

Full Length Research Paper

Effect of convulsant drugs in GDH activity and oxygen consumption in mouse brain

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Abstract

Glutamate (GLU), the major excitatory neurotransmitter in central nervous system, participates in several pathologies such as epilepsy and also in intracellular signaling functions via its metabolism through glutamate dehydrogenase (GDH, E.C. 1.4.1.3.). Considering that GDH is a key enzyme for GLU biosynthesis and modulation and, an indirect γ -aminobutyric acid (GABA) source, here we studied the effect of some chemoconvulsants such as pentilene tetrazole (PTZ), thiosemicarbazide (TS) and bicuculline (BIC) on mouse brain GDH activity *in vitro* and *ex vivo*, to explore the possible participation of GDH in seizures mechanism. Convulsants influence in oxygen consumption *in vitro* and *ex vivo* was also investigated, since glutamate is a glucogenic amino acid and anoxia is a primary cause of convulsions. *In vitro*, all convulsants decreased GLU oxidative deamination and oxygen consumption when α Ketoglutarate (α K) reductive amination was catalyzed. *Ex vivo*, all convulsants increased α K reductive amination and oxygen consumption with this substrate. Results suggest that, during seizures, GDH increase GLU levels which induced changes in oxygen consumption. Changes on GDH activity may result from changes on GABA levels through T-GABA activity modulation and/or in energy requirements caused by convulsions, making GDH an important regulation point of GLU and indirectly of GABA levels, thus of neuronal excitability.

Keywords: GDH; Convulsant; Glutamate; GABA; Oxygen consumption.

INTRODUCTION

The importance of glutamate (GLU) in normal central nervous system (CNS) synaptic function, and the observation that increased amounts of GLU in the synaptic cleft lead to neurotoxicity has been well established (Choi et al., 1987). In addition, GLU participates in several CNS pathologies such as amyotrophic lateral sclerosis (Bruijn et al., 1997; Howland et al., 2002; Rothstein et al., 1995), Alzheimer's disease (Masliah et al., 2000; Noda et al., 1999), Parkinson's disease (Lievens et al., 2001a), Huntington's disease (Behrens et al., 2002; Lievens et al., 2001b), Glioma (Ye et al., 1999), stroke (Fukamachi et al., 2001; Namura et al., 2002; Rao et al., 2001a; Rao et al., 2001b) and

epilepsy (Sepkuty et al., 2002; Tanaka et al., 1997; Watase et al., 1998)

It is considered that GLU has a role in the initiation, propagation and maintenance of epileptic activity (Urbanska et al., 1998), potential therapeutic role for drugs affecting glutamatergic mechanisms in epilepsy has not yet been defined but this is an issue attracting interest. One major hypothesis links alteration in brain energy metabolism with the pathophysiologic mechanism underlying glutamate excitotoxicity associated with seizure disorders (Malthankar-Phatak et al., 2006). A critical enzyme in energy and neurotransmitter metabolism, which contributes to the alterations in the metabolic status leading to the excitotoxic effects of glutamate, is Glutamate Dehydrogenase (GDH, EC 1.4.1.3.) (Yu et al., 1982).

GDH catalyze the reversible deamination of L-glutamate to 2-oxoglutarate and ammonia with either

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NAD⁺ or NADP⁺ as coenzymes, it is a mitochondrial matrix enzyme limited to certain tissues in humans, including liver, kidney and brain. GDH activity is subjected to complex regulation by negative (GTP, palmitoyl-coenzyme A) and positive (ADP, leucine) allosteric effectors. Complex regulation allows GDH activity to be modulated by changes in energy state and substrate availability.

On the other hand, brain vulnerability to hypoxia, which is a well known cause of seizures, seems to be related with GLU. It has been proposed that brain sensitivity to ischemia is related to GLU N-methyl-D-aspartate (NMDA) receptors (Crepel et al., 2003), and GLU may be a substrate during acute ischemia in stroke (Rink et al., 2011). GDH represents a link between amino-acid and carbohydrate metabolism, since GLU is a ketogenic acid that may be oxidized through the Krebs Cycle. Thus, changes in GDH activity may be reflected as modifications in oxygen consumption.

In the recent past we have showed that GDH participates in the induced effect of anticonvulsants such as pyridoxal phosphate, aminooxyacetic acid and hydroxylamine, including changes in oxygen consumption (Vega Rasgado et al., 2012). In this work we explore the effect of chemically induced seizures on GDH activity in mice's brain *in vitro* as well as *ex vivo*, and its aftermaths on oxygen consumption, since it is considered that if GDH participates in seizures mechanism, then convulsions induced by different chemical agents would modify its enzymatic activity, which in turn would have repercussions on oxygen consumption.

METHODS

Animals

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

CD1 albino male mice with a mean weight of 25 g, fed *ad libitum* on a stock laboratory diet (49.8%carbohydrates, 23.5% protein, 3.7% fat, minerals and added vitamins and amino acids; w/w), were used for the experiments. The animals were maintained on a 12-h light-dark cycle.

Reagents

Pentylentetrazole (PTZ) and Bicuculline (BIC) were purchased from Sigma Chemical co. (USA). Thiosemicarbazide (TS) was obtained from JT Baker (USA).

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

Brain GDH Activity

Tissue Processing

Animals without treatment (*in vitro* assays) or after the treatment (*ex vivo* assays), were sacrificed and whole wet brains were removed. 25% (wt/vol) homogenates were prepared with a Glas-Col tissue homogenizer in a 5% Triton X-100 solution. After centrifugation (12,500 RPM/45 min.), GDH activity was determined in the supernatant using a spectrophotometric assay according to the Strecker method (Strecker, 1953). Enzymatic activity assays were performed for the GLU oxidative deamination (forward reaction) and reductive amination of α K (backward reaction).

Oxidative Deamination of Glutamate

Reaction medium was composed by phosphate buffer 0.05 M pH=7.6, NAD (0.036 M) and brain supernatant (see Tissue Processing). Reaction started by adding GLU (0.5 M pH=7.0).

Reductive Amination of α ketoglutarate

Reaction medium was composed by phosphate buffer 0.05 M pH=7.6, NADH (0.0113 M), NH₄SO₄ (0.3 M) and brain supernatant (see Tissue Processing). Reaction started by addition of α ketoglutarate (0.04 M pH=6.8).

In vitro Effect of Convulsant Drugs in Brain GDH Activity

Different concentrations of PTZ, TS and BIC (between 10 μ M and 1mM) were added to the supernatant obtained from brain homogenates. GDH activity was determined 10, 20, 30 and 60 min after reaction was started.

Ex vivo Effect of Convulsant Drugs in Brain GDH Activity

PTZ (90 mg/kg), TS (20 mg/kg) and BIC (3 mg/kg) were administrated IP (n=5). Once seizures appeared, animals were sacrificed and brains were quickly removed and processed for GDH activity determination as described in brain GDH activity.

Used dosages were selected based on our own experience as those that produce seizures but at the same time animals can be protected using different anticonvulsant drugs.

Brain Oxygen Consumption

Tissue Processing and Oxygen Consumption measurement

Animals were sacrificed, and once brains were excised, a

25% (wt/vol) homogenates in sucrose 0.25 M were prepared. Homogenates were centrifuged at 500g during 10 minutes. Supernatants were used to determine oxygen consumption by a polarographic method using a biological oxygen monitor YSI 5300. Determinations were performed using the same medium of reaction used for determination of oxidative deamination of GLU and reductive amination of α K catalyzed by GDH (see Oxidative Deamination of Glutamate and Reductive Amination of α ketoglutarate).

In vitro Effect of Convulsant Drugs in Oxygen Consumption

To the supernatant obtained from animals without treatment as described above were added concentrations of 10, 100 and 1000 μ M of PTZ, TS and BIC and oxygen consumption was measured.

Ex vivo Effect of Convulsant Drugs in Oxygen Consumption

Groups of five animals received PTZ (90 mg/kg), TS (20 mg/kg) and BIC (3 mg/kg) IP and once animals presented convulsions, they were sacrificed and brains were quickly removed and once processed oxygen consumption was determined.

GABA- α -oxoglutarate aminotransferase (GABA-T) activity.

Groups of five animals received PTZ (90 mg/kg), TS (20 mg/kg) and BIC (3 mg/kg). Once animals presented seizures, they were sacrificed and brains were quickly removed and 25% (wt/vol) homogenates in a 5% Triton X-100 solution were prepared. Homogenates were centrifuged at 12,500 RPM for 45 min. and supernatants were used as an enzymatic source in the GABA-T assay (Bergmeyer, 1983). GABA, α ketoglutaric acid, 0.15 M potassium phosphate buffer (pH=8) and tissue homogenates were incubated in 37°C for 30 min, followed by the addition of NADP⁺. The amount of NADPH generated in the brain tissue for 20 min measured by spectrophotometer at 340 nm as an activity of GABA-T.

Effect of Convulsant Drugs in GABA Levels.

Mice (groups of 5 animals each) received chemoconvulsants (PTZ 90 mg/kg, TS 20 mg/kg and BIC 3 mg/kg) and just after seizures appeared, they were sacrificed and brains removed in order to prepare homogenates in ethanol 80%. After centrifugation (500g,

5 min), and once precipitated was washed with ethanol 75%, lipids were extracted from supernatants with chloroform and GABA was determined in aqueous phase, employing an enzymatic method. A partially purified lyophilized from *Pseudomonas fluorescens*, containing both GABA-T and Succinic Semialdehyde Dehydrogenase (SSDH) was used, following reduction of NADP in the spectrophotometer.

Protein Levels

Protein concentration was also determined in supernatant by Lowry method (Lowry et al., 1951)

Statistical Analyses

All results, expressed as percentage with regard to the control from at least four determinations ($n \geq 4$). GDH activity was compared between groups using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons. A p value of 0.01 was considered as statistically significant.

RESULTS

Convulsant Drugs and GDH Activity In vitro

We investigated the effects of PTZ, TS and BIC on brain homogenate GDH activity *in vitro*.

Results figures 1 and 2, show that PTZ increased very slightly but significantly the reductive amination of α -ketoglutaric acid (α K) only at the concentration of 1 mM (Figure 1a), whereas at these same concentration, decreased GLU oxidative deamination about 8% (Figure 2a). Neither TS nor BIC changed substantially α K utilization (Figure 1b and 1c), but decreased GDH activity by about 20 and 35% respectively when GLU was the substrate (Figure 2b and 2c).

Convulsant Drugs and GDH Activity Ex vivo

Analyzing the possible participation of GDH in seizures, the effect of PTZ, TS and BIC on brain GDH activity was also investigated *ex vivo*. An increase in α K reductive amination catalyzed by GDH was found in all cases (Figure 3a). For GLU oxidative deamination could be considered that PTZ and TS did not affect GDH activity, but BIC decreased it 13.5% (Figure 3b).

Convulsant Drugs and Oxygen Consumption In vitro

As observed in GDH activity, *in vitro* effects of convulsant

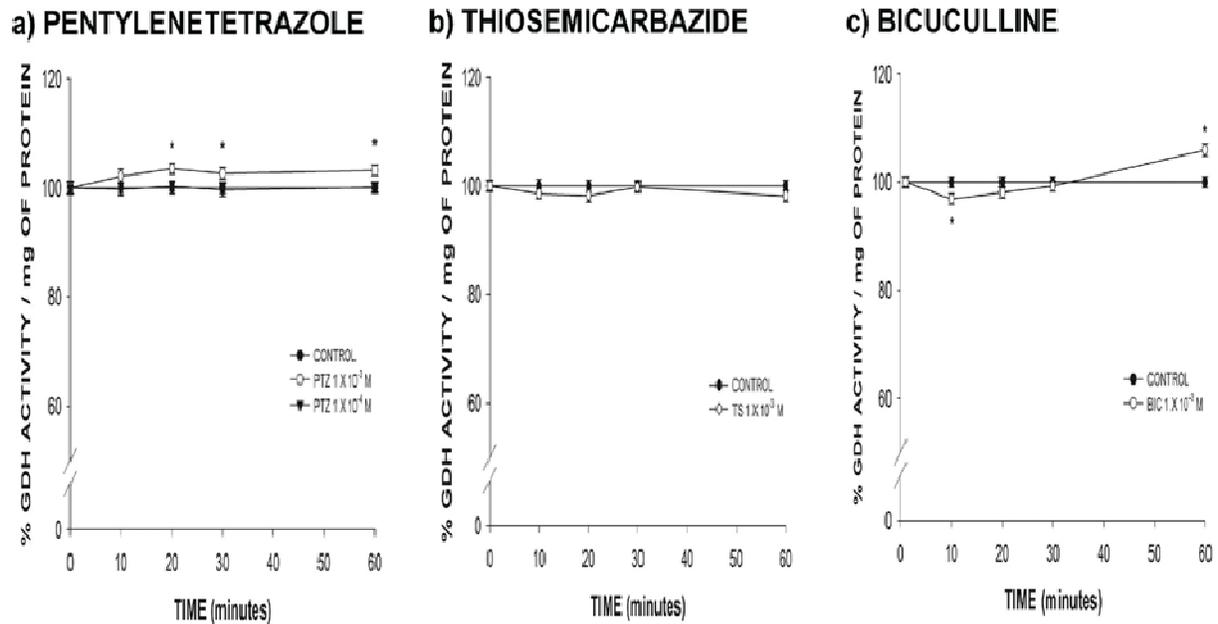


Figure 1. EFFECT IN VITRO OF SOME CONVULSANT DRUGS IN REDUCTIVE AMINATION OF α KETOGLUTARATE CATALYZED BY MOUSE BRAIN GLUTAMATE DEHYDROGENASE. Glutamate dehydrogenase activity (GDH) was determined in mouse brain homogenates (1:5 w:v on phosphates 0.05 M pH=7.6 buffer) with different concentrations of pentylenetetrazole (PTZ), thiosemicarbazide (TS) and bicuculline (BIC), using α -ketoglutaric acid as substrate, with a spectrophotometric method (see "Materials and Methods"). Results are means + SEM (n>4). ANOVA test was applied and * used to indicate statistically different groups as compared with the control ($p < 0.01$, Tuckey's test).

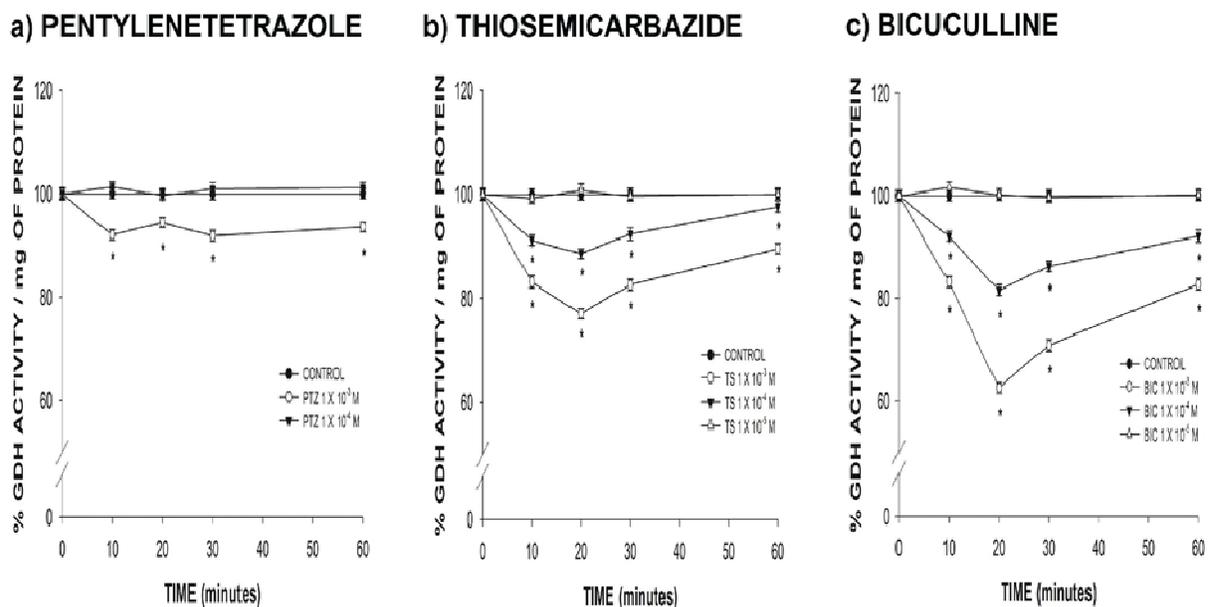


Figure 2. EFFECT IN VITRO OF SOME CONVULSANT DRUGS IN GLUTAMATE OXIDATIVE DEAMINATION CATALYZED BY MOUSE BRAIN GLUTAMATE DEHYDROGENASE. Glutamate dehydrogenase activity (GDH) was determined in mouse brain homogenates (1:5 w:v on phosphates 0.05 M pH=7.6 buffer) with different concentrations of pentylenetetrazole (PTZ), thiosemicarbazide (TS) and bicuculline (BIC), using glutamic acid as substrate, with a spectrophotometric method (see "Materials and Methods"). Results are means + SEM (n>4). ANOVA test was applied and * used to indicate statistically different groups as compared with the control ($p < 0.01$, Tuckey's test).

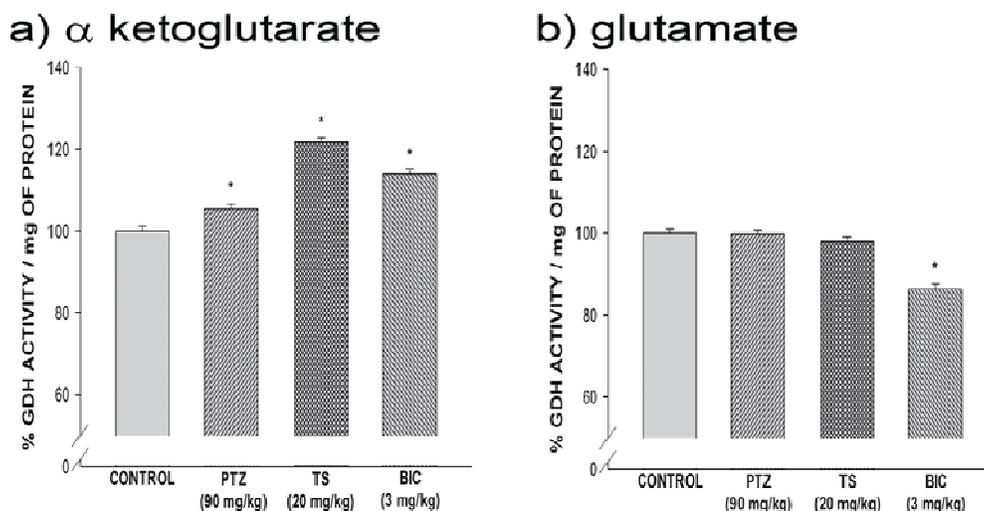


Figure 3. EFFECT EX VIVO OF SOME CONVULSANT DRUGS IN MOUSE BRAIN GLUTAMATE DEHYDROGENASE ACTIVITY. Animals received pentylenetetrazole (PTZ, 90 mg/Kg), thiosemicarbazide (TS, 20 mg/kg) and bicuculline (BIC, 3 mg/Kg). Once convulsants induced seizures, glutamate dehydrogenase activity (GDH) was determined in brain homogenates (1:5 w:v on phosphates 0.05 M pH=7.6 buffer), using α -ketoglutaric acid (a) and glutamate (b) as substrates, with a spectrophotometric method (see "Materials and Methods"). Results are means + SEM (n>4). * Statistically significant differences as compared with the control ($p < 0.01$, Student's t test).

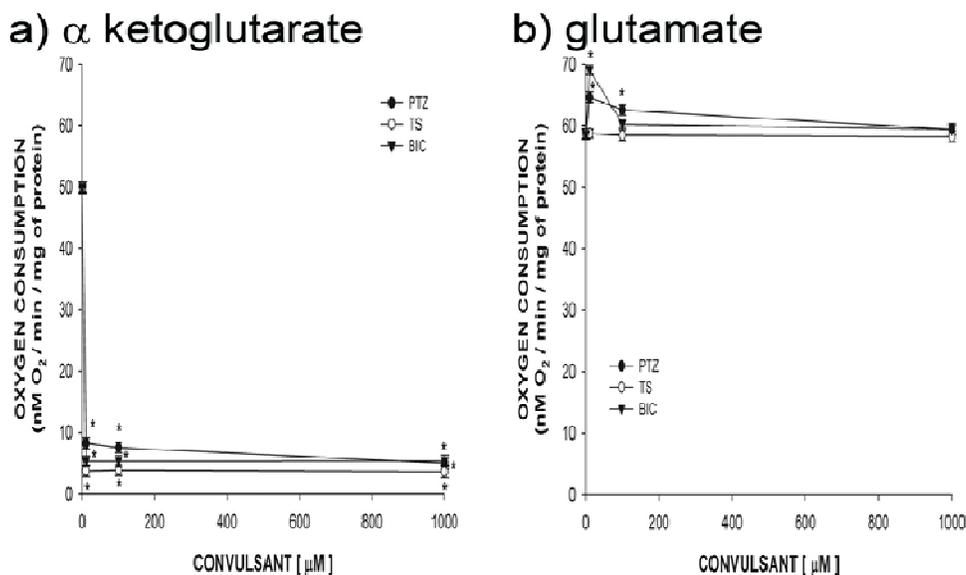


Figure 4. EFFECT IN VITRO OF SOME CONVULSANT DRUGS IN OXYGEN CONSUMPTION. Different concentrations of pentylenetetrazole (PTZ), thiosemicarbazide (TS) and bicuculline (BIC) were added and oxygen consumption was determined in mouse brain homogenates (25% wt/vol in sucrose 0.25 M) by a polarographic method using α -ketoglutarate (a) and glutamate (b) as substrates (see "Materials and Methods"). Results are means + SEM (n>4). ANOVA test was applied and * were used to indicate statistically different groups as compared with the control ($p < 0.01$, Tuckey's test).

drugs on oxygen consumption depend on the substrate used. Results show that all anticonvulsants tested decreased approximately by 90% oxygen consumption, even with the smallest concentrations tested, when α K

was the substrate (Figure 4a). With GLU as substrate, this parameter was essentially not modified by convulsant drugs used (Figure 4b).

Convulsant Drugs and Oxygen Consumption *Ex vivo*

Ex vivo, all convulsants increased (3 to 4 fold) oxygen consumption when α K was the substrate (Figure 5a). With GLU as substrate discrete increases (PTZ) as well as decreases (TS and BIC) in oxygen consumption were found (Figure 5b).

Effect of Convulsant Drugs in GABA- α -oxoglutarate aminotransferase (GABA-T) activity

As can be appreciated in Figure 6, administration of different convulsants caused in all cases significant increase on brain GABA-T activity.

Effect of Convulsant Drugs in GABA Levels.

Results, expressed as percentage versus to the control (Figure 7), show that PTZ does not modify GABA brain levels, whereas TS decreased about 30% and BIC increased around 13 % respectively this parameter.

DISCUSSION

Differential GDH activity profile in patients with temporal lobe epilepsy (Malthankar-Phatak et al., 2006), the association of CNS hyperexcitability with GDH gain of function mutations (Raizen et al., 2005), the alteration of GDH activity in rats with genetic absence epilepsy (Dutuit et al., 2000) and audiogenic seizure-susceptible mice (Cordero et al., 1994), the relation of Myoclonic absence epilepsy with photosensitivity and a gain of mutation in GDH (Bahi-Buisson et al., 2008), changes on GDH activity during insulin-induced seizures (Abdul-Ghani et al., 1989), the increased GDH activity in actively spiking areas of human epileptic cerebral cortex (Sherwin et al., 1984) and in focally epileptic human brain (Sherwin, 1999) are some experimental results which suggest that GDH may participate in seizure induction mechanisms. However, the way and circumstances of participation of GDH in seizures mechanism have not been studied yet, so here we try to investigate this issue.

Our results show the relevance of analyzing both GDH enzymatic directions, oxidative deamination of GLU and also α K reductive amination, since effects produced by chemoconvulsants assayed were different depending on available substrate. Results obtained *in vitro* also differ significantly from the obtained *ex vivo*, since although in both cases convulsants act on GDH, results *in vitro* show just the influence of convulsants on GDH activity but *ex vivo* results reflect different metabolic effects of these drugs which finally lead to changes on GDH activity.

PTZ decreased GDH activity when GLU was the substrate, which is in coincidence with reports from

Lacoste (Lacoste et al., 1988), TS and BIC induced inhibition of GLU oxidative deamination (Figure 2b and 2d). The overall consequence of these *in vitro* effects on GDH activity would be an increase in GLU levels, which may be associated with their convulsant properties.

If it is considered that the mechanism of action of PTZ is associated with its ability to decrease GABAergic synaptic transmission suppressing both ionotropic GABA_A and metabotropic GABA_B receptor (Corda et al., 1992; De Sarro et al., 2000; Getova et al., 1998), that TS is a classic inhibitor of glutamate decarboxylase (GAD) enzymatic activity which decreases GABA synthesis, and BIC is a GABA_A receptor antagonist, it could be proposed that seizures induced by drugs that involve GABA-mediated events generate an increase in GLU levels by a decrease on GDH dependent GLU oxidative deamination, these effects seem not to be related with GABA_A or GABA_B receptors activity modulation.

Ex vivo results are apparently different to those observed *in vitro*, since an increase in α K reductive amination catalyzed by GDH was found in all cases. However, the final effect of the increase on GDH activity with α K as substrate also would be an increase in GLU levels, which enforce the hypothesis of GDH participation on seizures mechanism by increasing brain levels of this excitatory neurotransmitter.

Differences between *in vitro* and *ex vivo* results could be explained by changes in energy requirements generated during seizures, since energetic needs are a very important factor on regulation of GDH activity. This possibility was evaluated by measuring changes in oxygen consumption while GDH reaction was catalyzed for reductive amination of α K and oxidative deamination of GLU, *in vitro* as well as *ex vivo*. It must be remembered that GLU is a glucogenic aminoacid and the reaction catalyzed by GDH represents a pathway to get energy by α K oxidation in the Krebs cycle. So it was considered that changes in GDH activity produced by convulsant drugs may have repercussion on oxygen consumption.

As Figure 4 shows, *in vitro*, all convulsants diminished oxygen consumption during reductive amination of α K catalyzed by GDH, indicating a decreased brain metabolic activity, meaning that there is no need of additional energy. So, generation of α K is not required, which could be an explanation for the convulsant induced decrease of GLU *in vitro*, which would lead to a decrease of α K levels. On the other hand, *ex vivo* results (Figure 5) indicate that all convulsants increased oxygen consumption when α K was the substrate, representing a rise on brain metabolism with additional energy requirements during seizures. However, this additional energy does not seem to be supported by GDH, since GLU produced by enhanced reductive amination of α K by the convulsant drugs *ex vivo* does not increase oxygen consumption, which suggest that this molecule is not being used to ensure no shortage of α K. In other words, a relation between GDH activity and

a) α ketoglutarate

b) glutamate

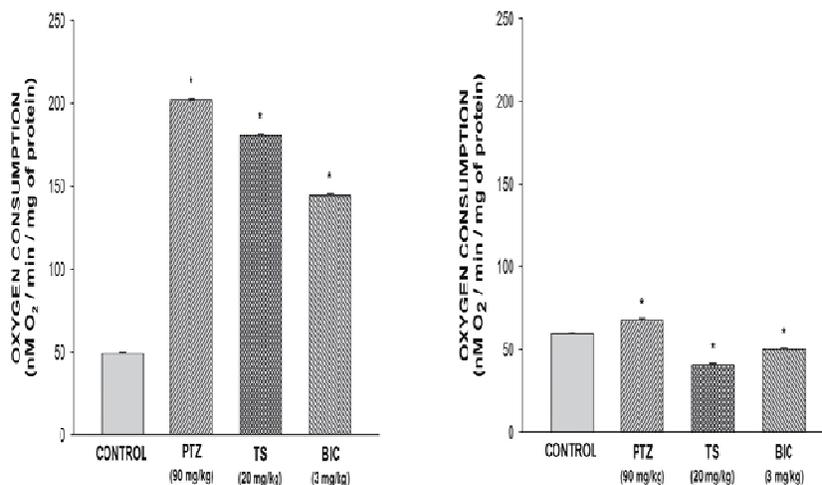


Figure 5. EFFECT EX VIVO OF SOME CONVULSANT DRUGS IN OXYGEN CONSUMPTION Animals received pentylenetetrazole (PTZ, 90 mg/Kg), thiosemicarbazide (TS, 20 mg/kg) and bicuculline (BIC, 3 mg/Kg). Once convulsants induced seizures, oxygen consumption was determined in brain homogenates (25% wt/vol in sucrose 0.25 M), by a polarographic method using α -ketoglutarate (a) and glutamate (b) as substrates (see "Materials and Methods"). Results are means + SEM (n>4). * Statistically significant differences as compared with the control (p<0.01, Student's t test).

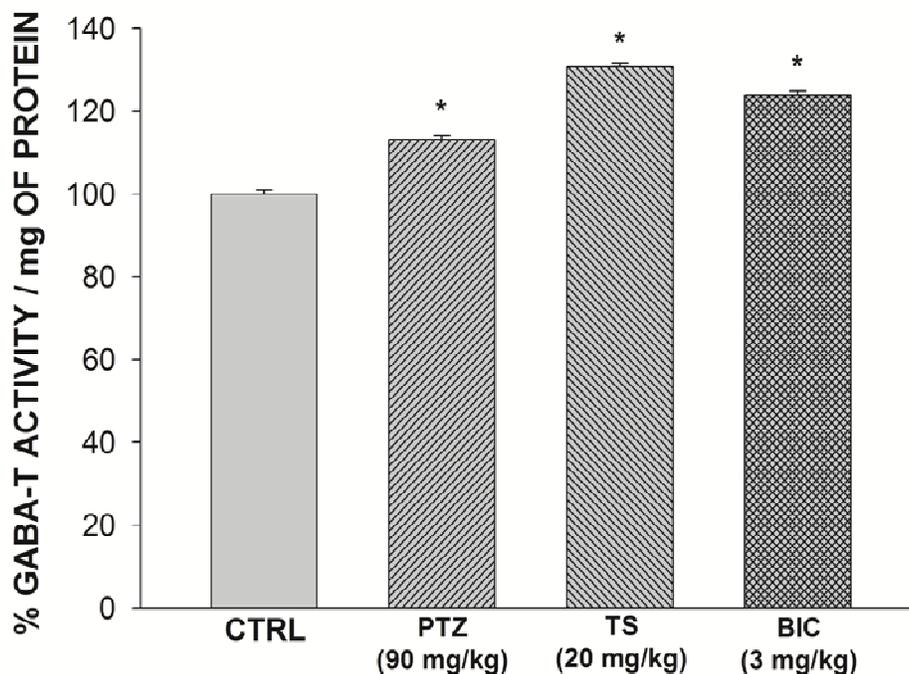


Figure 6. EFFECT OF SOME CONVULSANT DRUGS IN MOUSE BRAIN GAMMA AMINOBUTYRIC ACID TRANSAMINASE (T-GABA) ACTIVITY Animals received pentylenetetrazole (PTZ, 90 mg/Kg), thiosemicarbazide (TS, 20 mg/kg) and bicuculline (BIC, 3 mg/Kg). Once convulsants induced seizures, gamma aminobutyric acid transaminase (GABA-T) was determined in brain homogenates (1:5 w:v on phosphates 0.05 M pH=7.6 buffer) with a spectrophotometric method (see "Materials and Methods"). Results are means + SEM (n>4). * Statistically significant differences as compared with the control (p <0.01 Student's t test).

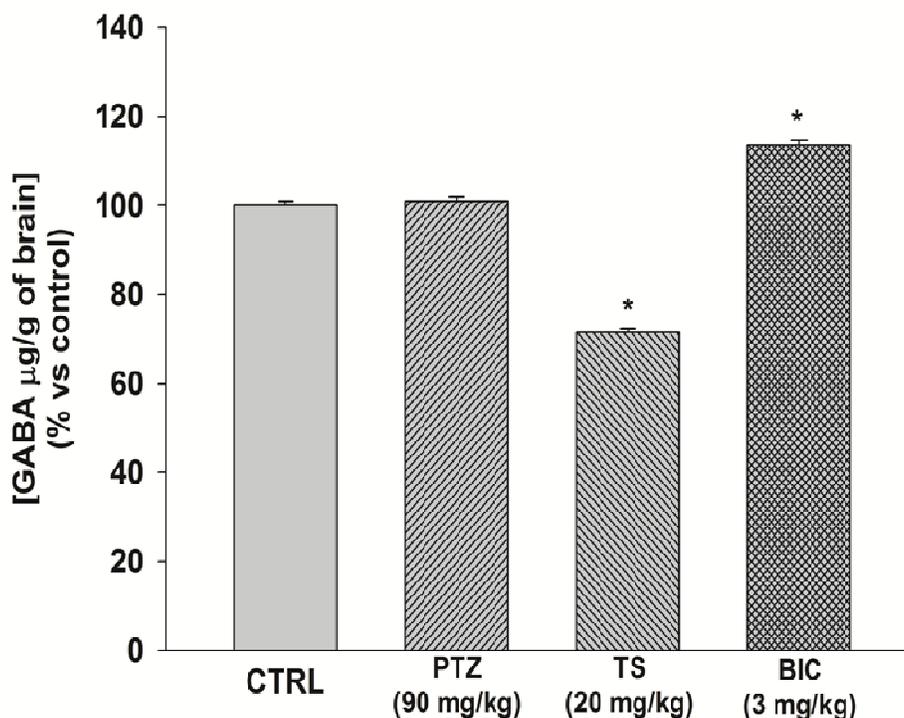


Figure 7. EFFECT OF SOME CONVULSANT DRUGS IN MOUSE BRAIN GAMMA AMINOBUTYRIC ACID (GABA) LEVELS. Animals received pentylenetetrazole (PTZ, 90 mg/Kg), thiosemicarbazide (TS, 20 mg/kg) and bicuculline (BIC, 3 mg/Kg). Once convulsants induced seizures, gamma aminobutyric acid (GABA) levels were determined in brain homogenates with an enzymatic method (see "Materials and Methods"). Results are means + SEM (n>4). * Statistically significant differences as compared with the control ($p < 0.01$ Student's t test).

oxygen consumption is observed *in vitro* as well as *ex vivo*, but during seizures brain GDH does not seem to be an energy source.

αK generated by GDH could be possibly used as a substrate for transamination reaction, which is a common pathway in brain metabolism. Particularly, the possibility of αK utilization for GABA transamination was examined, since it would have important repercussions in GABA brain levels, hence in neuronal excitability. This point was examined by analyzing the effect of convulsants on GABA transaminase (GABA-T) activity, enzyme that catalyzes GABA degradation. As can be appreciated on figure 6, all anticonvulsants increased GABA-T activity, which suggests that GDH would be indirectly modulating GABA levels through regulation of GABA-T activity.

GABA levels in animals treated with chemoconvulsants used were also studied. Decreased levels of GABA were produced by TS (Figure 7), which could be explained not only by its effect on GAD, but also by the increase on GABA-T activity described before. Modifications on GABA levels by PTZ were not found (Figure 7), which is in coincidence with reports of Löscher (Löscher and Frey,

1977). Increase on GABA levels caused by BIC would be the result of blockade of GABA-A receptors caused by this convulsant, leading to its accumulation although GABA-T activity is increased and a decrease on GABA levels would be expected.

Altogether, our results showed that in contrast with the widely held view that GDH acts to deaminate GLU and channel the carbon skeleton into the Krebs cycle, during seizures induced by interferences with GABAergic transmission this enzyme catalyzes preferably the reductive amination of αK , increasing brain GLU levels. Besides, changes in GDH activity have repercussions on oxygen consumption. Through modulating GABA-T activity, GDH may also regulate, indirectly, GABA levels. Thus, GDH may participate in neuronal excitability regulation and may represent a key enzyme in seizures mechanism.

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