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Effects of anti-epileptic drugs on glutamine synthetase activity in mouse brain

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1 Glutamine synthetase (GS) is a key enzyme in the regulation of glutamate neurotransmission in the central nervous system. It is responsible for the conversion of glutamate to glutamine, and for the detoxification of ammonia.

2 We have investigated the effects of single and repeated intraperitoneal administration of a range of established and new anti-epileptic drugs on GS activity in mouse brain.

3 Four hours after the final dose, animals were sacrificed and the brains removed for analysis of GS activity.

4 Both single and repeated doses of phenytoin and carbamazepine were found to reduce enzyme activity ($P<0.05$).

5 Single doses of phenobarbitone, felbamate and topiramate were without effect, however repeated administration of these drugs dose-dependently reduced GS activity ($P<0.05$).

6 Single and repeated doses of sodium valproate, vigabatrin, lamotrigine, gabapentin, tiagabine, levetiracetam and desglycinyl-remacemide were found to have no effect on GS activity.

7 The reduction in enzyme activity demonstrated is unlikely to be related to the anti-epileptic actions of these drugs, but may contribute to their toxicity.

Keywords: Anti-epileptic drugs; epilepsy; glutamine synthetase

Abbreviations: AED, anti-epileptic drug; CBZ, carbamazepine; DGR, desglycinyl-remacemide; FBM, felbamate; GBP, gabapentin; GS, glutamine synthetase; LEV, levetiracetam; LTG, lamotrigine; MSO, methionine sulphoximine; PB, phenobarbitone; PHT, phenytoin; TGB, tiagabine; TPM, topiramate; VGB, vigabatrin; VPA, sodium valproate

Introduction

Glutamine synthetase (GS; EC 6.3.1.2) is a key enzyme in the regulation of glutamate neurotransmission in the central nervous system (CNS). It catalyzes the synthesis of glutamine from glutamate, and is responsible for the detoxification of ammonia in the brain (Meister, 1974). GS is crucial for nitrogen homeostasis, as the glutamine formed by this enzyme is a constituent of proteins and serves as a nitrogen source for a number of biosynthetic pathways (Meister, 1980). It has been demonstrated to be specifically located in the glial fraction of the brain, primarily in astrocytes (Martinez-Hernandez *et al*., 1977; Norenberg, 1979; Norenberg & Martinez-Hernandez, 1979). Glutamine which is synthesized in astrocytes *via* GS is then transported into neurones, where it serves as a precursor for the formation of the neurotransmitters glutamate and γ-aminobutyric acid (GABA; Waniewski & Martin, 1986; Waniewski, 1992). GS is of particular importance in the brain, as although glutamate has a number of metabolic enzymes, the only known pathway for the synthesis of glutamine is *via* GS (Cooper *et al*., 1983).

It is well documented that systemic administration of methionine sulphoximine (MSO), the classical inhibitor of GS, precipitates seizures in many species (Peters & Tower, 1959; Sellinger *et al*., 1984; Swanson *et al*., 1990). The convulsant activity of MSO has been attributed to its inhibition of GS (Meister, 1980), where an increase in glutamate and enhanced excitation at neuronal sites is thought to be responsible for seizure activity. It has been demonstrated that genetically epilepsy-prone rats have a significantly lower brain GS activity than control rats, and this has been proposed to result in the increased seizure susceptibility exhibited by these animals (Carl *et al*., 1993). Seizure-prone gerbils have also been shown to have a lower brain GS activity than normal gerbils (Laming *et al*., 1989), which adds further support to the link between a reduction in GS and seizure activity.

In addition, it has been reported that the anti-epileptic drug (AED) sodium valproate (VPA) stimulates GS activity in rat cortical and cerebellar homogenates (Nolan *et al*., 1985; Phelan *et al*., 1985). In light of this finding that GS may be involved in AED action, and the evidence suggesting a role in seizure generation, we have investigated the effects of single and repeated administration of a range of established, new and experimental AEDs on enzyme activity in mouse brain (preliminary data published in abstract form; Fraser *et al*., 1998).

Methods

Male ICR mice (25–30 g) were obtained from Harlan Olac (Bicester, U.K.) and were housed in a controlled temperature and humidity environment with day/night cycle conditions and access to food and water *ad libitum*. Animals were kept for a minimum period of 7 days prior to use to allow for acclimatization. All experimental work was governed by the Animals (Scientific Procedures) Act, 1986 (U.K.). Radiolabelled glutamate (L-[^{14}C(U)]-glutamic acid) was obtained from NEN Research Products (Stevenage, U.K.). All chemicals

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(reagent grade) were obtained from Sigma Chemical Company (Poole, U.K.).

Drugs

The established anti-epileptic drugs phenobarbitone (PB; 5-Ethyl-5-phenyl-2,4,6-trioxohexahydropyrimidine), phenytoin (PHT; 5,5-diphenyl-2,4-imidazolidinedione), carbamazepine (CBZ; 5H-dibenz[b,f]azepine-5-carboxamide), and VPA (2-propylpentanoic acid) were purchased from Sigma Chemical Company (Poole, U.K.). The new anti-epileptic drugs were obtained from the following companies: vigabatrin (VGB; D,L-4-aminohex-5-enoic acid) Hoechst Marion Roussel (Uxbridge, U.K.), lamotrigine [LTG; 6-(2,3-dichlorophenyl)-1,2,4-triazine-3,5-diamine] Glaxo Wellcome Research and Development (Stevenage, U.K.), felbamate (FBM; 2-phenyl-1,3-propanediol dicarbamate) Schering-Plough Research Institute (Kenilworth, NJ, U.S.A.), gabapentin [GBP; 1-(aminomethyl)-cyclohexaneacetic acid] Parke-Davis Pharmaceutical Research (Ann Arbor, MI, U.S.A.), topiramate [TPM; 2,3 : 4,5-bis-O-(1-methylethylidene)-β-D-fructopyranose sulphamate] The R.W.Johnson Pharmaceutical Research Institute (Spring House, PA, U.S.A.), tiagabine [TGB; (R-)-(−)-1-[4,4-Bis(3-methyl-2-thienyl)-3-butenyl]-3-piperidine-carboxylic acid, hydrochloride] Novo Nordisk A/S (Bagsvaerd, Denmark), levetiracetam [LEV; (S)-α-ethyl-2-oxo-pyrrolidine acetamide] UCB Pharma (Chemin du Foriest, Belgium), and desglycinyl-remacemide [DGR; (±)-1-methyl-1,2-diphenylethylamine] Astra Charnwood (Loughborough, U.K.).

Drug administration

All drugs were prepared daily for intraperitoneal (i.p.) injection. VPA, VGB, LTG, GBP, TGB, LEV, and DGR were dissolved in 0.9% saline. PB, PHT, CBZ and TPM were prepared as a suspension in 0.5% Tween 80 (Polyoxyethylene sorbitan mono-oleate). FBM was suspended in 30/70 (v/v) polyethylene glycol 400/water. For each drug study, mice were randomized into seven groups ($n=12$/group). The following doses of drugs were administered: PB, CBZ, TGB – 0.1, 0.3, 1, 3, 10 and 30 mg kg^{-1}; PHT, LTG, TPM, DGR – 0.3, 1, 3, 10, 30 and 100 mg kg^{-1}, FBM, GBP, LEV – 1, 3, 10, 30, 100 and 300 mg kg^{-1} and VPA, VGB – 3, 10, 30, 100, 300 and 1000 mg kg^{-1}. The seventh group (control) received the appropriate vehicle alone. Four hours after the first dose, six animals from each group were sacrificed, and their brains removed and stored at −70°C until required. In the six remaining mice from each group, treatment was continued twice daily (08.00 h and 16.00 h) for 5 days, with the exception of VGB which was administered once daily. At 4 h after the final dose of the repeated treatment regimen, the remaining animals were sacrificed and their brains removed and stored at −70°C until required.

Glutamine synthetase assay

This method was devised from modifications of the method of Pishak & Phillips (1979). A 100 mM and a 5 mM imidazole-HCl buffer were prepared monthly and used throughout the assay. A test assay solution consisting of 4 mM 2-mercaptoethanol and 40 mM adenosine triphosphate (ATP) in 100 mM imidazole-HCl buffer was prepared daily. A blank assay solution was prepared by omitting ATP from the test solution. A [^{14}C] L-glutamic acid incubation medium, prepared monthly, consisted of (in mM): L-glutamic acid (specific activity = 0.12 mCi mmol^{-1}) 40, $MgCl_2 \cdot 6H_2O$ 60, NH_4Cl 16, and ouabain 4, in 100 mM imidazole-HCl buffer. Mouse brains were homogenized and sonicated using a polytron (PT 1200, Philip Harris Scientific, Lichfield, U.K.) in 4 volumes (v/w) of 5 mM imidazole-HCl buffer. Samples were centrifuged (Heraeus Biofuge 15R, Heraeus Equipment Ltd., Brentwood, U.K.) at 800 × g for 10 min at 4°C. The supernatant was decanted and its protein content determined by the BIORAD method (described by Sills *et al*., 1997). The supernatant was adjusted to a protein concentration of 2 mg ml^{-1} with 5 mM imidazole-HCl buffer. A 50 μl volume of the adjusted supernatant was added to 25 μl of the test assay solution and 25 μl of the [^{14}C] L-glutamic acid incubation medium. Assays were performed in duplicate with a blank assay included for each sample. Samples were mixed and incubated for 15 min at 37°C. The reaction was terminated by the addition of 1 ml of ice cold deionized water. The incubated samples were mixed and applied to single use tandem chromatography columns (BIORAD, Hemel Hemstead, U.K.). The first column contained 0.8 × 3.5 cm Dowex AG1-X8 acetate form resin, 200 – 400 mesh (BIORAD, Hemel Hempstead, U.K.), pre-washed five times with 1N acetic acid, followed by five washes with deionized water. This was placed directly over a second column containing 0.8 × 3.5 cm Amberlite CG-50 hydrogen form resin, mesh 100 – 200 (Sigma Chemical Company, Poole, U.K.), pre-washed five times with 1N NaOH, then five times with 1N HCl, followed by five washes with deionized water. One ml of ice cold deionized water was added to each column, but was not collected. Newly synthesized glutamine was eluted directly into plastic scintillation vials using 5 × 1 ml aliquots of deionized water. Ten ml of Ultima Gold XR scintillation fluid (Canberra Packard, Pangbourne, U.K.) was added to each vial and the disintegrations per minute (d.p.m.) were counted in a liquid scintillation counter (2000CA TRI-CARB, Canberra Packard, Pangbourne, U.K.). The radioactive content of samples was compared to known radioactive standards, corrected for background and blank sample counts, and related to protein content and reaction time. GS activity was expressed as nmol mg protein^{-1} min^{-1}.

Statistical analysis

Analysis was performed using MINITAB for Windows (version 10.1) on an Elonex PC-5120/1 microcomputer. Results were calculated as the percentage of mean control values. Group results were then expressed as mean percentages ± s.e.mean. Drug treated groups were compared to control using one-way analysis of variance with Dunnett's correction for multiple comparisons.

Results

All results are summarized in Tables 1 and 2. Both single and repeated treatments with PHT and CBZ (Figure 1) significantly ($P<0.05$) reduced the activity of GS 4 h after the final dose. No results are shown for repeated 100 mg kg^{-1} PHT, as treatment was discontinued in this group due to adverse effects. Single doses of PB, FBM and TPM (Figure 2) were without effect on enzyme activity, although repeated administration of these drugs significantly ($P<0.05$) reduced GS activity at 4 h post-administration. Single and repeated treatments with VPA, VGB, LTG, GBP, TGB, LEV, and DGR were without effect on mouse brain GS activity at 4 h post-administration.

Table 1 Effect of established antiepileptic drugs on mouse brain glutamine synthetase activity

	Glutamine synthase activity					
	Acute (nmol mg protein^{-1} min^{-1} ± s.e.mean)			*Chronic* (nmol mg protein^{-1} min^{-1} ± s.e.mean)		
	Control	*Drug-treated*	*(% of control)*	*Control*	*Drug-treated*	*(% of control)*
Phenobarbitone (0–30 mg kg^{-1})	18.88 ± 0.5	17.2 ± 0.4	–	20.02 ± 1.1	11.11 ± 0.5	↓ to 55%
Phenytoin (0–100 mg kg^{-1})	27.11 ± 1.9	13.11 ± 0.5	↓ to 47%	16.65 ± 0.6	11.54 ± 0.2	↓ to 69%
Carbamazepine (0–30 mg kg^{-1})	30.85 ± 1.2	22.81 ± 1.5	↓ to 74%	17.34 ± 0.8	11.95 ± 0.9	↓ to 69%
Valproate (0–1000 mg kg^{-1})	14.13 ± 0.8	14.50 ± 0.5	–	14.61 ± 1.2	13.89 ± 0.8	–

Table 2 Effect of new antiepileptic drugs on mouse brain glutamine synthetase activity

	Glutamine sythetase activity	
	Acute	*Chronic*
Vigabatrin (0–1000 mg kg^{-1})	–	–
Lamotrigine (0–100 mg kg^{-1})	–	–
Felbamate (0-300 mg kg^{-1})	–	↓ to 55% of control
Gabapentin (0–300 mg kg^{-1})	–	–
Topiramate (0–100 mg kg^{-1})	–	↓ to 52% of control
Tiagabine (0–30 mg kg^{-1})	–	–
Levetiracetam (0–300 mg kg^{-1})	–	–
Desglycinyl-remacemide (0–100 mg kg^{-1})	–	–

Discussion

GS is the enzyme responsible for the metabolism of glutamate to glutamine, and for the detoxification of ammonia in the brain (Meister, 1974). It provides the only known pathway for the synthesis of glutamine in the CNS (Cooper *et al*., 1983). The glutamine produced serves as a precursor for the synthesis of glutamate and GABA, therefore, GS is partly responsible for replenishing the supply of these neurotransmitters (Waniewski, 1992). Inhibition of this enzyme with MSO has been demonstrated to precipitate seizures in many species (Peters & Tower, 1959; Sellinger *et al*., 1984; Swanson *et al*., 1990), and a reduction in GS activity has been proposed to be related to the seizure susceptibility of genetically epilepsy prone animals (Laming *et al*., 1989; Carl *et al*., 1993). It has previously been reported that the AED VPA stimulates GS activity in rat brain homogenates (Nolan *et al*., 1985; Phelan *et al*., 1985). In light of the importance of GS in regulating glutamate neurotransmission, and it's possible involvement in seizure generation and anti-epileptic drug action, we have investigated the effects of single and repeated administration of a range of AEDs on the activity of GS in mouse brain.

Both single and repeated treatments with PHT and CBZ, and repeated administration of PB, FBM and TPM significantly reduced the activity of GS. Given, that an inhibition of this enzyme results in a diminished capacity for the brain to metabolize, and thereby detoxify glutamate and ammonia, it is unlikely that the reduction in activity demonstrated contributes to the anti-epileptic actions of these drugs. Accumulation of glutamate and ammonia would be expected to facilitate neuronal excitation, and precipitate seizures, as seen with the GS inhibitor MSO. It is therefore more likely that the inhibition of GS may contribute to the toxicity of these compounds. Interestingly, those drugs with an inhibitory action on the enzyme are those which most frequently exhibit CNS-related toxicity, such as ataxia, sedation, dizziness and cognitive impairment (Rogawski & Porter, 1990; Dichter & Brodie, 1996). Both single and repeated treatments with VPA, VGB, LTG, GBP, TGB, LEV and DGR were found to have no effect on the activity of GS, suggesting that an action on the enzyme is not involved in the anti-epileptic or toxic mechanisms of these drugs. The failure to demonstrate the VPA-induced potentiation reported by other groups (Nolan *et al*., 1985; Phelan *et al*., 1985) may have been due to differences in experimental design. The other groups added VPA directly to the enzyme incubation medium and observed an *in vitro* response, whereas in our study we examined the *ex vivo* effects of the AEDs on enzyme activity.

It is well documented that anti-epileptic drug treatment may worsen seizures, either by aggravating pre-existing ones, or by inducing new seizure types (Troupin & Ojemann, 1975; Osorio *et al*., 1989; Murphy *et al*., 1991; Loiseau, 1998; Perucca *et al*., 1998). This phenomenon is referred to as paradoxical intoxication and is most commonly reported in patients treated with PHT (Osorio *et al*., 1989; Murphy *et al*., 1991; Perucca *et al*., 1998), and CBZ (Troupin & Ojemann, 1975; Perucca *et al*., 1998). Experiments on glial cell cultures have suggested that at higher PHT concentrations, the cells appear to be less able to regulate extracellular potassium (White *et al*., 1985). This has been proposed to partially explain the excitatory effects of the drug seen clinically at high therapeutic doses. It has also been reported that PB (Perucca *et al*., 1998), FBM (Wolff *et al*., 1994) and TPM (Elger *et al*., 1998) may increase seizure frequency in patients. From this evidence, it is possible that the occurrence of paradoxical intoxication may be related to the reduction in GS activity exerted by these drugs, in particular CBZ and PHT, the two most commonly linked to paradoxical intoxication, and the only AEDs found to have both acute and chronic effects on GS activity in this study.

As with the incidence of paradoxical intoxication, the tendency to reduce enzyme activity is more prominent in the established AEDs (Table 1), with a reduction in GS only evident following repeated administration with two of the newer drugs, FBM and TPM (Table 2). The most marked reduction in enzyme activity was evident following single and repeated dosing with two of the established AEDs, CBZ and PHT, which are known to exert their anti-epileptic effects *via* blockade of voltage-dependent sodium channels (Rogawski & Porter, 1990). Interestingly, LTG, a new AED whose anti-epileptic mechanism of action is also thought to be mediated

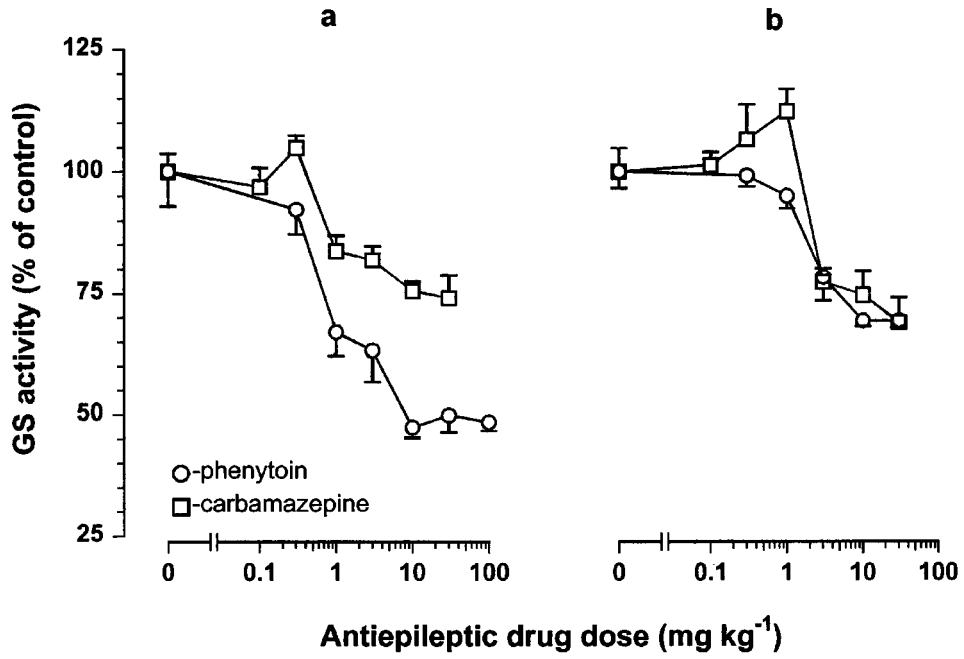

Figure 1 Effect of phenytoin (0–100 mg kg^{-1}) and carbamazepine (0–30 mg kg^{-1}) on mouse brain glutamine synthetase (GS) activity at 4 h after acute (a; single dose) and chronic (b; twice daily for 5 days) administration. Results ($n=6$) are expressed as the percentage of mean control values and error bars denote s.e.mean.

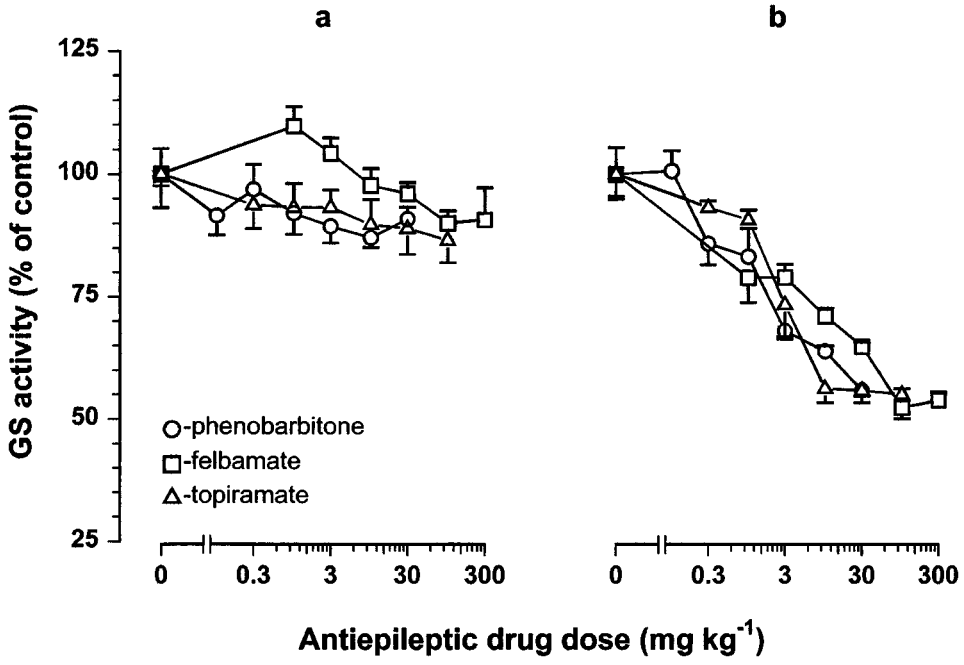

Figure 2 Effect of phenobarbitone (0–30 mg kg^{-1}), felbamate (0–300 mg kg^{-1}) and topiramate (0–100 mg kg^{-1}) on mouse brain glutamine synthetase (GS) activity at 4 h after acute (a; single dose) and chronic (b; twice daily for 5 days) administration. Results ($n=6$) are expressed as the percentage of mean control values and error bars denote s.e.mean.

via blockade of sodium channels (Cheung *et al*., 1992), had no effect on the activity of GS. Similarly, DGR, the experimental drug with proposed sodium channel blocking effects, (Wamil *et al*., 1996) failed to influence the activity of the enzyme. It is unclear why CBZ and PHT reduced GS activity following a single dose, while no other drug examined had acute effects. The mechanism of the inhibition may differ between the AEDs with acute effects and those with effects only after chronic treatment. It is possible that CBZ and PHT are interacting with the active site of the enzyme, resulting in direct inhibition which is evident following a single dose. PB, FBM and TPM, the AEDs which had effects following only repeated administration may be reducing enzyme activity *via* an indirect mechanism, such as an effect on protein synthesis or gene expression. The possibility that these AEDs are actually reducing the amount of enzyme present and not simply inhibiting it cannot be excluded without further investigation. The inhibition may also be indirect, in that it is occurring

secondary to a change in the concentration of a neurotransmitter, or activity of another enzyme in the brain. Such effects may take time to develop, therefore only becoming apparent following repeated administration. Each of these possibilities require further detailed investigation in order to determine the nature of the inhibition of GS.

In conclusion, the reduction in GS activity following both single and repeated treatments with PHT and CBZ, and repeated treatments with PB, FBM and TPM is unlikely to be related to the anti-epileptic actions of these drugs. It is possible that inhibition of this enzyme may contribute to the toxicity of these drugs, particularly CNS-related adverse effects. It is also possible that this reduction in GS activity may be contributing to the phenomenon of paradoxical intoxication seen clinically with these drugs in some patients. However, further work is needed to substantiate these observations.

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