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Original research paper

Modulation of brain glutamate dehydrogenase as a tool for controlling seizures

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Glutamate (Glu) is a major excitatory neurotransmitter involved in epilepsy. Glu is synthesized by glutamate dehydrogenase (GDH, E.C. 1.4.1.3) and dysfunction of the enzymatic activity of GDH is associated with brain pathologies. The main goal of this work is to establish the role of GDH in the effects of antiepileptic drugs (AEDs) such as valproate (VALP), diazepam (DIAZ) and diphenylhydantoin (DPH) and its repercussions on oxygen consumption. Oxidative deamination of Glu and reductive amination of α-ketoglutarate (αK) in mice brain were investigated. Our results show that AEDs decrease GDH activity and oxygen consumption *in vitro*. In e*x vivo* experiments, AEDs increased GDH activity but decreased oxygen consumption during Glu oxidative deamination. VALP and DPH reversed the increase in reductive amination of αK caused by the chemoconvulsant pentylenetetrazol. These results suggest that AEDs act by modulating brain GDH activity, which in turn decreased oxygen consumption. GDH represents an important regulation point of neuronal excitability, and modulation of its activity represents a potential target for metabolic treatment of epilepsy and for the development of new AEDs.

Keywords: GDH, antiepileptics, oxygen consumption, GABA, glutamate

The main therapeutic indication of antiepileptic drugs (AEDs) is the management of seizures in epileptic patients. However, they are widely used for different psychiatric indications (1). However, pharmacological treatment improvements in terms of clinical outcomes have fallen short of expectations.

Glutamate (Glu) is a major excitatory neurotransmitter and is known to be the direct biosynthetic precursor of gamma amino-butyric acid (GABA). Due to its neurotoxic potential, Glu may be involved in the pathogenesis of epilepsy, since it seems to play a critical role in the initiation and spread of seizure activity (2).

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The considerable role in epilepsy of inexcitable elements of the central nervous system (3–6), urges us to consider it as a disease of energy metabolism rather than neuronal discharge, requiring metabolic control (*7,* 8). Thus, antiepileptic drugs that act on metabolic pathways are of great interest and should have been better studied (9). Considering epilepsy from this new perspective, Glu, which participates in the control of energy homeostasis, plays a dual role as it is both a signaling molecule and a metabolite at the border between carbohydrate and protein metabolisms.

Glutamate dehydrogenase (GDH, EC 1.4.1.3), a key enzyme in the metabolism of Glu, is a member of the family of enzymes that catalyze the oxidative deamination of L-Glu to α-ketoglutarate (αK) using NAD^+ and/or NADP as coenzymes (forward reaction, Fig. 1). GDH also catalyzes the reductive amination of αK to Glu (reverse reaction, Fig. 1). Disorders associated with GDH activity defects are restricted to the brain, suggesting that it is of particular importance in the biology of the central nervous system (CNS). Evidence in support of the participation of GDH in epilepsy includes: (*i*) the enzyme isolated from a patient with a variant form of multisystem atrophy displayed pronounced reduction of one of the GDH isoproteins (10); (*ii*) GDH activity is significantly decreased in the temporal cortex and hippocampus of patients with temporal lobe epilepsy (11); (*iii*) in patients with hyperinsulinism/hyperamonemia syndrome, generalized seizures may be related to effects of different GDH mutations in brain tissue (12, 13), some of them in the GTP binding site of GDH (14, 15); (*iv*) in active areas of the human epileptic cerebral cortex, GDH activity is increased (16); and (*v*) in focally epileptic human brain, GDH activity is increased (16). We have recently shown that the anticonvulsant and convulsant properties of some drugs are related to changes in GDH activity (17, 18).

In an attempt to explore the role of GDH in the epileptic network, we evaluated the effects of clinically relevant antiepileptic drugs on GDH activity and oxygen consumption in mouse brain using *in vitro* and *ex vivo* techniques. We hypothesize that if AEDs modify GDH activity, then this enzyme would represent a possible regulation point for the metabolic control of epilepsy.

Knowing that convulsant drugs modify GDH activity as well as oxygen consumption (18), it was also investigated if AEDs could reverse seizure effects on these parameters. With this aim, pentylenetetrazole, a non-competitive GABA antagonist specifically used in seizure assays as a method of assessing the excitability of CNS and GABA activity, was employed.

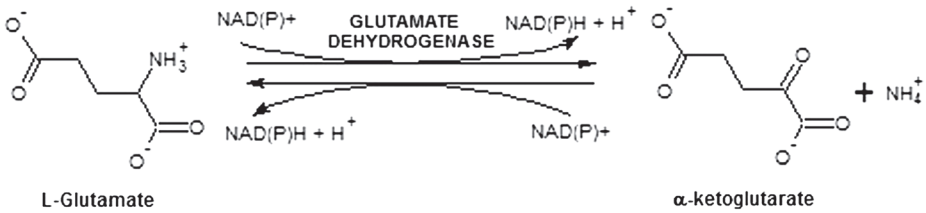

Fig. 1. Reaction catalyzed by glutamate dehydrogenase.

EXPERIMENTAL

Animals

Experiments were conducted in accordance with the Helsinki Guide for the Care and Use of Laboratory Animals, adopted and promulgated by the EU Directive 2010/63/EU for animal experiments, and were approved by the institutional committee of ethics of the Escuela Nacional de Ciencias Biológicas México.

Albino male mice with a mean mass of 25 g, fed *ad libitum* a stock laboratory diet (49.8 % carbohydrates, 23.5 % protein, 3.7 % fat, minerals, added vitamins and amino acids), were used for the experiments. The animals were maintained under a 12-h light-dark cycle.

Reagents and assays

Diazepam (DIAZ), valproic acid (VALP) and diphenylhydantoin (DPH) were purchased from Sigma Chemical Co. (USA).

Standard analytical grade laboratory reagents were obtained from Merck (Germany) or Sigma-Aldrich Chemical Co. (USA).

Protein concentrations were determined using the Lowry method.

Brain GDH activity determination

Tissue processing. – Animals were sacrificed and whole wet brains were removed. Homogenates (25 %, *m*/*V*) were prepared with a Glas-Col tissue homogenizer in a 5 % Triton X-100 solution. After centrifugation at 12,500 rpm for 45 min, GDH activity was determined in the supernatant using a spectrophotometric assay according to the Strecker method (19). Enzymatic activity assays were performed to assess Glu oxidative deamination (forward reaction) and reductive amination of αK (reverse reaction).

Oxidative deamination of glutamate. – The reaction medium contained 0.05 mol L^{-1} phosphate buffer (pH 7.6), 0.036 mol L^{-1} NAD and brain homogenate supernatant. The reaction was initiated by adding Glu (0.5 mol L^{-1}, pH 7.0), and the absorbance at 340 nm was measured each min for five min using a spectrophotometer.

Reductive amination of α-ketoglutarate. – The reaction medium contained 0.05 mol L^{-1} phosphate buffer (pH 7.6), 0.0113 mol L^{-1} NADH, 0.3 mol L^{-1} $(NH_4)_2SO_4$ and brain supernatants. The reaction was initiated by addition of αK (0.04 mol L^{-1}, pH 6.8) and the change in absorbance at 340 nm was measured each min for five min using a spectrophotometer.

Effects of selected antiepileptic drugs on brain GDH activity

In vitro *effects.* – Different concentrations of VALP, DPH and DIAZ (0.1 to 1 μmol L^{-1}) were added to the supernatant obtained from the mouse brain homogenates. GDH activity was determined at 10, 20, 30 and 60 min after the reaction was initiated.

Ex vivo *effects.* – Anticonvulsant doses of VALP (170 mg kg^{-1}), DPH (40 mg kg^{-1}) or DIAZ (10 mg kg^{-1}) were administered *i.p.* to groups of 5 animals. After 1 h of exposure, animals were sacrificed and brains were quickly removed and processed for GDH activity determination.

In a separate set of experiments, animals received AEDs at the dose indicated above. After 1 hour of exposure, convulsions were induced *via* administration of pentylenetetrazole (PTZ, 95 mg kg^{-1}). Twenty min later, animals were sacrificed and GDH activity was determined. The enzymatic activity was compared with that of animals that received only AEDs.

Oxygen consumption determination

Tissue processing. – Animals were sacrificed, brains were excised, and homogenates (25 %, *m/V*) were prepared in 0.25 mol L^{-1} sucrose. The homogenate samples were centrifuged at 3,500 rpm for 10 minutes. The supernatants were used to determine oxygen consumption using a polarographic method with a biological oxygen monitor YSI 5300 (Yellow Springs Instrumental Co., Inc. USA). Determinations were performed using the same reaction medium that was used for the determination of oxidative deamination of Glu and reductive amination of αK catalyzed by GDH.

Effects of antiepileptic drugs on oxygen consumption

In vitro *effects.* – To the supernatant obtained from untreated control animals, 10, 100 and 1,000 μmol L^{-1} of VALP, DPH and DIAZ were added and oxygen consumption was measured.

Ex vivo *effects.* – Groups of five animals received VALP (170 mg kg^{-1}), DPH (40 mg kg^{-1}) or DIAZ (10 mg kg^{-1}) *i.p.;* the animals were sacrificed after 1 h of exposure. The brains were quickly removed and processed for determination of oxygen consumption.

Statistical analysis

All results were normalized against the control and expressed as the mean ± SE values of at least four determinations ($n \geq 5$ animals per group). GDH activity was compared between groups using a one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test. Differences with p value ≤ of 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Despite its wide use, the mechanisms of VALP action are not fully understood. The results shown in Fig. 2 suggest that VALP may act by modifying GDH activity, since decreased activity was observed in Glu oxidative deamination as well as in reductive amination of αK *in vitro.*

Results described herein indicate that DPH also influences GDH activity by decreasing reductive amination of αK after 60 minutes of incubation (Fig. 2a) and Glu oxidative deamination (Fig. 2b). Considering that DPH prevents the spread of seizure activity by stabilizing the neuronal membrane, a minor effect of DPH on GDH activity *in vitro* was expected, since convulsive activity was not induced in these experiments.

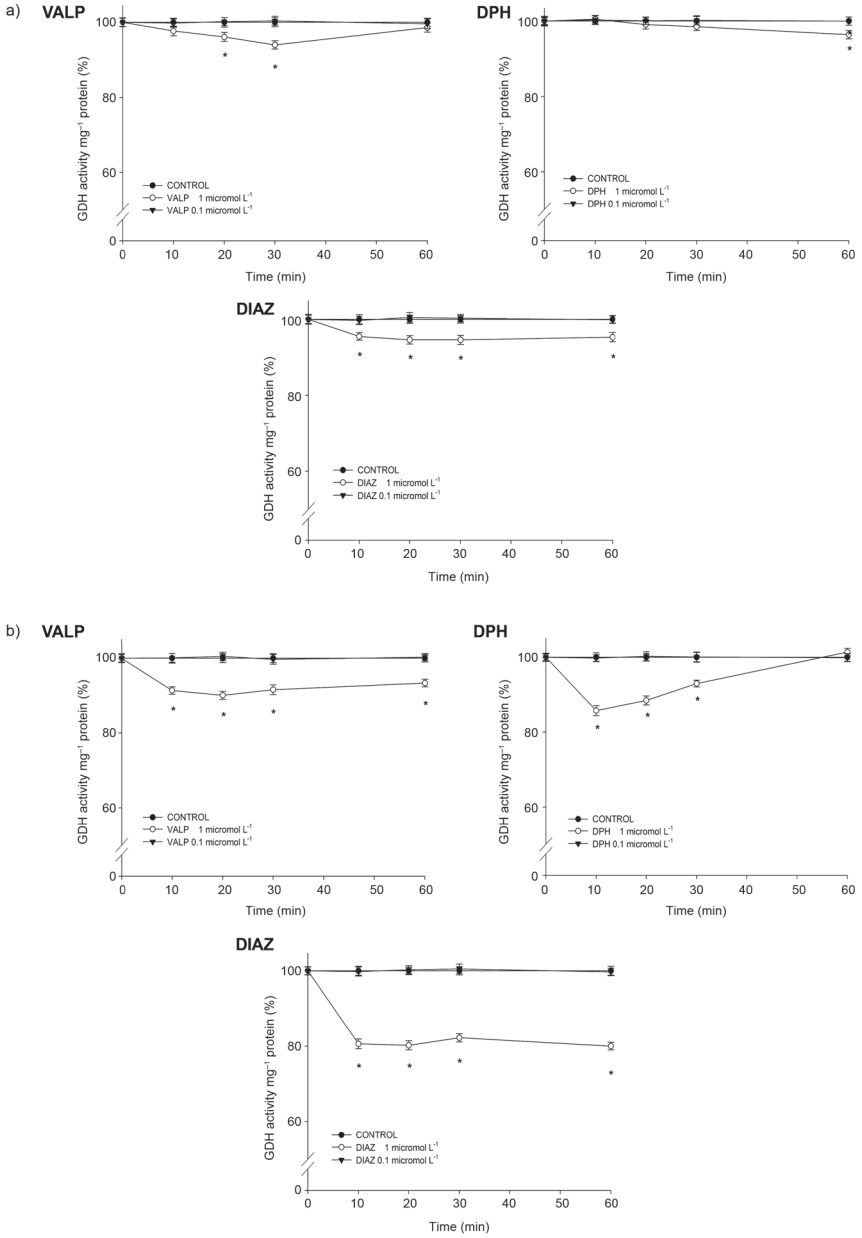

Fig. 2. *In vitro* effects of selected antiepileptic drugs on mouse brain glutamate dehydrogenase activity. Results are presented as the mean ± SEM ($n > 4$). An ANOVA test was applied, and the asterisk (*) indicates statistically different groups compared to the control ($p \leq 0.05$, Tukey's test). a) Reductive amination of α ketoglutarate (α ketoglutarate GDH L-glutamate); b) Oxidative deamination of L-glutamate (L-glutamate GDH α-ketoglutarate).

Results in Fig. 2 also demonstrated that decreased GDH activity observed after the treatment with DIAZ may be related to its antiepileptic properties.

When Glu was used as the substrate for oxidative deamination by GDH, the results demonstrated that all AEDs assayed decreased GDH activity (Fig. 2b). This is important because it would lead to accumulation of Glu in the system. This additional Glu may be used as substrate for GABA synthesis.

The AEDs tested affected GDH activity not only *in vitro* but also *ex vivo*, since the evaluation showed that exposure to AEDs slightly but significantly modified αK reductive amination. DPH increased GDH activity whereas VALP and DIAZ treatment decreased GDH activity (Fig. 3a). Contrary to the results observed *in vitro (*with the exception of DIAZ), AEDs increased the oxidative deamination of Glu, as shown in Fig. 3b. It is plausible that the main cause of the differences observed between the *in vitro* and *ex vivo* results are due to differences in brain energy requirements, *i.e.*, metabolic regulation influenced *ex vivo* GDH activity.

Previous results obtained with convulsant drugs (18) support the hypothesis that GDH participates in the initiation of seizures and that the inhibition of GDH activity may constitute one of the many possible mechanisms of action of these drugs. If these hypotheses are correct, an increase in Glu utilization by GDH would lead to decreased Glu levels, causing low neuronal excitability, which may contribute to the antiepileptic properties of VALP and DPH. Experiments analyzing the role of GDH in the protective effects induced by AEDs on seizures caused by PTZ exposure revealed several interesting findings that support these hypotheses (Fig. 4). Firstly, PTZ increased GDH activity by approximately 20 % when αK was used as a substrate (Fig. 4a), but the enhanced GDH activity induced by exposure to the convulsant was reversed by AEDs, with GDH activity returning to levels lower than those observed in the control group (Fig. 4a). Secondly, PTZ produced no effect when Glu was used as substrate, whereas VALP and DPH treatment increased Glu oxidative deamination in the presence of seizures induced by PTZ, whereas DIAZ had no effect on PTZ exposure (Fig. 4b).

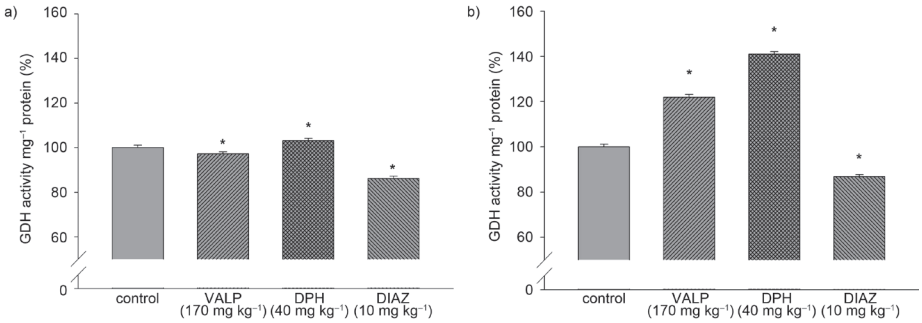

Fig. 3. *Ex vivo* effects of select antiepileptic drugs on mouse brain glutamate dehydrogenase activity. Results are presented as the mean ± SEM ($n > 4$). * denotes statistically significant differences compared to the control ($p \leq 0.05$ Student's *t* test). a) Reductive amination of α-ketoglutarate (α-k) α-ketoglutarate $\xrightarrow{\text{GDH}}$ L-glutamate; b) Oxidative deamination of L-glutamate (L-glu) L-glutamate $\xrightarrow{\text{GDH}}$ α-ketoglutarate).

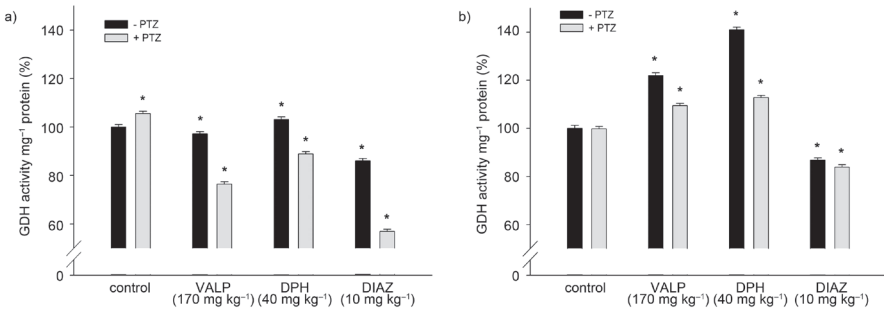

Fig. 4. Influence of pentylenetetrazole on *ex vivo* effects of selected antiepileptic drugs on mouse brain glutamate dehydrogenase activity. Results are presented as the mean ± SEM ($n > 4$) and were compared with those of animals that received only the corresponding antiepileptic. An ANOVA test was applied and an asterisk (*) is used to indicate statistically different groups compared to the control ($p \leq 0.05$, Tukey's test). a) Reductive amination of α-ketoglutarate (α-k) α-ketoglutarate $\xrightarrow{\text{GDH}}$ L-glutamate; b) Oxidative deamination of L-glutamate (L-glu) L-glutamate $\xrightarrow{\text{GDH}}$ α-ketoglutarate).

Reversion of PTZ effect on αK reductive amination caused by AEDs, with GDH activity to levels lower than those observed in the control group (20 to 30 %, Fig. 4a), would result in decreased Glu levels. On the other hand, though PTZ exposure decreased the oxidative deamination of Glu by 15 to 30 % in animals previously protected by VAL or DPH treatment, GDH activity was still increased when compared to the control group. Thus, AEDs treatment shifted the equilibrium of the GDH reaction to oxidative deamination of Glu, which reduced Glu levels and neuronal excitability.

The effect of AEDs on oxygen consumption in brain supernatants (highly enriched in mitochondria) was dependent on the substrate used. When αK reductive amination was catalyzed by GDH, all AEDs diminished oxygen consumption (Fig. 5a). During oxidative

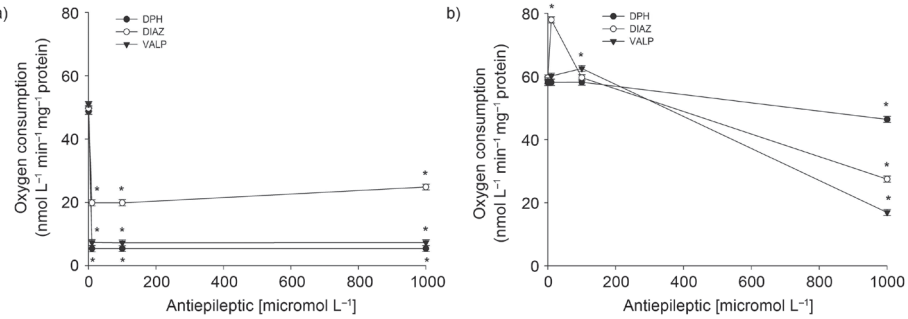

Fig. 5. *In vitro* effects of selected antiepileptic drugs on oxygen consumption. Results are presented as the mean ± SEM ($n > 4$). An ANOVA test was applied, and an asterisk (*) was used to indicate statistically different groups compared to the control ($p \leq 0.05$, Tukey's test). a) Reductive amination of α-ketoglutarate (α-k) α-ketoglutarate $\xrightarrow{\text{GDH}}$ L-glutamate; b) Oxidative deamination of L-glutamate (L-glu) L-glutamate $\xrightarrow{\text{GDH}}$ α-ketoglutarate).

deamination of Glu, oxygen consumption also decreased, but only in case of high concentrations (1 mmol L^{-1}) of AEDs, the highest concentration of AEDs tested *in vitro* (Fig. 5b).

The decreased oxygen consumption *in vitro* caused by AEDs when reductive amination of αK was catalyzed by GDH (Fig. 5a), indicates diminished brain metabolic activity. Because additional energy was not required under these conditions, generation of αK was unnecessary. This could explain the decrease of GDH activity observed when Glu was used as the substrate (Fig. 2b).

Ex vivo, with the exception of VALP, AEDs did not modify oxygen consumption when αK was used as substrate for GDH (Fig. 6a), but all of them decreased oxygen consumption when oxidative deamination of Glu was catalyzed by GDH (Fig. 6b). These results imply that αK generated by the high GDH activity under these conditions was not introduced to the TCA cycle because metabolic activity was not required.

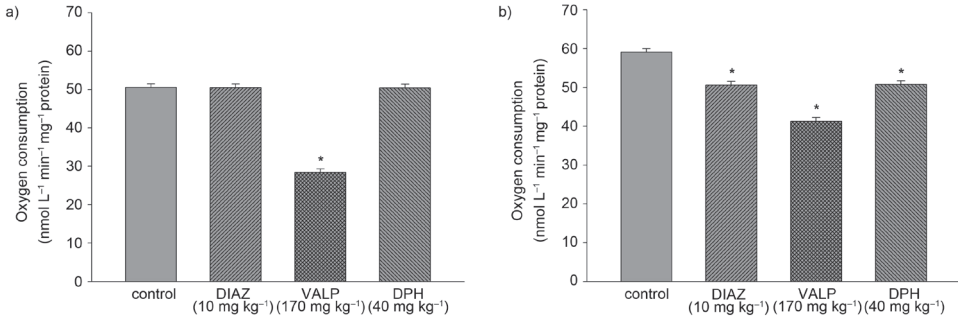

Fig. 6. *Ex vivo* effects of selected antiepileptic drugs on oxygen consumption. Results are presented as the mean ± SEM ($n > 4$). * denotes statistically significant differences compared to the control ($p \leq 0.05$, Student's *t* test). a) Reductive amination of α-ketoglutarate (α-k) α-ketoglutarate $\xrightarrow{\text{GDH}}$ L-glutamate; b) Oxidative deamination of L-glutamate (L-glu) L-glutamate $\xrightarrow{\text{GDH}}$ α-ketoglutarate).

In accordance with previous findings, our hypothesis that GDH participates in the mechanism of the initiation of seizures is supported by the data presented herein. In a previous work, we demonstrated that anticonvulsants such as pyridoxal phosphate, aminooxyacetic acid and hydroxylamine modify GDH activity *in vitro*, with apparent linkage to changes in oxygen consumption (17), just like antiepileptic drugs of broad clinical use tested herein. Described effects of anticonvulsants and antiepileptic drugs tested on GDH activity could decrease the Glu levels but also diminish oxygen consumption *ex vivo* when Glu oxidative deamination was catalyzed by GDH. It must be realized that effects of AEDs on GDH activity and oxygen consumption are opposite to those previously reported for chemoconvulsants such as pentylenetetrazole, thiosemicarbazide and bicuculline (18).

CONCLUSIONS

Overall, the results presented here suggest that VALP, DPH and DIAZ share a common and not yet described target: GDH. Treatment with these AEDs could modulate GDH activity, which induced concomitant changes in oxygen consumption. Clearly, GDH represents an important regulation point of brain Glu and GABA levels and thus contributes to the regulation of neuronal excitability. We propose a new mechanism for the metabolic treatment of epilepsy: modulation of GDH activity, as observed in mouse brain.

Abbreviations, acronyms, symbols. – AEDs – antiepileptic drugs, Glu – glutamate, CNS – central nervous system, αK – α-ketoglutarate, DIAZ – diazepam, NAD(H) – nicotinamide adenine dinucleotide, AEDS – diphenylhydantoin, PTZ – pentylenetetrazole, GABA – gamma amino-butyric acid, VALP – valproate, GDH – glutamate dehydrogenase, TCA – tricarboxylic acid cycle.

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