Valproate causes reduction of the excitatory amino acid aspartate in nerve terminals

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HIGHLIGHTS

• The nerve terminal levels of the excitatory amino acid aspartate are reduced by valproate treatment.
• Aspartate levels are reduced in both excitatory and inhibitory terminals.
• The aspartate level in inhibitory terminals is about twice of that in excitatory terminals.

ABSTRACT

Valproate is well established in the treatment of epilepsy and psychiatric disorders, yet the main mechanism of action remains to be determined. Here we show that valproate may reduce neurotransmission of the excitatory amino acid, aspartate. By electron microscopic immunogold cytochemistry we demonstrate a 63–68% reduction in the level of aspartate in excitatory nerve terminals at 30 min after an acute dose of valproate. The level of glutamate in the same terminals was unchanged by valproate treatment. In inhibitory terminals, valproate caused a 65% decrease in the aspartate level, whereas the GABA level was not significantly changed. In summary, the present study shows that valproate reduces the nerve terminal content of the excitatory neurotransmitter aspartate. This points to a new mechanism of action for valproate: reduced neuronal excitation through reduced aspartergic neurotransmission.

1. Introduction

Valproate is one of the most widely used drugs in neurology and psychiatry, and has been in the treatment of epilepsy for more than 40 years. Yet its main mechanism of action is still unclear. Valproate has a broad spectrum of effects against both generalised and partial seizures, as well as against bipolar disorders [37]. Several mechanisms have been proposed to explain the clinical action of valproate, including inhibition of voltage-gated sodium channels [30,39], inhibition of neuronal energy metabolism [20] and effects on neurotransmitter amino acids [31,32]. At therapeutically relevant concentrations, it is unclear if direct inhibition of voltage-gated sodium channels contributes to the clinical effect of valproate (for review, see Refs. [19,26]). Instead, increased GABAergic transmission is believed to be highly important both for the acute and long-term effects of valproate. It is well known that GABA accumulates in the brain after valproate treatment [1,10,25,28,29], and this increase in GABA is thought to occur selectively in GABAergic terminals [25].

Valproate also causes the brain levels of the excitatory transmitter aspartate to decrease [21,38]. The effect of valproate on the distribution of aspartate between different subcellular neuronal compartments, however, remains unknown. If valproate reduces aspartate levels in nerve terminals, the transmitter releasing compartment, this would suggest reduced excitatory synaptic activity and in turn point to an additional mechanism underlying the clinical action of valproate.

2. Materials and methods

Animal handling was in strict accordance with local and national ethical guidelines. Male Wistar rats (n = 3 in each group) were given an intraperitoneal (i.p.) injection of sodium valproate (400 mg/kg) or saline (0.9%). At 30 min after the injection, the rats were anaesthetised with pentobarbital (100 mg/kg, i.p.) and transcardially perfused with a mixture of 2.5% glutaraldehyde and 1% paraformaldehyde in phosphate buffer (pH 7.4). The brains were
gently removed, and the hippocampal CA3 region was dissected out and embedded in Lowicryl HM20 as previously described [2]. After embedding, ultrathin sections (80–100 nm) were cut and labelled with the 435 l-aspartate (1:300), 607 L-glutamate (1:3000) or 990 GABA (1:300) antisera. These antisera have been well characterised [4,11–13,17,18]. To avoid possible cross-reactivities, the glutamate, aspartate and GABA antisera were used with the addition of soluble complexes (0.2 mM) of glutaraldehyde/formaldehyde-treated l-aspartate plus glutamine, or l-asparagine, l-glutamate plus GABA, or l-glutamate, l-aspartate plus β-alanine, respectively.

As a specificity test, ultrathin test sections containing various amino acids conjugated to brain protein by form- and glutaraldehyde were labelled along with the tissue sections. These test systems showed that the primary antibodies only labelled the conjugate containing the amino acid against which the antibodies were raised (not shown). The primary antibodies were visualised with colloidal gold conjugated goat anti-rabbit IgG (British Biocell International; Cardiff, UK). The sections were studied in a Tecnai 12 electron microscope. To visualise aspartate and glutamate/GABA in the same nerve terminals, we performed double labelling experiments, in which the ultrathin sections were first treated with the aspar- tate antibodies and then with the glutamate or GABA antibodies. Between the first and the second step, the sections were subjected to formaldehyde vapour (80 °C, 1 h) to prevent interference between the sequential incubations [36]. Secondary antibodies coupled to 10 and 15 nm gold particles were used in the first and second step, respectively. To verify that the double labelling protocol produced the same results as does separate antibody labelling, we also performed one series of specific and glutamate single labellings. Electron micrographs of sections labelled for aspartate/glutamate were randomly taken from the CA3 stratum radiatum and, of the sections labelled for aspartate/GABA, from the granule- or pyramidal cell layer. The terminals to be included in the study were chosen at low magnification, where the 10 nm aspartate gold particles were not visible. All quantitative analyses were performed by a blinded observer (CM). The densities (number of gold particles/μm²) of aspartate and glutamate gold particles in excitatory nerve terminals, dendritic spines and stem dendrites, and the densities of aspartate and GABA gold particles in inhibitory terminals, were calculated as described [23]. In excitatory nerve terminals, gold particle densities were separately determined in the cytosol and mitochondria, while in the other tissue profiles gold particle densities were recorded in the cytosol. Background labelling over empty resin in each section was subtracted. Excitatory terminals were defined as those making asymmetric synapses with dendritic spines. Inhibitory terminals were defined as those making symmetric synapses with stem dendrites or granule cell bodies and containing GABA immunogold particles. The results were statistically evaluated by a non-parametric Mann–Whitney U-test, two tailed (SPSS). Data are given for two independent labelling experiments. For each experiment, sections from 3 controls and 3 valproate-treated hippocampi were analysed.

3. Results

To determine if valproate regulates the nerve terminal pool of aspartate, we used valproate-treated rats and saline-treated controls to quantify aspartate immunoreactivities in excitatory terminals, inhibitory terminals, dendritic spines and stem dendrites in the stratum radiatum of CA3 hippocampus. As observed previously in the CA1 stratum radiatum [11,17], we found that aspartate immunogold particles were located together with glutamate immunogold particles in excitatory terminals (Fig. 1). In response to valproate, aspartate levels were profoundly decreased in excitatory terminals (Fig. 2A vs. C). Quantitative analyses of two independent labelling experiments showed that, in excitatory terminals, valproate reduced the density of gold particles representing aspartate by 68% and 63% respectively (Fig. 2E), while the glutamate level was not significantly reduced (Fig. 2E). The quantitative data from both experiments are reported in the legend of Fig. 2. The density of aspartate immunogold particles in dendritic spines paralleled the changes detected in the opposing nerve terminals. Aspartate levels decreased from 29.5 ± 4.0 gold particles/μm² in the control group to 7.8 ± 2.2 gold particles/μm² in the valproate treated group (p < 0.05, Mann–Whitney U-test, two tails). Glutamate levels in dendritic spines were not altered by valproate (Fig. 2). The area of excitatory nerve terminals was not affected by valproate treatment (0.23 ± 0.04 μm² in the control group vs. 0.27 ± 0.01 μm² in the valproate treated group; average area ± SEM, n = 3 animals; p > 0.05 (Mann–Whitney U-test, two tails)). In stem dendrites, aspartate was significantly reduced by valproate treatment in one labelling experiment but not in the other (for quantitative values, see legend Fig. 2). The mitochondrial density of aspartate gold particles was 42% lower in valproate treated excitatory terminals than in control terminals, but this difference did not reach statistical significance (39.0 ± 7.1 vs. 22.6 ± 12.2 gold particles/μm²; average number of gold particles ± SEM, n = 3 animals; p > 0.05, Mann–Whitney U-test, two tails). The densities of glutamate gold particles were largely unchanged (79.7 ± 6.4 vs. 62.1 ± 20.4 gold particles/μm²; average number of gold particles ± SEM, n = 3 animals; p > 0.05, Mann–Whitney U-test, two tails).

As reported previously [13] we found that inhibitory nerve terminals contained strong aspartate labelling (Fig. 3A). Like in excitatory nerve terminals, valproate reduced the labelling for aspartate in inhibitory terminals (Fig. 3B). Immunogold quantification of two independent immunolabellings showed that, in these terminals, the density of aspartate gold particles was reduced by 65% in response to valproate treatment, while the GABA level was unchanged (Fig. 3C). The level of aspartate in inhibitory terminals was approximately twice as high as in excitatory terminals (Fig. 3C vs. Fig. 2E).

4. Discussion

The present study demonstrates that valproate causes a decrease in the nerve terminal pool of aspartate, suggesting that valproate affects the release of aspartate. Moreover, in excitatory
nerve terminals, only aspartate and not glutamate, was significantly reduced. The decreased nerve terminal levels of aspartate described in this study are likely to cause diminished aspartergic neurotransmission, as the degree of vesicular filling is dependent on the cytosolic concentration of neurotransmitter [41]. This is in line with previous studies showing that valproate selectively inhibits the release of aspartate in preference to glutamate [3,6]. Further supporting the notion that valproate decreases release of aspartate from terminals, aspartate and glutamate content in dendritic spines paralleled the levels in nerve terminals. Spines can take up aspartate from the synaptic cleft through EAAT3, which is a glutamate/aspartate transporter concentrated at the postsynaptic edge and perisynaptic sites in hippocampal spines [15]. This suggests that the amino acid levels in the spines to a large degree reflect uptake of amino acids released from the nerve terminals. Thus, at the synapse, reduced release of aspartate would lead to reduced aspartate content in spines.

Our finding that valproate caused reduced aspartate levels, is in agreement with previous data from the hippocampus [5,27]. Using valproate at doses of 200–400 mg/kg, the latter studies found that valproate reduced hippocampal aspartate levels by about 20–30%, while Johanssen et al. [19] and Kukino and Deguchi [21] found a ~45% drop in aspartate levels in the whole brain after valproate treatment (400 mg/kg).

In the present study, the decrease in aspartate levels were not accompanied by significant changes in glutamate and GABA, suggesting that valproate selectively regulates the release of aspartate over that of glutamate and GABA. These are important findings as

Fig. 2. Valproate reduces the level of the excitatory amino acid aspartate in glutamatergic nerve terminals. Electron micrographs showing aspartate (small gold particles) and glutamate (large gold particles) labelling of nerve terminals (ter) making asymmetric synapses with dendritic spines (sp) (A and C) and of stem dendrites (dend) (B and D) in the stratum radiatum of CA3 hippocampus from saline-treated controls (A and B) and valproate-treated rats (C and D). Scale bars = 100 nm. Quantitative assessment of aspartate and glutamate in excitatory terminals (E) and stem dendrites (F). The values indicated by the bar charts are mean number of gold particles/μm² ± SEM for one experiment of n = 3 rats (26–32 excitatory terminals; 9–15 stem dendrites per animal). *Aspartate values in valproate treated terminals (9.5 ± 2.9) were significantly lower than the values in control terminals (30.1 ± 2.5; p < 0.05, Mann–Whitney U-test, two tails (SPSS)). The glutamate values in control terminals (63.3 ± 10.9) and valproate treated terminals (47.2 ± 8.9), as well as aspartate and glutamate values in stem dendrites (16.0 ± 6.3 and 13.5 ± 3.7 in controls vs. 10.0 ± 5.4 and 15.3 ± 2.1 in valproate treated animals) were not significantly altered by valproate treatment (p > 0.05, Mann–Whitney U-test, two tails). The figure show data from one double immunolabelling, but a single labelling experiment, where aspartate and glutamate labelling was performed separately, gave similar results. Aspartate values in valproate treated terminals (10.0 ± 4.5) were significantly lower than the values in control terminals (27.0 ± 11.4; p < 0.05, Mann–Whitney U-test, two tails), while glutamate values in control terminals (47.9 ± 11.6) and valproate treated terminals (32.4 ± 4.3) were not significantly different (p > 0.05, Mann–Whitney U-test, two tails). In the latter experiment, aspartate labellings in stem dendrites was significantly reduced by valproate treatment (27.1 ± 11.8 in controls vs. 7.1 ± 6.0 in valproate treated brain; p < 0.05, Mann–Whitney U-test, two tails). Glutamate (15.4 ± 1.1 in controls and 13.3 ± 5.9 in valproate treated brain) labellings in stem dendrites was unchanged by the treatment (p > 0.05, Mann–Whitney U-test, two tails).
they substantiate previous results showing release of aspartate at excitatory [11,17] and inhibitory [13] synapses, and support the notion that the release of aspartate and other neuroactive amino acids could be regulated by different mechanisms [34]. Aspartate released into the extracellular space selectively activates the NMDA type of glutamate receptors [8]. These receptors are located at most excitatory synapses in the hippocampus [40], but also at inhibitory synapses [13]. The attenuated release of aspartate caused by valproate could therefore act to reduce NMDA receptor signalling and account for part of the antiepileptic or mood stabilising effect of valproate. Interestingly, electrophysiology experiments have demonstrated that valproate decreases NMDA receptor responses via a presynaptic mechanism [7,9]. Further supporting the idea that valproate targets aspartate release, valproate inhibits the increased aspartate release and seizures known to occur in epileptic El-mice [16]. Also, in pentylentetrazol kindled rats, aspartate release during seizures is reduced by valproate [24].

What is the mechanism for the valproate-induced decrease in nerve terminal aspartate? Valproate inhibits the intramitochondrial enzyme α-ketoglutarate dehydrogenase [19], resulting in reduced concentration of oxaloacetate and thereby of aspartate, which is formed from oxaloacetate via the aspartate aminotransferase reaction. It should be noted that the labelling density of aspartate in inhibitory terminals was approximately twice of that in excitatory terminals. This is in agreement with several studies showing higher aspartate levels in GABAergic neurons than in glutamatergic neurons [12,14], and probably have a metabolic explanation: one of the rate limiting enzymes of the oxidative metabolism is α-ketoglutarate dehydrogenase [22,33]. In GABAergic neurons, the GABA-shunt can circumvent the step catalysed by α-ketoglutarate, facilitating the flux through to oxaloacetate. The conversion of oxaloacetate to citrate, however, is dependent of acetyl-CoA formed by pyruvate dehydrogenase, whose capacity is as limited as that of α-ketoglutarate dehydrogenase [33]. Thus

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**Fig. 3.** The level of the excitatory amino acid aspartate in inhibitory nerve terminals is reduced after valproate treatment. Electron micrographs showing aspartate (small gold particles) and GABA (large gold particles) labelling in inhibitory nerve terminals (ter) on granule cell bodies (G) in the CA3 hippocampus from a saline treated (control) (A) and a valproate treated rat (B). Scale bars = 100 nm. Quantitative assessment of aspartate and GABA in inhibitory terminals (C). The values indicated by the bar charts are mean number of gold particles/μm² ± SEM in n = 3 rats (20–25 terminals from each animal were analysed). *Aspartate values in valproate treated terminals (24.5 ± 5.2) were significantly lower than the values in control terminals (63.7 ± 12.8; p < 0.05, Mann–Whitney U-test, two tails), while GABA values in control terminals (22.0 ± 6.40) and valproate treated terminals (28.0 ± 2.26) were not significantly different (p > 0.05, Mann–Whitney U-test, two tails). The data are from one set of immunolabelling, but another independent double labelling experiment gave similar results. Aspartate values in valproate treated terminals (21.4 ± 6.5) were significantly lower than the values in control terminals (62.3 ± 14.0; p > 0.05, Mann–Whitney U-test, two tails), while GABA values in control terminals (79.4 ± 6.3) and valproate treated terminals (72.3 ± 4.5) were not significantly different (p > 0.05, Mann–Whitney U-test, two tails).
oxaloacetate, and subsequently aspartate, accumulates in these neurons. Valproate treatment inhibits not only α-ketoglutarate, but also the GABA shunt enzyme, GABA-transaminase, preventing further accumulation of oxaloacetate and aspartate through both pathways.

In conclusion, we show that valproate treatment leads to a decrease in the nerve terminal content of the excitatory amino acid aspartate in hippocampus, suggesting that valproate acts through reducing NMDA receptor mediated excitatory signalling in the brain.

References