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Reduction of epileptiform activity by valproic acid in a mouse model of Alzheimer's disease is not long-lasting after treatment discontinuation

Ziyatdinova, Sofya

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Corresponding Author: Prof. Asla Pitkanen,

Corresponding Author's Institution: University of Eastern Finland

First Author: Sofya Ziyatdinova, MSc

Order of Authors: Sofya Ziyatdinova, MSc; Jayashree Viswanathan, PhD; Mikko Hiltunen, PhD, Professor; Heikki Tanila, MD, PhD, Professor; Asla Pitkanen, MD, PhD, Academy Professor

Abstract: Patients with Alzheimer's disease are at increased risk for unprovoked seizures and epilepsy compared with age-matched controls. Experimental evidence suggests that neuronal hyperexcitability and epilepsy can be triggered by amyloid-β (Aβ), the main component of amyloid plaques. Previous studies demonstrated that the administration of an anticonvulsant and histone deacetylase inhibitor, valproic acid, leads to a long-lasting reduction in Aβ levels. Here we used an APdE9 mouse model of Alzheimer's disease with overproduction of Aβ to assess whether treatment with valproic acid initiated immediately after epilepsy onset modifies the occurrence of epileptiform activity. We also analyzed whether the effect is long-lasting and associated with antiamyloidogenesis and histone modifications. Male APdE9 mice (15 wk old) received daily intraperitoneal injections of 30 mg/kg valproic acid for 1 wk. After a 3-wk wash-out, the same animals received injections of a higher dose of valproic acid (300 mg/kg) daily for 1 wk. Long-term video-electroencephalography monitoring was performed prior to, during, and after the treatments. Aβ and total histone H3 and H4 acetylation levels were measured at 1 month after the final valproic acid treatment. While 30 mg/kg valproic acid reduced spontaneous seizures in APdE9 mice (p<0.05, chi-square), epileptiform discharges were not reduced. Administration of 300 mg/kg valproic acid, however, reduced epileptiform discharges in APdE9 mice for at least 1 wk after treatment discontinuation (p<0.05, Wilcoxon test), but there was no consistent long-term effects on epileptiform activity after treatment withdrawal. Further, we found no long-lasting effect on Aβ levels (p>0.05, Mann-Whitney test), only a meager increase in global acetylation of histone H3 (p<0.05), and no effects on H4 acetylation (p>0.05). In conclusion, valproic acid treatment of APdE9 mice at the stage when amyloid plaques are beginning to develop and epileptiform activity is detected reduced the amount of epileptiform activity, but the effect disappeared after treatment discontinuation.
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• VPA suppressed EDs in APdE9 mouse model of AD
• VPA had no long-lasting effect on epileptiform activity in APdE9 mouse
• VPA had no long-lasting antiamyloidogenic effects in APdE9 mouse
• VPA triggered a mild long-lasting increase in histone 3 acetylation

*Highlights (for review)*
Reduction of Epileptiform Activity by Valproic Acid in a Mouse Model of Alzheimer’s Disease is not Long-lasting after Treatment Discontinuation

Sofya Ziyatdinova¹, Jayashree Viswanathan², Mikko Hiltunen², Heikki Tanila¹,³, Asla Pitkänen¹

¹A.I. Virtanen Institute, University of Eastern Finland, Kuopio, Finland
²Institute of Clinical Medicine-Neurology, University of Eastern Finland, Kuopio, Finland
³Department of Neurology, Kuopio University Hospital, Kuopio, Finland

Corresponding author: Asla Pitkänen, MD, PhD, Department of Neurobiology, A.I. Virtanen Institute for Molecular Sciences, University of Eastern Finland, PO Box 1627, FI-70211 Kuopio, Finland, Tel: +358-50-517 2091, Fax: +358-17-16 3025, E-mail: asla.pitkanen@uef.fi

Key words: Alzheimer’s disease - Amyloid-β - Epilepsy - Epileptiform discharge - Histone acetylation - Soluble amyloid-β oligomers - Valproic acid
Abstract

Patients with Alzheimer’s disease are at increased risk for unprovoked seizures and epilepsy compared with age-matched controls. Experimental evidence suggests that neuronal hyperexcitability and epilepsy can be triggered by amyloid-β (Aβ), the main component of amyloid plaques. Previous studies demonstrated that the administration of an anticonvulsant and histone deacetylase inhibitor, valproic acid, leads to a long-lasting reduction in Aβ levels. Here we used an APdE9 mouse model of Alzheimer’s disease with overproduction of Aβ to assess whether treatment with valproic acid initiated immediately after epilepsy onset modifies the occurrence of epileptiform activity. We also analyzed whether the effect is long-lasting and associated with antiamyloidogenesis and histone-modifications. Male APdE9 mice (15 wk old) received daily intraperitoneal injections of 30 mg/kg valproic acid for 1 wk. After a 3–wk wash-out, the same animals received injections of a higher dose of valproic acid (300 mg/kg) daily for 1 wk. Long-term video-electroencephalography monitoring was performed prior to, during, and after the treatments. Aβ and total histone H3 and H4 acetylation levels were measured at 1 month after the final valproic acid treatment. While 30 mg/kg valproic acid reduced spontaneous seizures in APdE9 mice (p<0.05, chi-square), epileptiform discharges were not reduced. Administration of 300 mg/kg valproic acid, however, reduced epileptiform discharges in APdE9 mice for at least 1 wk after treatment discontinuation (p<0.05, Wilcoxon test), but there was no consistent long-term effects on epileptiform activity after treatment withdrawal. Further, we found no long-lasting effect on Aβ levels (p>0.05, Mann-Whitney test), only a meager increase in global acetylation of histone H3 (p<0.05), and no effects on H4 acetylation (p>0.05). In conclusion, valproic acid treatment of APdE9 mice at the stage when amyloid plaques are beginning to develop and epileptiform activity is detected reduced the amount of epileptiform activity, but the effect disappeared after treatment discontinuation.
Introduction

Patients with Alzheimer’s disease (AD) are at increased risk for unprovoked seizures and epilepsy as compared with age-matched controls (Amatniek et al., 2006; Bernardi et al., 2010; Friedman et al., 2012; Hauser et al., 1986; Pandis and Scarmeas, 2012). Seizures can occur at an early stage in both familial and sporadic AD (Vossel et al., 2013), as well as in advanced AD (Mendez et al., 1994; Romanelli et al., 1990). Seizures or even subclinical epileptiform activity are associated with cognitive decline, more rapid disease progression, and more severe neurodegeneration in AD patients (Aldenkamp and Arends, 2004; Hommet et al., 2008; Kleen et al., 2013; Volicer et al., 1995; Vossel et al., 2013). Moreover, a reduction in seizure frequency or severity during treatment with antiepileptic drugs (AEDs) results in cognitive improvement in some AD patients (Vossel et al., 2013). Taken together, these data suggest that rather than a mere co-occurrence, epileptogenesis and co-morbidogenesis could have shared molecular mechanisms which result in pathologies compromising the outcome in AD.

Epileptiform activity and seizures are observed in several transgenic mouse models of AD (Born et al., 2014; García-Cabrero et al., 2013; Minkeviciene et al., 2009; Palop et al., 2007). Experimental evidence suggests that neuronal hyperexcitability and epilepsy in AD can be triggered by amyloid-β (Aβ), the main component of amyloid plaques (Minkeviciene et al., 2009). The most harmful Aβ species are not those that aggregate in plaques, but rather the soluble oligomeric-protofibrillar Aβ species that accumulate in synapses and cause functional impairment (Cleary et al., 2005; Selkoe, 2002). The accumulation of Aβ species in inhibitory synapses could be the initial cause of AD-related hyperexcitability (Palop et al., 2007; Palop and Mucke, 2009; Verret et al., 2012). Along with compromising inhibitory synaptic transmission, Aβ
can also increase neuronal excitability, as previous studies demonstrated clusters of hyperactive neurons surrounding Aβ plaques in the cortex and hippocampus (Busche et al., 2012, 2008). Whereas high nanomolar concentrations of Aβ in brain slices suppress synaptic potentiation, low picomolar concentrations of Aβ enhance it (Puzzo et al., 2008). In addition, pre-incubation of brain slices with soluble profibrillar Aβ triggers neuronal hyperexcitability and reduces the resting membrane potential (Minkeviciene et al., 2009). Furthermore, in an APPswe/PS1dE9 mouse model of AD, the first seizures occur at the age of 3 to 4 months, when Aβ is still largely in a soluble form (Minkeviciene et al., 2009). These data suggest amyloidogenesis as a candidate mechanism for epileptogenesis in AD.

Qing et al. (2008) reported that valproic acid (VPA), a commonly used antiepileptic drug (AED), reduced the accumulation of Aβ and the number of amyloid plaques in APP23 (APPswe) and APP23/PS45 (presenilin-1 with G384A mutation) mouse models of AD. Notably, the effect on reducing plaque formation persisted for at least 2 months after treatment discontinuation (Qing et al., 2008). Our previous study with a 3-5 days drug administration paradigm showed that AEDs, including VPA reduced spontaneous seizures and epileptiform discharges (EDs) in APDdE9 mice (Ziyatdinova et al., 2011). To test whether VPA would have an anti-amyloidogenic effect also in APDdE9 mouse, and whether it would result in chronic reduction in epileptiform activity [suggesting antiepileptogenic (AEG) effect] in addition to acute AED effect, we administered VPA at the reported anti-amyloidogenic (30 mg/kg) as well as at anticonvulsive doses (300 mg/kg). Moreover, we continued the treatment for 1 wk, and followed the persistence of the effect on epileptiform activity beyond the known biological half-life of VPA. Since VPA is a known inhibitor of histone deacetylase [HDAC; Monti et al. (2009)], we also
assessed whether the putative long-term anti-amyloidogenic and AEG effects of VPA would be associated with a prolonged HDAC inhibition.

**Materials and Methods**

*Figure 1* shows the study design.

**Animals**

Male heterozygous APPswe/PS1dE9 (APdE9) mice were used in the study (n=18). At the time of electrode implantation, the mice were 12 to 13 wk old (weight 25 ± 2 g). APdE9 colony founders were obtained from D. Borchelt and J. Jankowsky (Johns Hopkins University, Baltimore, MD, USA). Mice were created by coinjection of chimeric mouse/human APPswe and human PS1-dE9 (deletion of exon 9) vectors controlled by independent mouse prion protein promoter elements. The two transgenes cointegrate and cosegregate as a single locus *(Jankowsky et al., 2004)*. The line was originally maintained in a C3HeJxC57BL/6J hybrid background. At the time of the present study, the mice had been backcrossed to C57BL/6J for 13 generations.

The mice were housed in individual cages in a controlled environment (constant temperature, 22 ± 1°C, humidity 50-60%, and lights on 07:00-19:00 h). Food and water were available *ad libitum*.

All animal procedures were performed in accordance with the guidelines of the European Community Council Directives 86/609/EEC and approved by the Animal Experiment Board in Finland (license ESAVI-2010-05581/Ym-23).
Electrode implantation

The occurrence of epileptiform activity was assessed based on long-term video-EEG monitoring (Ziyatdinova et al., 2011). For electrode implantation, the mice were deeply anesthetized with sodium pentobarbital (60 mg/kg, intraperitoneal [ip]). Two stainless steel screw electrodes (diameter 1 mm, Microbiotech/se AB, Sweden) were fixed bilaterally into the skull above the frontal association cortex [2.7 mm anterior to bregma, ± 2 mm lateral to bregma (Franklin and Paxinos, 2008)] and served as recording electrodes. Two other screw electrodes fixed bilaterally into the occipital bone above the cerebellum served as reference and ground electrodes (Fig. 2A). A miniature connector (Plastics One, Roanoke, VA, USA) was fixed to the screw and skull with acrylic cement (Selectaplus CN, Dentsply De Trey GmbH, Dreieich, Germany). The mice were allowed to recover for 9 ± 2 d before initiation of the video-EEG recording.

Administration of vehicle and antiepileptic drug

After baseline video-EEG recording, the mice were randomly divided into VPA (n=12) and vehicle (n=6) groups. To assess the effect of VPA on epileptiform activity and amyloidogenesis, 15-wk-old APdE9 mice received daily intraperitoneal injections of 30 mg/kg VPA for 1 wk (“Low dose” trial, see Fig. 1). After a 3-wk wash-out period, the same mice (now 20 wk old) were injected daily with 300 mg/kg VPA for 1 wk (“High dose” trial, see Fig. 1). For both experiments, VPA (valproic acid sodium salt, P4543, Sigma-Aldrich, Germany) was dissolved in 0.9% NaCl and injected (ip) in a volume of 5 ml/kg once per day (in the morning). Vehicle-treated animals (n=6) were injected with 0.9% NaCl (5 ml/kg, ip).
Video-electroencephalography monitoring

To investigate the occurrence of spontaneous epileptiform activity, all mice underwent a continuous (24/7) 1-wk baseline video-EEG monitoring before treatment initiation. The 1-wk low-dose VPA treatment (30 mg/kg) was then started and video-EEG recordings of the mice were obtained during the treatment period and for 1 wk after discontinuing treatment. Three weeks later, the high-dose VPA trial (300 mg/kg) was performed using a similar monitoring protocol (Fig. 1). To assess whether the effect of VPA was long-lasting (i.e., antiepileptogenic), additional 1-wk video-EEG monitoring was initiated 3 wk after discontinuing the high-dose treatment (Fig. 1).

Video-EEG monitoring was performed as described previously (Nissinen et al., 2000). Mice were housed individually in Plexiglas cages where they could move freely and were connected to a Nervus EEG Recording System (sampling rate 256 Hz, high-pass filter 0.5 Hz, low-pass filter 100 Hz; Nervus magnus 32/8 Amplifier [Taugagreining, Iceland]). In parallel with the EEG recording, video recordings were obtained for all mice 24 h/day with a video-camera (WV-BP330/GE, Panasonic, Japan) positioned in front of the cage. A wide-angle lens permitted simultaneous videotaping of up to eight animals. A WFL-II/LED15W infrared light (Videor Technical, GmbH, Germany) was used to obtain video recordings during lights-off (19:00-7:00).

Analysis of seizures and epileptiform discharges

Each EEG file was analyzed visually by scanning through the EEG recording on the computer screen. An electrographic spontaneous seizure was defined as a high-amplitude (>2x baseline) rhythmic discharge clearly representing an abnormal EEG pattern (repetitive spikes,
spikes-and-wave discharges, and slow waves) that lasted for ≥5 s (Fig. 2B). An electrographic epileptiform discharge (ED) was defined as rhythmic transients (≥1 s, but <5 s) containing spikes and uniform sharp-waves (Fig. 2C). The number of EDs per week was calculated for each animal for each experimental condition. The response to VPA was calculated as the [frequency of EDs on VPA treatment vs. baseline] x 100%. A "responder" was defined as a mouse with 50% or fewer EDs during therapy compared to its own baseline.

**Sampling of brain tissue**

Upon completion of the experiment (i.e., 4 wk after discontinuation of the 1-wk high-dose VPA treatment), mice were killed by decapitation, and their brains were removed from the skull and rinsed in ice-cold 0.9% NaCl for 1 min. The brains were split at the midline. The left hemisphere was dissected on ice into five tissue blocks: thalamus, hippocampus, frontal cortex, parietal cortex, and temporal cortex, which were snap-frozen in dry ice and stored at -70°C.

**Enzyme-linked immunosorbent assay for amyloid-β levels**

Brain tissue samples from the parietal cortex were weighed and homogenized in 10x volume of Dulbecco’s phosphate-buffered saline (PBS) (Sigma-Aldrich), containing complete inhibitor mixture (Roche Diagnostics, Germany). Samples were centrifuged at 45,000 rpm (Beckman Ultrafuge) for 2 h at 4°C. The supernatant was diluted at 1:2 and used to analyze soluble levels of Aβ42. The remaining pellet was resuspended in 8x of the original volume of 5 M guanidine-HCl/50 mM Tris-HCl, pH 8.0 and mixed on a shaker for 3 h at room temperature. Samples were then diluted at 1:25 with reaction buffer (Dulbecco’s PBS with 5% bovine serum
albumin, 0.03% Tween-20, supplemented with protease inhibitor cocktail) and centrifuged at 16,000x g for 20 min at 4°C. Decanted supernatant was further diluted 1:100 with dilution buffer. Diluted samples were then used to analyze insoluble Aβ42 species. The Aβ40 and Aβ42 levels were estimated using enzyme-linked immunosorbent assay (ELISA) kits (Invitrogen) in accordance with the manufacturer’s instructions. The Aβ40 and Aβ42 levels were standardized to brain tissue weight and expressed as picograms of Aβ per gram ± SEM.

**Assessment of H3 and H4 histone acetylation**

**Histone extraction.** Histone proteins were extracted from the frontal cortex of APdE9 mice that had been treated with saline (n=5) or with VPA (n=11), using the EpiQuik™ Total Histone Extraction Kit. The analysis was performed 1 month after discontinuing the high-dose treatment. Briefly, the tissue was homogenized using a Cell Crusher™ tissue pulverizer (Cellcrusher, Cork, Ireland). The homogenate was dissociated in prelysis buffer by passing through a 24G needle 10 times and centrifuged at 10,000 rpm for 1 min at +4°C. The pellet was resuspended in lysis buffer and incubated on ice for 30 min. The tissue was centrifuged at 12,000 rpm for 5 min, and the supernatant fraction was transferred to a new tube. An appropriate amount of Balance-dithiothreitol buffer was immediately added to the supernatant. The concentration of extracted histone protein was measured using a BCA Protein Assay Reagent kit (Pierce Chemical, Thermo Scientific, Rockford, IL).

**Analysis of total H3 and H4 acetylation.** Total histone H3 and H4 acetylation levels were measured using the EpiQuik™ Total Histone H3 Acetylation Detection Fast Kit (Fluorometric) and EpiQuik™ Total Histone H4 Acetylation Detection Fast Kit (Fluorometric) according to the
manufacturer's instructions (Epigentek, Brooklyn, NY, USA). Briefly, 50 to 200 ng histone protein was added to assay wells already coated with high-affinity anti-acetylated H3 or H4 antibodies. The wells were incubated with a labeled secondary antibody, followed by addition of the detection reagent. Fluorescence was measured using an Envision® multilabel reader (Perkin Elmer, Waltham, MA, USA) at an excitation of 530 nm and emission of 590 nm.

**Preliminary analysis of the effect of VPA on histone binding at promoters of genes involved in memory and synaptic plasticity.** As VPA had a barely detectable effect on H3 acetylation in chronic samples and no effect on H4 acetylation (see Results), we wanted to confirm the previous data showing that VPA has an effect on H3 and H4 acetylation. For this experiment, we assessed histone acetylation in adult male BL6alpha2 mice (Jackson Laboratories, Bar Harbor, ME, USA) treated with 200 mg/kg VPA or saline (n=3 in each group) twice daily for 3 days. Mice were killed for analysis at 18 h after administration of the last VPA dose.

The cortex was homogenized using the Cell Crusher™ tissue pulverizer. PBS supplemented with protease inhibitors (Roche; PBS+PI) was added to the crushed tissue and dissociated by passing through a 24G needle 10 times. The tissue was cross-linked by adding paraformaldehyde to a final concentration of 1% and incubating for 10 min at room temperature. The tissue was centrifuged at 1000x g for 5 min and the supernatant discarded. The tissue was resuspended in PBS+PI and centrifuged at 1000x g for 5 min at +4°C. The pellet was resuspended in Farnham Lysis Buffer (5 mM PIPES pH 8.0, 85 mM KCl, 0.5% NP40) supplemented with protease inhibitors and passed through a 24G needle 10 times for nuclear extraction. The tissue was centrifuged at 1000x g for 5 min at +4°C. The pellet was resuspended
in 400 µl RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate) supplemented with protease inhibitors and sonicated using the SONICS vibra cell VCX750 sonicator (Sonics & Materials Inc., Newton, CT, USA). The sheared chromatin was centrifuged at 12,500 x g for 5 min at +4°C.

The ChromataChIP™ Kit (Novus Biologicals, Littleton, CO, USA) was used for chromatin immunoprecipitation. Briefly, the sheared chromatin was incubated overnight at +4°C with 5 µg of the following antibodies from EMD Millipore (Billerica, MA, USA): AcH3, AcH4, AcH4K12, and HDAC2. The antibody complexes were precipitated using magnetic beads (Novus Biologicals) and the cross-linking was reversed. The DNA was purified and analyzed using quantitative polymerase chain reaction (qPCR) with the primers mentioned below. The signal was calculated relative to the input. Primers: RPL30 - ribosomal protein 30: 5’ – AGCAACCAACTACCGCAGAC and 5’ – TCTAGGCCCGACAGAGGATTG; BDNF - brain-derived neurotrophic factor: 5’ – GCGCGGAATTCTGATTCTGGTAAT and 5’ – GAGAGGGCTCCACGCTGCCTTGACG; CDK5 - cyclin dependent kinsase 5: 5’ – CGCAGCCTGTTGGACTTTGT and 5’ – GCGTTGCAGAGGAGGTGGTA; NR2A - N-methyl D-aspartate receptor subtype 2 isoform A: 5’ – TCGGCTTGGACTGATACGTG and 5’ – AGGATAGACTGCCCTGCAC; β-TUB - β-tubulin: 5’ – TCCAGGGATGAAGAATGAGG and 5’ – TGAGCACTGGTAGGGAGCTT.

**Statistical analysis**

Statistical analysis was performed using SPSS 14.0 (IBM Software, New York, NY, USA) and Microsoft Excel (Redmond, WA, USA). The difference in the number of mice with seizures in the VPA and vehicle groups was evaluated using the chi-square test. Change in the number of
EDs was assessed separately for saline and VPA treated groups using Wilcoxon signed-rank test. Intergroup differences in ED frequency and cortical Aβ levels were evaluated using the Mann-Whitney U-test. Also, chromatin immunoprecipitation, qPCR results, and total histone measurements were tested for statistical significance using the Mann-Whitney U-test. The correlation between number of spontaneous EDs during the last recording session and the level of Aβ or the H3 or H4 acetylation was investigated using Spearman Rank Correlation coefficient. A p value less than 0.05 was considered statistically significant.

**Results**

**Mortality**

One mouse died from a seizure on day 6 of wash-out after 30 mg/kg VPA treatment. It had experienced four seizures during the baseline and four during the VPA treatment, and had frequent high-amplitude spikes in interictal EEG. Another mouse (saline group) died between recordings 1 and 2, and had no documented seizures.

**Treatment with valproic acid reduced the occurrence of spontaneous seizures in APdE9 mice**

*Baseline 1.* Two of the 6 mice (33%) randomized to vehicle and 4 of 12 mice (33%) randomized to VPA treatment had spontaneous seizures during the 1-wk baseline video-EEG recording (p>0.05, chi-square test) that preceded the initiation of vehicle or low-dose VPA treatment (30 mg/kg/d). The seizure duration was 37 ± 21 s in the vehicle group and 29 ± 11 s in the VPA group (p>0.05, Mann-Whitney test). Among the mice that displayed seizures, the
number of seizures was $1.5 \pm 0.7$ per week (range, 1-2) in the vehicle group and $2 \pm 1.4$ per week (range, 1-4) per week in the VPA group.

**Low-dose VPA treatment (30 mg/kg).** Three of 6 mice (50%) in the vehicle group and 1 of 12 (8%) mice in the VPA group had spontaneous seizures during the 1-wk video-EEG monitoring during treatment ($p<0.05$, chi-square test). The antiepileptic effect of VPA disappeared during the 1-wk wash-out as 2 of 6 mice (33%) in the vehicle group and 2 of 12 (17%) mice in the VPA group had spontaneous seizures ($p>0.05$, chi-square test). The number of seizures observed during the monitoring period was too low for a more detailed evaluation of the drug effect.

**Baseline 2.** None of the 5 mice (0%) in the vehicle group and 3 of 11 mice (27%) in the VPA group had spontaneous seizures during the 2nd video-EEG recording ($p>0.05$, chi-square test). Seizure duration did not differ from that at baseline 1 ($p>0.05$, Wilcoxon test).

**High-dose VPA treatment (300 mg/kg).** None of the 5 mice in the vehicle group and none of the 11 mice in the VPA group had spontaneous seizures during treatment with the higher VPA dose. No seizures occurred during the 1-wk wash-out period. Monitoring at 4 wk post-treatment revealed that none of the 5 mice (0%) in the vehicle group and 2 of 11 mice (18%) in the VPA group had spontaneous seizures ($p>0.05$, chi-square test; Supplementary Table 1).

Treatment with valproic acid reduced the occurrence of spontaneous epileptiform discharges in APdE9 mice
**Baseline 1.** Four of the 6 (67%) mice in the saline group and 11 of 12 mice (92%) in the VPA group had spontaneous EDs (p>0.05; chi-square test Fig.3A). The mean number of EDs per week was 76 ± 150 (range 11 - 379, median 13) in the saline group and 27 ± 72 (range 3 - 253, median 4) in the VPA group (p=0.18, Mann-Whitney test).

**Low-dose VPA treatment (30 mg/kg).** During low-dose VPA treatment, none of 4 (0%) animals in the saline group and 2 of 11 (18%) animals in the VPA group were responders with EDs reduced to ≤50% that at baseline (p>0.05; chi-square test, Fig. 3B). The mean number of EDs per week was 107 ± 230 in the saline group (range 1 – 576, median 14) and 22 ± 52 (range 2 – 183, median 4) in the VPA group. The change in the number of EDs was nonsignificant in both the saline and VPA groups (p>0.24, Wilcoxon test).

During wash-out, 2 of 11 (18%) mice in the VPA group had ≥50% reduction in the number of EDs compared to baseline, but these animals differed from those who were responders during the VPA treatment. On the other hands, 5 of 11 (45%) mice treated with VPA had a higher than 50% increase in number of EDs compared to baseline (Fig. 3C). The mean number of EDs per week was 93 ± 194 (range 2 – 488, median 16) in the saline group and 22 ± 40 (range 2 – 143, median 10) in the VPA group.

**Baseline 2.** All 5 (100%) mice in the saline group and 10 of 11 mice (91%) in the VPA group had spontaneous EDs (Fig. 4A). The mean number of EDs per week was 103 ± 187 (range 2 - 437, median 20) in the saline group and 36 ± 81 (range 1 - 274, median 7) in the VPA group (p=0.15, Mann-Whitney U test). The frequency of EDs at baseline 2 was equal or more than that at baseline 1 in 4 of 5 (80%) mice in the saline group and in 8 of 10 (80%) mice in the VPA group.
with EDs at baseline. Compared with the 1-wk wash-out period after low-dose VPA treatment, the frequency of EDs moderately increased (to ≤150% that during the wash-out) in 3 of 5 mice with EDs at wash-out in the saline group. Interestingly, the number of EDs was reduced by 50% to 100% that during the wash-out in 3 of 10 mice in the VPA group, suggesting recovery after the increase in EDs during VPA withdrawal. It should be noted, however, that two mice had an increase in the frequency of EDs to ≥150% that during the wash-out.

**High-dose VPA treatment (300 mg/kg).** During treatment with a high-dose of VPA, 2 of 5 (40%) of mice in the saline group and 5 of 10 (50%) of mice in the VPA group had EDs reduced to ≤50% of that at baseline (p>0.05; chi-square test, Fig. 4B). The mean number of EDs per week was 73 ± 133 (range 1 – 310, median 8) in the saline group and 20 ± 47 (range 1 – 158, median 2) in the VPA group. The change in the number of EDs was significant in the VPA group (p=0.013, Wilcoxon test), but not in the saline group (p=0.14).

During the wash-out, 1 of 5 (20%) mice in the saline group had EDs reduced to ≤50% that at baseline. In the VPA group, 7 of 10 (70%) had EDs reduced to ≤50% that at baseline (p>0.05; chi-square test, Fig. 4C). The mean number of EDs per week was 63 ± 103 in the saline group (range 1 – 245, median 14) and 14 ± 32 (range 1 – 107, median 3) in the VPA group (p=0.12, Mann-Whitney U test). The change in the number of EDs compared to baseline 2 was significant in the VPA group (p=0.009, Wilcoxon test), but not in the saline group (p=0.3).

During the 4th week after discontinuation of the treatment, the EDs remained reduced (<50% of that at baseline) in only 3 of 10 (30%) of the VPA-treated animals. In 4 of 10 (40%) VPA-treated mice, the number of EDs was ≥100% that at baseline. In the saline group, 2 of 5
mice had the number of EDs increased to \( \geq 150\% \) of that at baseline in (Fig. 4D). The mean number of EDs per week was 97 ± 154 (range 5 – 367, median 23) in the saline group and 19 ± 22 (range 2 – 67, median 5) in the VPA group. The change in the number of EDs compared to baseline 2 was nonsignificant in both groups (p>0.9, Wilcoxon test).

During the entire follow-up, the number of EDs was higher in mice with spontaneous seizures (n=11) than in mice without seizures (n=7) under all conditions investigated, including baselines 1 and 2, low-dose or high-dose VPA treatment, and wash-out periods (p<0.05, Mann-Whitney U test). The number of EDs did not change with age, since in the saline group, there was no difference in the number of EDs at different time points, corresponding to different ages (repeated measure ANOVA, F (6, 24)=0.97, p=0.38).

Valproic acid administration had no long-lasting effects on cortical amyloid-β levels

Amyloid-β levels were measured in parietal cortex sampled at 1 month after discontinuation of the high-dose VPA treatment. An ELISA assay revealed no difference in the concentrations of soluble or insoluble Aβ42 between vehicle (n=5) and VPA-treated (n=11) APdE9 mice (p>0.05, Mann-Whitney U test). Soluble and insoluble Aβ levels did not differ between mice with (n=10) or without (n=6) spontaneous seizures. Aβ levels were not significantly different between responders (n=5) and non-responders (n=4) to 300 mg/kg VPA treatment (p>0.05, Mann-Whitney U test). Also, Aβ levels did not differ between mice that showed a long-term reduction in the frequency of EDs after VPA treatment (n=3) and those that did not (n=7) (p>0.05, Mann-Whitney U test).
Finally, the number of spontaneous EDs during the last recording session did not significantly correlate with the level of Aβ (p>0.05, Spearman test).

**Valproic acid triggered a mild long-lasting increase in H3 but not in H4 acetylation**

To assess the long-term epigenetic effects of VPA, we measured global acetylation of H3 and H4 in the frontal cortex of mice at 4 wk after discontinuation of the 1-wk high-dose VPA treatment. We found a mild increase in global H3 acetylation and no change in H4 acetylation in VPA-treated mice as compared to vehicle-treated mice (Fig. 5).

The number of spontaneous EDs during the last recording session did not significantly correlate with the H3 or H4 acetylation levels (p>0.05, Spearman test).

**Short-term valproic acid administration increases histone acetylation at promoters of genes involved in memory and plasticity**

To confirm the methodology used and previous data showing that VPA has an acute effect both on H3 and H4 acetylation, we assessed whether VPA-induced histone modifications would target genes involved in memory and plasticity. Chromatin immunoprecipitation revealed increased H3 acetylation at CDK5 and NR2A promoters (both p<0.05) and increased H4K12 acetylation at CDK5 and β-TUB promoters (both p<0.05) in the mouse cortex after treatment with VPA (200 mg/kg) for 3 d (Supplementary Figure 1). A tendency toward increased acetylation of histones H3 (at promoters of BDNF4 and β-TUB), H4 (at promoters of CDK5, BDNF4, NR2A and β-TUB), and H4K12 (at promoters of BDNF4 and NR2A) was also
observed. Binding of HDAC2 to the *CDK5* promoter was significantly increased (p<0.05). H4K12 acetylation at the promoter for the housekeeping gene *β-TUB* was also significantly increased (p<0.05).

**Discussion**

Recently the Working Group of the International League Against Epilepsy revised the terminology related to the term “epileptogenesis” and provided recommendations for conducting antiepileptogenesis studies (Pitkänen et al., 2013). Epileptogenesis refers to the development and extension of tissue capable of generating spontaneous seizures, resulting in (a) the development of an epileptic condition and/or (b) progression of epilepsy after the condition is established. Disease-modification has two components: antiepileptogenesis and co-morbidity modification. Antiepileptogenesis treatment can be given prior to or after epilepsy onset. When antiepileptogenesis treatment is administered prior to epilepsy onset, it prevents or delays the development of epilepsy. If seizures occur, they may be fewer in frequency, shorter, or of milder severity. When such a treatment is given after the diagnosis of epilepsy, it can alleviate seizure severity, prevent or reduce the progression of epilepsy, or change the seizures from drug-resistant to drug-sensitive. Cure is achieved when there is a complete and permanent reversal of epilepsy such that no seizures occur after treatment withdrawal.

The present study was designed to evaluate whether VPA has an antiepileptogenic effect in an AD mouse model that overproduces Aβ when treatment is started after epilepsy onset, and whether the effect is associated with antiamyloidogenic and histone-modifying
effects. We found that treatment with a clinically relevant dose of VPA reduced EDs. The antiepileptic effect was present for 1 wk after treatment discontinuation, but vanished thereafter. Treatment with VPA had a mild long-lasting effect on H3 acetylation but no long-lasting effects on Aβ levels.

VPA reduces epileptiform activity in APdE9 mice but the effect is not long-lasting after treatment discontinuation

Approximately 30% of 14-wk-old APdE9 mice had spontaneous seizures lasting for about 30 s, and 80% of mice showed EDs lasting for about 1 s. Thus, the cohort of APdE9 mice included in the study exhibited a similar epilepsy phenotype as those in our previous studies (Minkeviciene et al., 2009; Ziyatdinova et al., 2011). However, as in our previous study the seizure frequency was low, compromising the use of spontaneous seizures as an outcome measure. Therefore, we also investigated EDs as an outcome measure.

Experimental evidence has suggested that Aβ can trigger neuronal hyperexcitability and epileptiform activity (Minkeviciene et al., 2009; Palop et al., 2007; Palop and Mucke, 2009; Verret et al., 2012). Previous data have also shown that VPA has a long-lasting antiamyloidogenic effect both in vivo and in vitro (Qing et al., 2008; Su et al., 2004). Therefore, we expected that VPA treatment would reduce amyloidogenesis, and consequently, epileptiform activity in our AD mouse. Moreover, these effects would last beyond the elimination of the drug from the animal [about 9-18 h in mice, (Perucca, 2002)], indicating that VPA treatment would be antiepileptogenic (i.e., by reducing the amyloid pathology maintaining
seizure susceptibility) and not merely antiepileptic (i.e., by directly suppressing the neuronal excitability).

We initiated the treatment with 30 mg/kg VPA. There is no information about the ED$_{50}$ of VPA in AD mouse models. Thirty mg/kg of VPA is a low dose compared to the ED$_{50}$ value of VPA in the maximal electroshock seizure test in normal mice [~260 mg/kg, (Bialer et al., 2004)]. Even this dose, however, was shown to have antiamyloidogenic effects in APP23 and APP23/PS45 AD mouse models (Qing et al., 2008). Because soluble Aβ oligomers are thought to be the most harmful form of Aβ, we began the intervention at the age of 15 wk, when most of the Aβ is still in the soluble form in our mouse model (Garcia-Alloza et al., 2006). In the second treatment session started 1 month later, when Aβ is present in both soluble and insoluble forms, we administered 300 mg/kg VPA to increase our chance of achieving a long-term effect as the higher dose of VPA inhibits Aβ production for at least 15 h in the APP$_{V717F}$ mouse (Su et al., 2004).

Our results demonstrated that the lower dose of VPA (30 mg/kg/day) reduced seizures but not EDs in the APdE9 mouse. The antiepileptic effect disappeared after treatment discontinuation. As expected based on our previous study (Ziyatdinova et al., 2011), the higher dose of VPA (300 mg/kg/day) reduced EDs during the treatment, and importantly even during the 1-wk post-treatment follow-up. The EDs remained reduced to below 50% of that at baseline in 3 of 10 animals, even on the 4th wk post-VPA. Although the treatment effects should be assessed animal by animal rather than as group means (see Pitkänen et al., 2013), we cannot exclude the possibility that spontaneous fluctuations in the ED frequency of the 3 responders
contributed to this finding, as 4 of 10 mice showed a dramatically increased number of EDs during the same follow-up period. Taken together, we conclude that VPA had no long-lasting effects on epileptiform activity in APdE9 mice after treatment discontinuation.

**VPA treatment had no long-lasting effects on the levels of soluble and insoluble Aβ42 in the cortex**

While the level of soluble Aβ can change rapidly, insoluble Aβ (plaque-bound Aβ) is very stable and can be used to measure delayed antiamyloidogenic effects of VPA treatment (Lord et al., 2009). At 1 month after treatment discontinuation, we detected no difference in soluble or insoluble Aβ42 levels in the cortex between saline- and VPA-treated animals. This is inconsistent with an earlier report showing that amyloid plaques were reduced for at least 1 – 2 months after VPA treatment in APP23 mice (Qing et al., 2008). One possible explanation for the discrepancy between the studies could relate to the difference in the mouse models used. The APdE9 mouse used in the present study produces mainly parenchymal amyloid plaques comprising Aβ42 (Jankowsky et al., 2004), while the APP23 mouse produces mainly Aβ40 as well as substantial vascular amyloid (Calhoun et al., 1999). In addition, plaques appear at the age of 3 to 4 months in APdE9 mice, whereas plaques appeared at the age of 6 months in the APP23 mice (Qing et al., 2008). Qing and colleagues (2008) also studied a double-transgenic APP23/PS45 mouse that exhibits rapid production of amyloid plaques. However, they started the treatment already at 6 wk of age when the mice do not yet have amyloid plaques. It is well established that many drugs prevent amyloid production but few if any treatment is effective
against established plaques. It is also possible that the antiamyloidogenic effects of VPA in APdE9 mice are not long-lasting, but disappear within 1 month after treatment discontinuation. Whether the duration of the antiamyloidogenic effects of VPA depend on the mutation carried by the mouse or human remains to be further explored. Finally, the last VPA dose used may have been too high compared to the anti-amyloidogenic dose used by Qing and co-authors (30 mg/kg, single dose vs. 300/mg/kg for 1 wk). However, had the 30 mg/kg dose been anti-amyloidogenic, the effect should have persisted till the time of sacrifice of the mice in the present study.

**VPA administration increases histone acetylation at promoters of genes involved in memory and plasticity**

To assess the long-term effects of VPA-induced histone acetylation and its possible association with antiepileptogenesis, we measured the global H3 and H4 acetylation levels in the frontal cortex of mice at 4 wk after low- and high-dose VPA treatment. We found that global H3 acetylation was slightly increased, but H4 acetylation was at control level, suggesting a transient effect of VPA on histone acetylation as suggested previously (Halsall et al., 2012; Perisic et al., 2010).

Because VPA and its analogs are well-established HDAC inhibitors (Göttlicher et al., 2001), we wanted to (a) exclude the possibility of false negative findings in our chronic samples due to methodological reasons and (b) assess the effects of VPA treatment on the acetylation status of specific histones. Therefore, we prepared another cohort of mice and treated them...
with 200 mg/kg VPA for 3 d, a treatment protocol reported to affect histone deacetylation (Yu et al., 2013). We focused on the main histones H3 and H4, on lysine 12 of H4, which correlates with learning and memory (Peleg et al., 2010); HDAC2, which negatively regulates memory and plasticity (Gräff et al., 2012; Guan et al., 2009; Kimura et al., 2010; Oakley et al., 2006); and genes implicated in memory formation and synaptic plasticity namely, CDK5, BDNF4, and NR2A (Asztely et al., 2000; Desai et al., 1999; Fischer et al., 2002; Hollmann and Heinemann, 1994; Ohshima et al., 2005). We found increased H3, H4, and H4K12 acetylation at promoters of these genes after VPA treatment, which is the line with previous studies. Moreover, our finding of a significant increase in HDAC2 binding to the CDK5 promoter implies that some cellular mechanisms supersede HDAC inhibition in maintaining epigenetic markers at gene promoters. Histone acetylation at single genes may be regulated by gene-specific factors that might circumvent the actions of non-specific HDAC inhibitors, such as VPA on global histone hyperacetylation (Boudadi et al., 2013; Halsall et al., 2012; Van Lint et al., 1996).

To summarize, our study confirmed that 30 mg/kg VPA reduced spontaneous seizures but not EDs in an AD mouse model. Treatment with VPA at a dose of 300 mg/kg reduced EDs in AD mice for at least 1 wk after treatment discontinuation, but no consistent long-term ED reducing effect was detected at 4 wk after treatment withdrawal. Further, there were no long-term changes in Aβ levels and only a minor effect on H3 acetylation. Based on the outcome measures analyzed, VPA treatment had no long-term effects on epileptiform activity in the APdE9 mouse model of AD when started at the stage when the first amyloid plaques are beginning to develop and epileptiform activity has already developed.
Acknowledgments

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References


**Figure legends**

**Figure 1.** Study design. Timeline (in weeks) of the video-EEG monitoring prior to, during, and after low-dose (30 mg/kg dissolved in saline, ip, once a day) and high-dose (300 mg/kg, once a day) treatment with valproic acid (VPA) of APdE9 mice (n=12 in the beginning of the study). The control group of APdE9 mice (n=6) was treated with saline. An additional 1-wk video-EEG monitoring was performed at 3 wk after discontinuation of the high-dose VPA treatment to assess the long-lasting effects of VPA on seizures and epileptiform discharges (EDs). Thereafter, tissue was collected for histology and molecular assays.

**Figure 2.** EEG abnormalities in APdE9 mice. (A) A drawing showing the electrode locations. (B) A representative recording trace of a spontaneous seizure lasting 40 s (mouse #481). Arrows indicate the beginning and end of the seizure. (C) A representative example of an epileptiform discharge (ED, mouse #541). Arrows indicate the beginning and end of the ED. Abbreviations: CxL, left cortical channel; CxR, right cortical channel.

**Figure 3.** Effect of a lower dose of VPA (30 mg/kg/day) on epileptiform discharges (EDs). Panels on the left show data from the saline-treated and those on the right show data from VPA-treated animals. (A) Number of EDs per week at baseline (before treatment) in individual APdE9 mice in the saline group (left) and in the VPA group (right). Numbers underlined in red indicate mice with spontaneous seizures during the 1-wk video-EEG monitoring. (B) A change in the number of EDs per week in individual mice during the 1-wk saline treatment (left) or VPA 30
mg/kg treatment (right). Data are expressed as change (%) from the baseline (total number of EDs per week was used for the calculation). Pink bars show animals with an ED frequency ≥ 150% that at baseline, green bars show mice with ED frequency ≤ 50% that at baseline (responders), and grey bars indicate no change. (C) Change in number of EDs per week in individual mice during the 1-wk monitoring performed immediately after treatment discontinuation.

Figure 4. Effect of a higher dose of VPA (300 mg/kg/day) on epileptiform discharges (EDs). Panels on the left show data from the saline-treated and those on the right show data from VPA-treated animals. (A) Number of EDs per week at baseline (before treatment) in individual APdE9 mice in the saline group (left) and in the VPA group (right). Numbers underlined in red indicate mice with spontaneous seizures during the 1-wk video-EEG monitoring. (B) A change in the number of EDs per week in individual mice during the 1-wk saline treatment (left) or VPA 300 mg/kg treatment (right). Data are expressed as change (%) from the baseline (total number of EDs per week was used for the calculation). Pink bars show animals with the ED frequency ≥ 150% that at baseline, green bars show the mice with ED frequency ≤ 50% that at baseline (responders), and grey bars indicate no change. (C) Change in number of EDs per week in individual mice during the 1-wk monitoring performed immediately after treatment discontinuation. (D) Change in the number of EDs per week in individual mice at 3 wk post-treatment.
**Figure 5.** Histone acetylation at 4 wk after the discontinuation of VPA treatment. Graph shows total histone H3 and H4 acetylation in the frontal cortex of APdE9 mice treated with low and high doses of VPA (30 mg/kg VPA followed by 300 mg/kg 5 wk later, n=5-11). Note a slight increase in H3 acetylation (*p<0.05). Data are shown as mean ± SD.

**Supplement Figure 1.** Effects of VPA treatment on histone acetylation and HDAC2 binding at gene promoters. (A-D) Quantitative PCR results of chromatin immunoprecipitated with (A) Acetylated H3, (B) Acetylated H4, (C) Acetylated H4K12, and (D) HDAC2 at the promoters of genes involved in memory and synaptic plasticity, namely CDK5, BDNF4, NR2A, and the housekeeping gene β-TUB from the frontal cortex of mice treated with 200 mg/kg VPA twice a day for 3 d (n=3, *p<0.05). Data are shown as mean ± SD.
**Supplementary Table 1.** Frequency (number of seizures/1 wk) and mean duration (sec) of spontaneous seizures in APP/PS1 mice at different phases of the study.

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Abbreviations: n.d., not done; VPA, valproic acid.
Figure(s)
Click here to download high resolution image
Figure 5

[Bar chart showing normalized levels of total H3 acetylation and total H4 acetylation for Control and VPA conditions.]

- Control
- VPA