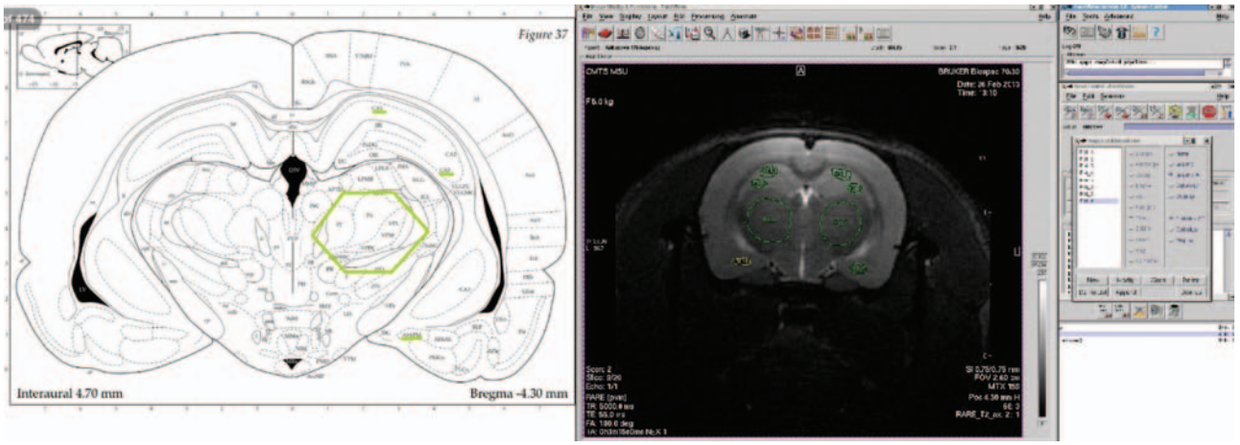

Fig. 2. Left—a scheme of brain structures [21], right—an interface of ParaVision 5.0 with highlighted structures: thalamus, amygdale and CA1 and CA3 regions of hippocampus. The slice is on a level of −4.30 mm from bregma.

cerebellum and ending at the olfactory bulbs. The distance between each cutting plane was 1 mm, with a thickness of 0.5 mm per slice.

The following areas were selected for study: the CA1 and CA3 regions of the hippocampus, thalamus (posterior thalamic nuclear group + parafascicular thalamic nucleus + ventral posterolateral thalamic VPA nucleus + ventral posteromedial thalamic nucleus + ventral posterior thalamic nucleus), striatum (caudate putamen), the lateral part of the globus pallidus (lateral globus pallidus) and the amygdale (amygdalohippocampal area).

Statistical processing of the results was performed using the statistical software Statistica 8.0. Comparisons were made by ϕ Fisher and the Mann-Whitney test. Differences were considered significant when $P < 0.05$ and $P < 0.01$. The results are expressed as means ± SEM.

RESULTS

Pregnancy Outcomes

The total number of pregnant females was $n = 4$ in the control group, $n = 9$ in the "VPA" group, $n = 4$ in the "PTZ" group, and $n = 3$ in the "VPA + PTZ" group. Pregnancy was considered a failure if the female did not give birth at all, gave birth to fewer infants than in the control group, or showed abnormal maternal behaviour; unfavourable pregnancy outcomes were observed in 0% of the control group, 87.5% of the VPA group, 50% of the "PTZ" group, and 100% of the "VPA + PTZ" group.

The average number of infants born was 11 in the control group, 10 in the "VPA" group, 9 in the "PTZ" group, and 3 in the "VPA + PTZ" group.

The gender composition of the offspring was also monitored. Majority of males were born to females treated with VPA (66.67% males out of a litter), whereas in the "PTZ" group males accounted for 53.85%, in the other two groups the percentage of males was almost identical: control 45.95%, "VPA+PTZ" group 44.44%.

MRI Investigation

MRI is a non-invasive and radiation-free tool to visualize the structure and function of the brain [20]. Visual examination of the MRI images showed increased T2 signal in the ventricles in the group without PTZ but injected with VPA, which because of the presence of liquor looked almost white. Strengthening and increasing of the light zone indicated expansion of the ventricles. Five out of 9 rats in the VPA group showed this pathology, but no such pathology was observed in the other groups. Figure 3 shows four similar brain slices from the rats of group "VPA," which illustrate the abovementioned pathology, and a fifth case is shown below in Figure 4 in comparison to the control: the upper row of images belongs to the

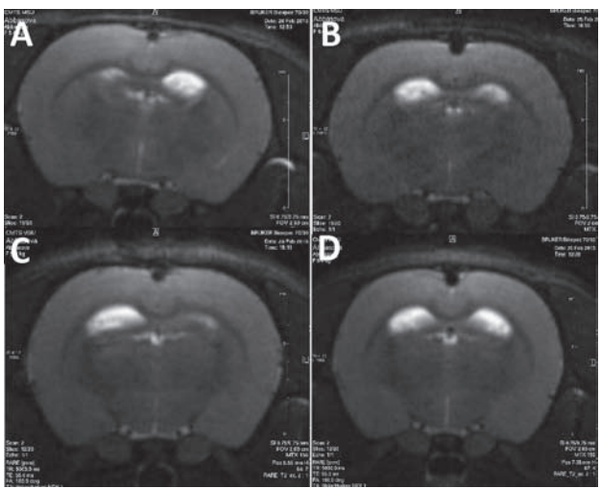

Fig. 3. Tomographic sections of the rat brain from a group of four rats treated with VPA, imaged in T2 RARE mode. Slices at about −1, 40 mm/−1.60 mm from the bregma.

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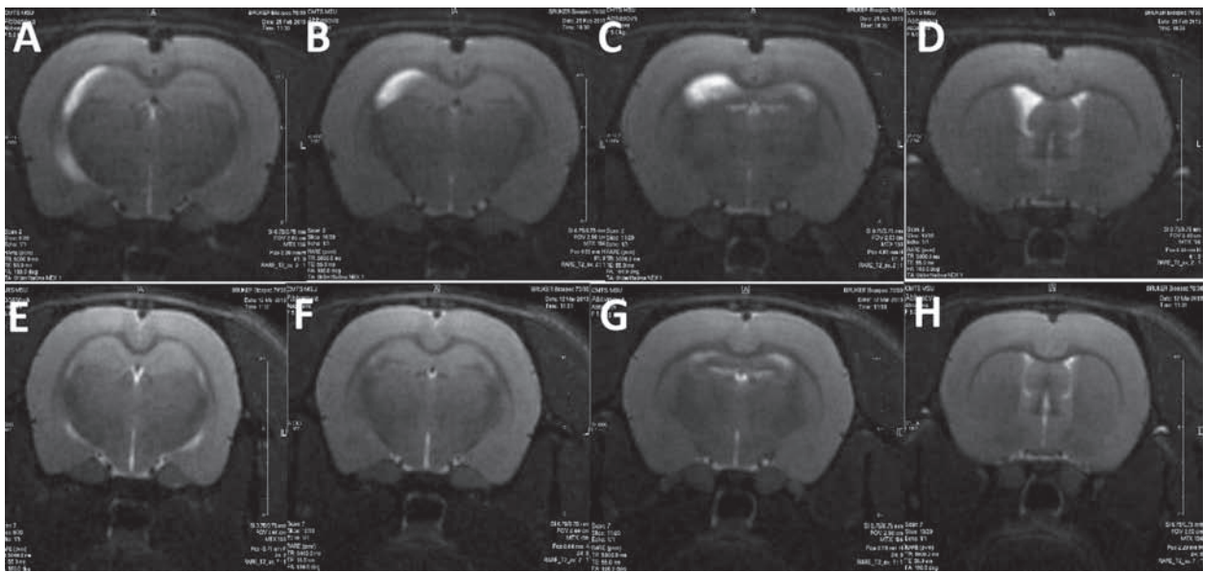

Fig. 4. (A)–(D) Tomographic images of rat brain from the VPA-treated group, imaged in T2 RARE mode. (E)–(H) Tomographic images from control group. Sections −3.80 mm/−0, 40 mm from the bregma.

"VPA" group (A)–(D), and the lower row to the control group (E)–(H).

T2 Maps

Increased signal on T2-weighted images is associated with various neurodegenerative processes, so by analysing this kind of data it was possible to assess whether there were any differences in the structure of brain tissue from animals in the experimental and control groups. The study was conducted on important structural limbic systems, the thalamus and basal ganglia. Increased T2 signal was observed in PTZ as well as in the VPA group, but was only statistically valid ($P < 0.05$) for the latter. T2 signal was amplified in the CA1 and CA3 regions of the hippocampus in the right hemisphere of 7.4% and 13.2% of T2 signal in msec, respectively, in the right half of the thalamus by 5.7% for the amygdale (left—6.7%, right—7.5%) and the striatum (3.3% and 6.8%) in both hemispheres (Fig. 5).

When comparing the VPA + PTZ and PTZ groups the difference between the T2 signal value was 4.5% (Fig. 6).

The PTZ and VPA groups differed in T2 signal by—2.8% in the right hemisphere CA1, 3.8% in the right amygdale and 3.0% in the right striatum (Fig. 7).

Thus, the administration of VPA during the first 7 days of prenatal development had a negative impact on the subsequent development of brain structures, causing an asymmetric change involving neurodegeneration of the hippocampus, expansion of the ventricles and changes in tissue structure in the CA1 and CA3 fields of the hippocampus, amygdale, striatum and thalamus. The strongest effect was found in the hippocampal region.

Volumetric Analysis

A reduction in the size of the hippocampal region, which is responsible for the formation of memory, emotions and

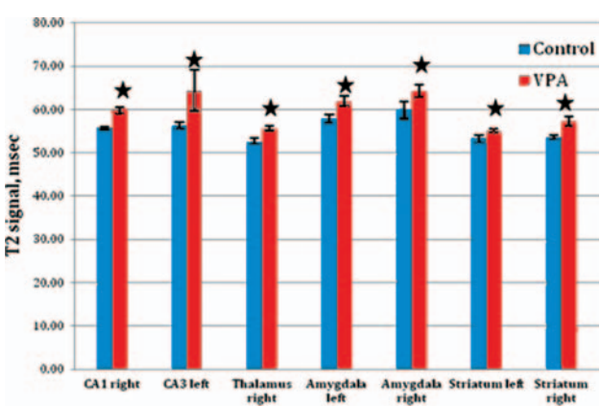

Fig. 5. Increase in T2 signal in the VPA group compared with the control group, $n(\text{control}) = 9$, $n(\text{VPA}) = 9$, $P < 0.05$. Data shown as mean ± sem.

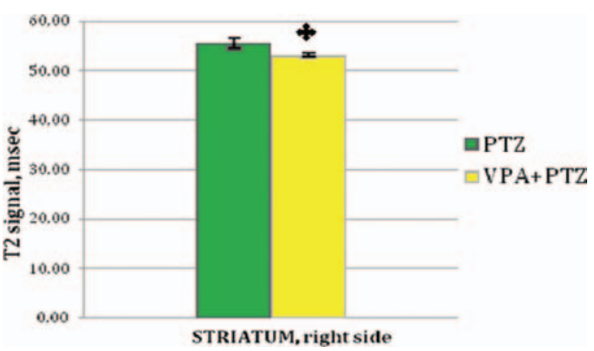

Fig. 6. T2 signal in PTZ group compared to the VPA + PTZ group, $n(\text{VPA}) = 9$, $n(\text{VPA} + \text{PTZ}) = 9$, $P < 0.05$ Data shown as mean ± sem.

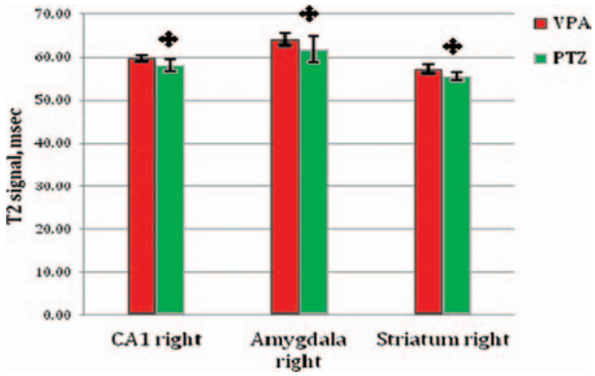

Fig. 7. T2 signal in PTZ compared to VPA group, $n(\text{PTZ}) = 9$, $n(\text{VPA}) = 9$. $P < 0.05$. Data shown as mean ± sem.

spatial orientation, is often observed in epileptic disorders. It is not surprising that we obtained significantly meaningful data about the reduction in the volume of the structure of the brain in the "PTZ" group—by 20.2% for the left hemisphere and 18.3% for the right. However, comparable degeneration of the hippocampus was observed in the VPA group, by 21.1% and 14.2%, again showing asymmetry of action. The "VPA + PTZ" group manifested no sum of the effects of PTZ and VPA (as it may be expected), and the possible neuroprotective effects of VPA in group VPA + PTZ, which were not observed when VPA was administered alone without PTZ: the volume of the hippocampus was reduced by 13.5–13.6% in both hemispheres.

Significant differences in change in hippocampal volume were found between the PTZ and PTZ + VPA groups.

Thus, all three factors had a negative impact on the development of brain structures when embryos were exposed in the first 7 days of prenatal development. The most influential factor was VPA, followed by PTZ, and then by the interaction between VPA and PTZ. The most sensitive structure studied was the hippocampus.

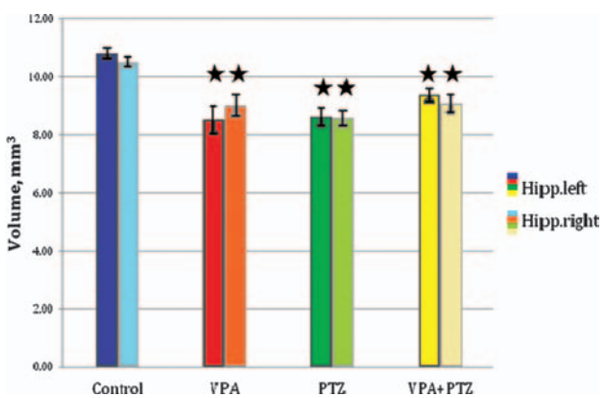

Fig. 8. Change in hippocampal volume of the right and left brain hemispheres for the control group compared with those in the treatment groups, n of each group is 9 (36 in total) $P < 0.05$. Data shown as mean ± sem.

DISCUSSION

Sodium valproate affects the outcome of pregnancy, as reflected by the abortion of embryos (for the 87.8% females from the VPA group and for the all females from group VPA + PTZ), and changes in maternal behaviour: females receiving valproate during the first 7 days of pregnancy did not feed their offspring and did not build a nest. This can be attributed to the fact that the normal process of implantation, in which the primary trophoblast cells trigger fibroblast cells in the uterus to differentiate into decidual cells [22], which produce extracellular matrix proteins such as laminin, collagen IV, vimentin, fibronectin and hyaluronan [23, 24], was disrupted. According to some histological studies, the endoderm and ectoderm layers were not differentiated, laminin was dispersed irregularly, and no collagen IV or vimentin expression was observed in embryonic rats exposed to valproate in utero [25].

We also showed a decrease in hippocampal volume in all treatment groups. This structure is responsible for memory, objective and subjective views of the world through the processing of external signals, and verbal memory, and reductions in its volume lead to memory loss and reduced verbal memory, which are signs of valproate syndrome and autism. This reduction is due to the neurodegenerative effect of PTZ on the brain [26–29]. The release of cytochrome-c and caspase-3 expression in the cytosol has been shown as a result of PTZ administration [30]. As shown herein, VPA acts in two ways: in a protective fashion when administered before PTZ and, conversely, in a detrimental fashion when used alone. The mechanisms underlying these manifestations remain unclear, and both aspects are currently under further investigation.

The neuroprotective action of valproate, when administered either before or after a seizure [31–35] enhances neurotrophic function through astrocytes [36] and neurogenesis [37] in the hippocampal region and the dentate gyrus [38, 39]. Valproic acid affects cell survival via the following factors: cyclic adenosine monophosphate (cAMP) responsive element binding protein (CREB), brain-derived neurotrophic factor (BDNF), bcl-2, and mitogen-activated protein (MAP) kinases [39–42].

VPA alters the development of organisms in general [43, 44], e.g. it inhibits histone deacetylases (HDAC) [45] and modifies processes of DNA methylation which correlate with DNA decondensation; it leads to increased expression of various genes, including proteins involved in apoptotic pathways (TRAIL/Apo2L and FAS/FAS-ligand, caspase-8) and proteins responsible for cell survival (heat shock proteins HSP90 and HSP70, p53, BDNF, bcl-2, Akt and the path through the induction Akt/mTOR/p70S6K phosphatidylinositol 3-kinase (PI3K) [46] suppress the transcription of cfos and c-jun genes [47]). In addition, it has been discovered that HDAC3 VPA leads to STAT3 phosphorylation, which in turn leads to the transcriptional activation of many genes involved in the immune response, oncogenesis, cell cycle control, development,

cell adhesion, and differentiation [46, 48]. VPA also alters the transcription of the gene Hoxa1, defects in which may lead to autism [49].

The reduction in T2 signal in the amygdale, a structure responsible for the formation of emotions, anxiety and fear, is consistent with manifestations in autistic children exposed to VPA in utero.

The reduction in T2 signal in the basal ganglia may affect the condition of the entire extrapyramidal system, including the striatum and globus pallidus. As a result, we can expect changes in learning ability and memory function, as observed in children exposed to VPA prenatally, which may be related to autism-like syndrome.

We have shown that Valproic acid in utero affects rats' brain more dramatically than their mother's seizure itself, altering the vitally important brain structures such as hippocampus, thalamus, striatum, the lateral part of the globus pallidus and the amygdale. This data may explain the correlation between autistic-like syndrome and epilepsy. However, to study the effects of VPA on cognitive function [50, 51] at different stages of pregnancy, on different models and in different tests and to create more links between MRI researches and behaviour is still important.

Conflict of Interest

There is no conflict of interest.

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