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Intrauterine valproate exposure is associated with alterations in hippocampal cell numbers and folate metabolism in a rat model of valproate teratogenicity

Alexander Semmler[a,*], Christian Frisch[b], Christiane Bleul[c], Desiree Smith[d], Laurent Bigler[e], Jean-Christophe Prost[e], Henk Blom[f], Michael Linnebank[g]

[a] Swiss Epilepsy Center, Clinic Lengg and Department of Neurology, University Hospital Zurich, Zurich, Switzerland
[b] University Bonn, Department of Epileptology, Germany
[c] Translational Genomics Research Institute, Phoenix, USA
[d] VU University Medical Center, Department of Internal Medicine and Metabolic Unit, Amsterdam, The Netherlands
[e] University of Zurich, Department of Chemistry, Switzerland
[f] University Medical Centre Freiburg, Laboratory of Clinical Biochemistry and Metabolism, Department of General Pediatrics, Adolescent Medicine and Neonatology, Germany
[g] Helios-Klinik Hagenbrock-Ambrock, Hagen, Germany

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ABSTRACT

Purpose: Valproate is one of the most commonly used anticonvulsive drugs. Despite its significant benefits, the teratogenicity of valproate is a relevant problem in the treatment of women of childbearing age. In addition to major congenital malformations, such as neural tube defects, reduced intelligence and attention after intrauterine valproate exposure are reported. Until now the mechanisms of teratogenicity of VPA are poorly understood and concepts how to reduce valproate teratogenicity are lacking.
Methods: In a rat model of valproate teratogenicity we examined hippocampal cell structure in 4 week old animals with a stereological approach. As potential mechanisms of VPA teratogenicity we examined histone acetylation by western blotting and metabolites of the folate metabolism as well as global DNA methylation by tandem mass spectrometry in the brain and liver tissue of newborn pups (p0).
Results: We found an increase in the number of neurons in the hippocampal areas CA1/2 ($p = 0.018$) and CA3 ($p = 0.022$), as well as a decreased number of astrocytes in CA1/2 ($p = 0.004$) and CA3 ($p = 0.003$) after intrauterine VPA exposure, as a possible indication of altered cell differentiation during intrauterine VPA exposure. Valproate exposure was also associated with an increase in 5-methyl-tetrahydrofolate (THF) ($p = 0.002$) and a decrease in 5-10-methenyl-THF in the brain of newborn pups, as well as a reduced homocysteine plasma level ($p < 0.001$). The described changes in hippocampal cell numbers and folate metabolism were only significant after high-dose intrauterine VPA exposure indicating a dose-dependent effect.
VPA exposure was not associated with changes in histone acetylation or global DNA methylation in brain tissue in newborn pups.
Conclusion: This study shows that intrauterine VPA exposure is associated with changes in hippocampal cell numbers in the CA1/2 and CA3 region and in folate metabolism.

1. Introduction

Valproate (VPA) is used in the treatment of epilepsy and psychiatric diseases worldwide and is one of the most used anticonvulsive drugs. Its considerable benefit is limited by toxicity and teratogenicity. The teratogenicity of VPA is a relevant problem in the treatment of women at childbearing age, and particularly high malformation rates are recorded for dosages of 1500 mg per day or greater. Neural tube, cardiac, facial, skeletal, and other defects are reported [1,2]. In addition to that, children exposed to VPA in utero, show a significant decrease in measures of intelligence and attention [3–6]. Therefore, intake of valproate during pregnancy is not recommended, especially in higher

* Corresponding author at: Swiss Epilepsy Center, Clinic Lengg and Department of Neurology, University Hospital Zurich, Bleuerstrasse 60, 8008 Zurich, Switzerland
E-mail address: alexander.semmler@kliniklengg.ch (A. Semmler).

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dosages. However, some women need to maintain the use of VPA during pregnancy for continued seizure control. Until now systematic data on the mechanisms of teratogenicity of VPA are lacking. Therefore it is unclear how to control or decrease the risk of intrauterine VPA exposure.

Consistent with its broad clinical effectiveness valproate has multiple cellular mechanisms of action which also could be responsible for its teratogenicity. In this study we aimed at investigating three possible mechanisms of VPA teratogenicity: interruption of folate metabolism [7,8], and two potential epigenetic changes, DNA hypomethylation [9], and inhibition of histone deacetylase (HDAC) activity [10,11].

It is already established that treatment with valproate is associated with lowered serum folate levels in adult epilepsy patients [12]. Moreover, intrauterine disturbance of folate metabolism is well known to impair brain development and lead to cognitive impairment [13,14]. In mice, folic acid pretreatment in pregnant mice significantly reduced VPA-induced neural tube defects in their offspring [15]. Therefore, disruption of folate metabolism might be a possible mechanism of valproate teratogenicity [16,17].

Folic acid metabolism also plays a critical role in the methylation of DNA [18]. We have recently shown that intrauterine VPA exposure is associated with alterations of two important metabolites of folate metabolism *S*-adenosyl-methionine (SAM) and its demethylated residue *S*-adenosyl- homocysteine (SAH) in the liver and brain tissue after intrauterine VPA exposure [8]. The SAM/SAH ratio represents the cellular methylation capacity [19]. In addition to that it is already shown that inhibition of intrauterine folate metabolism is associated with DNA hypomethylation and neural tube defects [20,21]. We therefore examined global DNA methylation in the brain tissue of pups after intrauterine VPA exposure.

Histones serve as epigenetic regulators of gene expression. Histone deacetylases are enzymes that remove acetyl groups from histones and thus alter their interaction with DNA [22,23]. In vitro and cell culture experiments have demonstrated that VPA can directly inhibit HDAC activity and cause hyperacetylation of histones [10,11], a mechanism probably relevant during intrauterine development.

We recently established a rat model of valproate teratogenicity by chronic delivery of VPA to dams via drinking water, thereby avoiding very high peak VPA doses achieved by repetitive VPA injections, which are related to severe side effects and limit interpretation of VPA teratogenic effects. In this model, we found a reduction of cortical volume induced by high VPA doses, while medium VPA doses induced slight hippocampal volume increases [24]. In the present study, we further investigated volumetric changes and changes in cell numbers by stereological examination of the hippocampus after intrauterine VPA exposure in juvenile animals 4 weeks of age.

2. Methods

Male and female parental Wistar rats aged 10 weeks (Charles River, Germany) were group-housed (4 per group) in the animal facility of the university hospital. The rat chow used in this study was Altromin Standard Diet, 1330 (Altromin, Soest, Germany). After 10 days of habituation, valproate (Sigma, Darmstadt) was diluted into the drinking water. Valproate metabolism and kinetics differ in humans and rodents with ten times higher metabolic rates in mice and rats [25]. Therefore, to reach a serum level between 50–100 μg/ml at least during the nightly active period without very high peak concentrations, VPA drinking water concentrations of 3.3, and 6.6 mg/ml were used. This results in daily valproate dosages in female pregnant rats of about 500, and 825 mg/kg [24]. Respective groups are termed MD (medium dose) group and HD (high dose) group in this manuscript. These dosages do not affect physical parameters as weight gain and food intake or behavior [24]. The higher dosage is close to the upper border of drug load applicable without induction of hepatic malfunction in the rat [26]. After seven days, drug application was interrupted, and one male animal was placed into each female group. Male rats were not exposed to VPA. 48 h later, males were taken out, females were single-housed, and valproate was applied again until delivery. Respective offspring was killed on postnatal day 0, as well as at the age of 4 and 8 weeks with a single overdose of thiopental. 1 ml blood was collected by cardiac puncture and stored in EDTA-containing vials. All animals were perfused transcardially with phosphate-buffered saline (PBS). Brain and liver were removed and immediately snap frozen for later analysis. Liver and brain tissue of pups (p0) and 8 week old animals (p56) were used for measurement of folate and homocysteine, DNA-methylation, and histone acetylation. The number of animals included in this biochemical analysis was n = 6 for each group (control, medium dose and high dose), and time point (p0 and p56) resulting in 36 animals totally. Male and female animals were included.

The animals used for brain histology were 4 weeks old (p28). The number of animals included for brain histology was n = 6 for each group (control, medium dose and high dose), resulting in 18 animals totally. Male and female animals were included. After perfusion with a PBS-Puffer, they were also perfused with a fixative containing paraformaldehyde 4%, and picric acid. Brains were removed and postfixated over night, cryoprotected in sucrose 30% over three days, frozen in carbon dioxide snow and stored at −80 °C.

The experiments were approved by the local Committee for Animal Experimentation. The ARRIVE guidelines and the principles of the Basel declaration including the 3R concept have been considered when planning the experiments.

2.1. Stereology

Serial, coronal 30 μm sections were cut and either mounted on slides or collected into a cryoprotectant to be stored at −30 °C until further processing.

For neural cell count, slides were Giemsa stained in accordance with the protocol provided by Iniguez et al. [27].

For astrocyte count slides were glial fibrillary acidic protein (GFAP)-stained. For GFAP-staining, the sections were transferred to tissue restrainers in a 12-well plate and washed three times for 10 min with Histo-tris buffered saline (TBS) (TBS; Triton-X100) and blocked for one hour at room temperature (Histo-TBS, 2.5% goat serum, 2.5% donkey serum). They were incubated over night at 4 °C with the primary antibody (guinea pig anti-GFAP, Acris Antibodies GmbH, BP5082 diluted 1:200 in 50% Histo-TBS and 50% blocking solution). After washing, they were incubated 2 h with the secondary antibody (anti-guinea pig CY2, Jackson, 706-255-148, diluted 1:200 in 50% Histo-TBS and 50% blocking solution) and washed again. Afterwards they were mounted with Hydromount (National Diagnostic) and stored protected from light at 4 °C.

2.2. Stereology measurement

The volume of CA 1/2, and CA3 was estimated on all of the sections using cavalieri estimator methods. Total cell numbers of these hippocampal sections were estimated by the optical fractionator method [28]. Definitions of the hippocampus region boundaries were similar to those illustrated in coronal section by Swanson. The primary data for both the Cavalieri estimator and the optical fractionator were obtained using Stereoinvestigator software (Microbrightfield Inc., Williston, VT, USA) and transferred to other computer programs for further analysis.