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ANTICONVULSANT AND BIOCHEMICAL EFFECTS OF INHIBITORS OF GABA AMINOTRANSFERASE AND VALPROIC ACID DURING SUBCHRONIC TREATMENT IN MICE

WOLFGANG LÖSCHER

Laboratory of Pharmacology and Toxicology, School of Veterinary Medicine, Free University of Berlin, Koserstraße 20, D-1000 Berlin 33, West Germany

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Abstract—Mice were treated with different doses of the GABA aminotransferase (GABA-T) inhibitors aminooxyacetic acid, γ-acetylenic acid, γ-vinyl GABA and ethanolamine-*O*-sulphate via the drinking water for periods of 1–12 days. All drugs caused marked elevations of whole brain GABA concentrations within 4 days of treatment which were associated with increases in the electroconvulsive threshold. However, the effect on seizure threshold could not be enhanced by an increase in the daily dosage of the GABA-T inhibitors and, especially with higher doses, tolerance to the anticonvulsant effect developed. At least in part, this finding may be attributed to a decrease in the activity of glutamic acid decarboxylase (GAD), the enzyme responsible for GABA synthesis. On the other hand, with valproic acid (VPA) no tendency towards a reduced anticonvulsant effectiveness during medication was observed. VPA caused only non-significant increases in cerebral GABA levels but elevated brain GAD activity significantly. No behavioral changes were seen following subchronic administration of GABA-T inhibitors and VPA except in cases where the daily fluid intake was markedly reduced. Our data suggest that the anticonvulsant efficacy of long term treatment with GABA-T inhibitors is limited by the development of compensatory mechanisms, such as reduction of GAD activity, which in turn reduce the amount of GABA available for synaptic transmission, though overall GABA concentrations in the brain are highly elevated. Drugs such as VPA which cause only moderate effects on GABA metabolism seem superior in this respect.

Over the past few years, evidence has accumulated which strongly suggests that an impairment of the central GABA system is involved in the pathophysiology and phenomenology of epilepsy [1–3]. Therefore, it was repeatedly proposed that increasing brain GABA or administration of a centrally active GABA-mimetic agent may be a specific and efficacious therapeutic approach. Brain GABA concentrations can be increased by inhibiting GABA-α-oxoglutarate aminotransferase (EC 2.6.1.19; GABA-T), the enzyme primarily responsible for GABA catabolism, and inhibitors of this enzyme have been shown to exhibit anticonvulsant properties in various experimental models of epilepsy (for review, see [4]). If efforts are to be made to activate the GABA-ergic system in patients with epilepsy, it is important to determine the long-term neurochemical, anticonvulsant and toxic effects of GABA-mimetic drugs. In this respect, recent reports have shown that subchronic administration of irreversible inhibitors of GABA-T in low doses by the oral route was an effective method to elevate substantially whole brain GABA content in rodents without producing evident toxic effects [5–7]. However, the anticonvulsant efficacy of continued oral treatment with such drugs has so far only been studied for isoniazid [8]. In the present paper, mice were treated with different doses of the GABA-T inhibitors aminooxyacetic acid (AOAA), γ-acetylenic GABA, γ-vinyl GABA and ethanolamine-*O*-sulphate (EOS) via the drinking water for periods from 1 to 12 days. During this period, the animals were studied for changes in brain GABA metabolism, seizure excitability in terms of the electroconvulsive threshold, and general behaviour. Valproic acid (VPA), a clinically useful antiepileptic drug also thought to act via the GABA system, was included in the study as a reference standard.

MATERIALS AND METHODS

Materials

γ-Acetylenic GABA and γ-vinyl GABA were kindly provided by Centre de Recherche Merrell International (Strasbourg, France). Valproic acid (VPA), used as the sodium salt, was a kind gift from Desitin-Werk Carl Klinke (Hamburg, West Germany). Aminooxyacetic acid hemihydrochloride (AOAA) was purchased from Ferak (Berlin, West Germany) and ethanolamine-*O*-sulphate (EOS) from Fluka AG (Buchs, Switzerland). The latter drug was cleaned by activated charcoal and recrystallized before use.

Methods

Animals. Male mice of the NMRI-strain (WIGA Versuchstierzuchtanstalt, Sulzfeld, West Germany), weighing 24–30 g, were used throughout these experiments. They were kept in groups of 10 in Makrolon cages at constant temperature (24–26°) and controlled humidity (approx. 50%) with a 12 hr light circle beginning at 7 a.m. Food (Altromin stan-

dard food, Altrogge, Lage, West Germany) and water were given *ad libitum*.

The drugs under study were administered via the drinking water for periods of 1, 4, 8 and 12 days, each drug being tested at 1–3 concentrations. The drug concentrations were chosen on the basis of previous experiments on the acute anticonvulsant and biochemical effects of the compounds [9], taking into account a daily water consumption of 210–230 ml/kg. Control mice were supplied in parallel with normal drinking water. Every day at 8 a.m. both the bottles and the mice were weighed and the daily drug intake was calculated in mg/kg. The bottles were then emptied and re-filled with freshly prepared drinking solutions. For each period of treatment and drug concentration, respectively, 20 mice were used for the determination of the electroconvulsive threshold and further 10 mice for the biochemical determinations.

Electroconvulsive threshold. After periods of 1, 4, 8 and 12 days the anticonvulsant efficacy of the medication was studied in groups of 20 mice by determination of the electroconvulsive threshold using a Lafayette A-625 B shocker. Determinations were carried out at 8:30 a.m. Stimulation was through eye electrodes for 0.2 sec, the serial resistance of the apparatus was set to 10 kΩ. The extension of the hind limbs was taken as the endpoint. The threshold was calculated with the "up and down" method of Kimball *et al.* [10] and is given as the voltage inducing the extensor phase in 50% of the mice (EV_{50}). All experimental groups were used for only one threshold determination.

Biochemical determinations. GABA concentrations and the activities of GABA-α-oxoglutarate aminotransferase (GABA-T; EC 2.6.1.19) and glutamic acid decarboxylase (GAD; EC 4.1.1.15) were assayed in homogenates of whole brain. Mice were sacrificed at 8:30 a.m. on the respective experimental days. For the determination of GABA, 5 mice were decapitated and their brains homogenized within 30 sec in 2 ml 80% ethanol (tubes immersed in a bath of methanol at −30°). After centrifugation at 4000 rpm for 10 min at −5°, 300 μl of the supernatant was evaporated to dryness by a stream of nitrogen. The residue was dissolved in 300 μl pyrophosphate buffer (0.1 M, pH 8.3) and GABA was measured in aliquots of 100 μl by the enzymatic "GABAase" method as described by Baxter [11]. For the determination of GAD and GABA-T, the brains of 5 mice were immediately homogenized in 4 ml ice cold water. GABA-T activity was measured by the method of Salvador and Albers [12] and GAD activity by the method of Lowe *et al.* [13] as described [9]. Untreated controls were used for each experimental day.

Side effects. Besides observation of general behaviour, prior to the determinations of the electroconvulsive threshold groups of 10 mice were tested for neurotoxic side effects by the chimney test of Boissier *et al.* [14]. In this test, neurological deficit is indicated by the inability of the mice to climb up backwards in a glass tube of 25 cm length within 30 sec.

Statistics. Arithmetical means and S.D. are given for the biochemical determinations. Significance of differences was calculated by comparing each treated group with the control group of the same day by the unpaired *t*-test.

RESULTS

The time course of the changes in seizure threshold and the concentration of GABA and GAD and GABA-T activities produced by subchronic administration of γ-vinyl GABA, γ-acetylenic GABA, EOS, AOAA and VPA is shown in Figs. 1–5.

γ-Vinyl GABA (Fig. 1) was administered in the drinking water at concentrations of 0.043 and 0.13%. Both concentrations reduced the daily liquid consumption by 20 and 40%, respectively. The daily drug intake calculated for the lower drug concentration was 70–80 mg/kg whereas in the case of the higher concentration the drug intake declined during the period of treatment from 170–200 mg/kg to 150–170 mg/kg. Within one day of medication with γ-vinyl GABA the activity of GABA-T was dose-dependently decreased to 40–60% of control and GABA levels were correspondingly increased. In

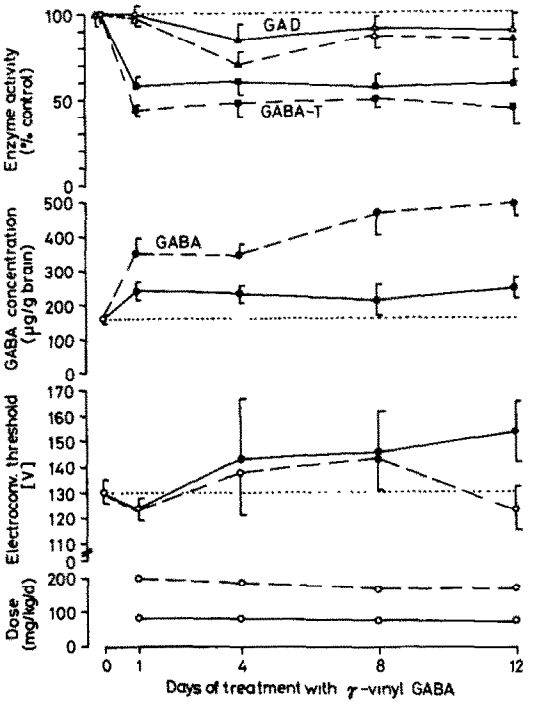

Fig. 1. Effect of continuous treatment with γ-vinyl GABA via the drinking water on whole brain GABA metabolism and the electroconvulsive threshold in mice. The concentrations of γ-vinyl GABA in drinking water were 0.043% (○——○) and 0.13% (○- - -○), respectively. For GABA, GAD and GABA-T the mean ± S.D. of 5–10 determinations is shown. Control enzyme activities ($n = 115$) were: GAD, 14.2 ± 1.14 μmoles/g/hr and GABA-T, 38.9 ± 3.1 μmoles/g/hr. The seizure threshold is given as the voltage inducing an extension of the hind limbs in 50% of the mice (EV_{50}). The vertical bars represent the confidence limits for 95% probability; 20 mice were used for each determination. Control values represent mean and confidence limits of 80 determinations. Significance of differences ($P < 0.05$) to control determinations of the same day is marked by filled symbols.

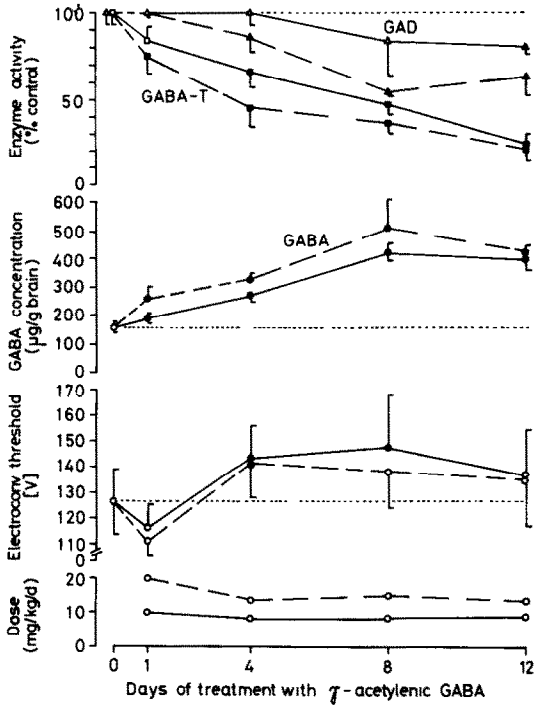

Fig. 2. Effect of continuous treatment with γ-acetylenic GABA via the drinking water on whole brain GABA metabolism and the electroconvulsive threshold in mice. The concentrations of γ-acetylenic GABA in drinking water were 0.0043 (O——O) and 0.0086% (O- - -O), respectively. For further details see legend to Fig. 1.

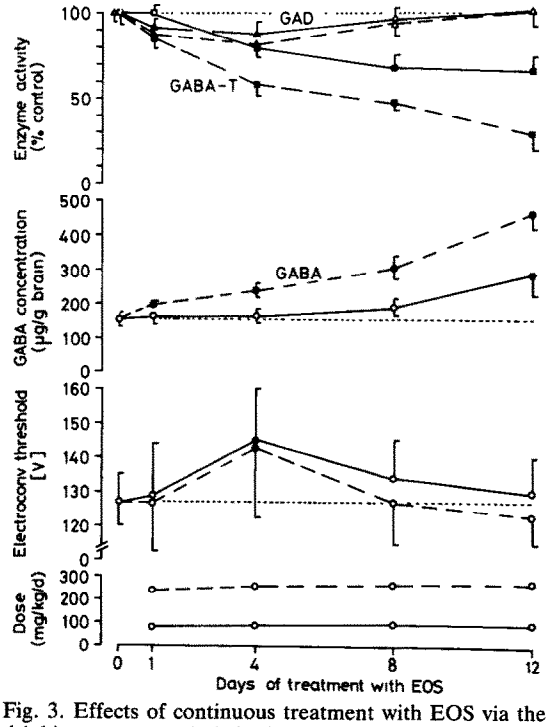

Fig. 3. Effects of continuous treatment with EOS via the drinking water on whole brain GABA metabolism and the electroconvulsive threshold in mice. The concentrations of EOS in the drinking water were 0.043 (O——O) and 0.13% (O- - -O), respectively. For further explanation see legend to Fig. 1.

addition, following both drug concentrations a slow decrease of GAD activity occurred after 4, 8 and 12 days of treatment. As regards the seizure threshold, a significant elevation was determined after 4 days of treatment with the 0.043% solution and the threshold was maintained elevated for the rest of the medication period. However, with the higher drug concentration a significant increase was only observed after 8 days and thereafter the threshold returned to controls though GABA levels remained markedly elevated. After 12 days of treatment with the 0.13% solution the body weight of the animals was reduced by 25% as compared to controls and the mice appeared sedated and in the chimney test the ability of 50% of the mice to climb up in the glass-tube was impaired. However, these symptoms could probably be explained by the reduced fluid intake.

γ-Acetylenic GABA was tested in a 0.0043 and 0.0086% solution (Fig. 2). From the lower drug concentration the average daily intake amounted to 8–10 mg/kg, whereas with the higher concentration, on account of a reduction in daily water consumption by about 20%, the drug intake decreased from 20 mg/kg/day on the first days to 14 mg/kg/day. This prohibited the use of higher concentrations, e.g. by the use of a 0.013% solution (not shown in Fig. 2) the water consumption was reduced by 80% and subsequently several mice died after 4–8 days of treatment. In the doses shown in Fig. 2, γ-acetylenic GABA caused a progressive inhibition of GABA-T, and GABA levels rose significantly. During the first 8 days both effects were more pronounced with the 0.0086% solution, however, after 12 days of administration both solutions gave rise to similar effects on GABA-T and GABA. The activity of GAD was dose-dependently reduced parallel to GABA-T activity and remained so for the duration of treatment. The electroconvulsive threshold was significantly elevated after 4 days of treatment, however, no difference could be determined between both drug solutions and the threshold increase declined towards controls on day 12 of drug administration. During the 12 days of treatment no obvious behavioural differences between controls and the γ-acetylenic GABA treated animals were observed. With the higher drug concentration a loss in body weight of about 5% was noted.

EOS (Fig. 3) was administered in a 0.043 and 0.086% solution. The daily water intake was not changed during treatment and the drug intake amounted to 80–100 and 230–300 mg/kg/day, respectively. With both drug concentrations a slow dose-dependent decline in GABA-T activity occurred, subsequently followed by an increase in cerebral GABA levels. A small but significant decrease in GAD activity was determined at day 1 and day 4 of treatment. With both drug solutions, the electroconvulsive threshold increased to a similar extent after 4 days but thereafter decreased towards control in spite of a further increment of GABA concentrations in the brain. No side effects were observed during the EOS administration and the body weight was not affected.

AOAA was tested at concentrations in the drinking water of 0.0022, 0.0043 and 0.0086%. With the

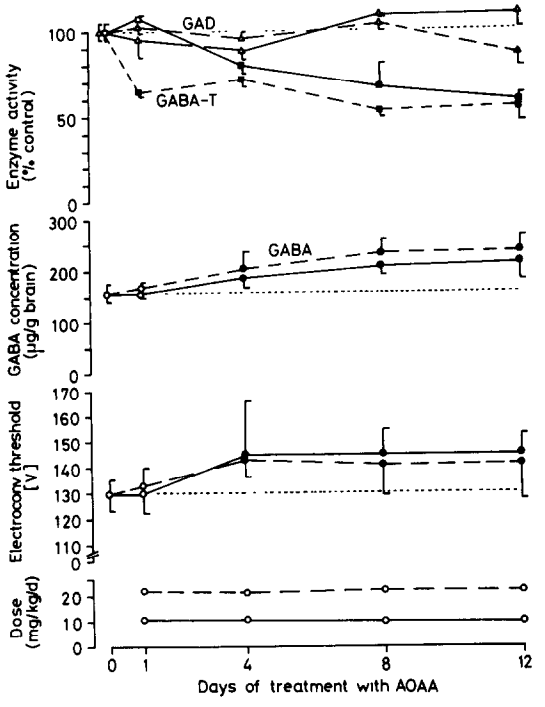

Fig. 4. Effect of continuous treatment with AOAA via the drinking water on whole brain GABA metabolism and the electroconvulsive threshold in mice. The concentrations of AOAA in drinking water were 0.0043 (O——O) and 0.0086% (O- - -O), respectively. For further explanation see legend to Fig. 1.

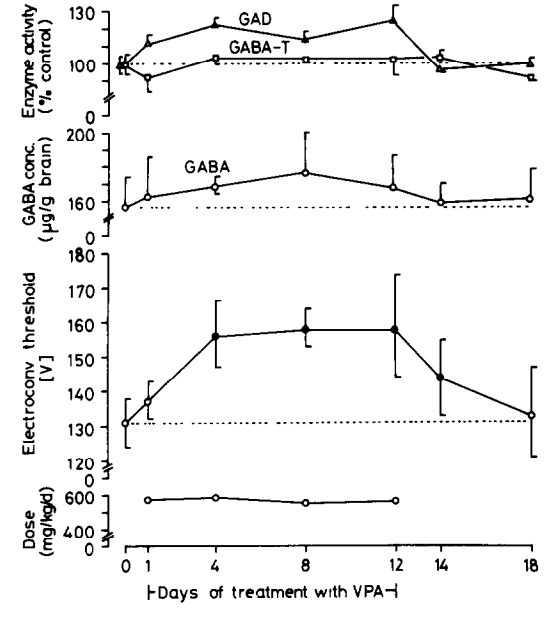

Fig. 5. Effect of continuous treatment with VPA via the drinking water on whole brain GABA metabolism and the electroconvulsive threshold in mice. The concentration of VPA in the drinking water was 0.26%. The treatment was terminated on day 12 after which the mice received drug-free water for further 6 days. For further explanation see legend to Fig. 1.

0.0022% solution the daily drug intake was 4–5 mg/kg, however, in this dose AOAA exerted no effects on GABA-T activity and GABA concentrations within 12 days of treatment. Thus, only the experiments with the two higher drug concentrations are shown in Fig. 4. With both drug solutions the daily water consumption was not affected and a drug intake of 9–11 and 19–23 mg/kg per day, respectively, was attained. The activity of GABA-T was significantly lowered within 1–4 days of medication and GABA levels raised moderately. A significant inhibition of GAD was only observed after 12 days of administration of the 0.0086% solution of AOAA. In both concentrations AOAA raised the threshold to a similar extent on day 4 and this effect was maintained over the period of treatment. No effects on behaviour and body weight were observed.

VPA (Fig. 5) was administered in the drinking water at a concentration of 0.26% with an average daily drug intake of between 500–580 mg/kg. By this concentration the daily water consumption of the animals was only reduced by an average of 10%. Over the period of treatment a slight non-significant increase in GABA concentrations was determined. The activity of GABA-T was not affected by VPA, but GAD activity rose significantly by 20–30%. Parallel to GAD increase an elevation of the electroconvulsive threshold took place within 4 days and this increase remained stable for the duration of treatment. After administration of VPA was terminated on day 12, some groups of mice were studied for carry-over effects. Two days after the cessation of medication the threshold was still significantly increased whereas after 6 days it had returned to control values. During administration of VPA no side effects or decreases in body weight were observed.

DISCUSSION

As recently shown for EOS in rats [7], chronic administration of irreversible inhibitors of GABA-T via the drinking water was found to be an effective method of producing marked elevation of whole brain GABA content in mice. With EOS, γ-vinyl GABA and γ-acetylenic GABA due to the cumulative effects on GABA degradation much lower doses were sufficient to increase GABA levels as compared to single dose experiments with these drugs [9, 15, 16]. AOAA proved relatively weak by the oral route in comparison to parenteral routes of administration [5]. The changes in GABA levels brought about by the inhibitors of GABA-T were accompanied by significant elevations of the electroconvulsive threshold. However, this effect could not be enhanced by an increase in the daily dosage contrary to the clear dose–response relations in terms of threshold elevations reported for acute experiments with these drugs [9]. Moreover, except in the case of AOAA, our experiments pointed to the development of tolerance to the anticonvulsant action of GABA-T inhibitors. This was most pronounced with EOS medication during which a significant elevation in seizure threshold was only obtained after 4 days and thereafter the threshold

returned to control values though brain GABA concentrations still increased.

To date, there have been only few reports concerning the anticonvulsant action of inhibitors of GABA degradation during continuous administration. Schechter *et al.* [15, 16] reported that repetetive intraperitoneal doses of γ-vinyl GABA and γ-acetylenic GABA showed cumulative effects with regard to audiogenic seizure protection in mice but they treated the animals only for 2 days. Administration of isoniazid with the drinking water for periods of 4 and 7 days led to significant increases in cerebral GABA levels but did not change the thresholds for electro- and chemoconvulsions in mice [9]. In acute experiments, however, similar doses of isoniazid exerted clear anticonvulsant effects [17].

With respect to clinically established antiepileptic drugs such as phenobarbital and phenytoin the development of tolerance is a well-known phenomenon and has been explained by both an acceleration of drug metabolism and a central adaptation to the permanent presence of the respective drugs [18]. As regards the present experiments, the question arises why in the course of treatment with GABA-T inhibitors the anticonvulsant efficacy could decrease despite GABA concentrations were maintained highly elevated or even further increased. One explanation would be that increases in electroconvulsive thresholds brought about by GABA-T inhibitors are not related to changes in whole brain GABA levels but rather to changes within the small GABA compartment localized in nerve terminals which is associated with GABA-ergic transmission. However, following acute administration of EOS, AOAA, gabaculine, γ-acetylenic GABA, γ-vinyl GABA and VPA, a fairly good correlation between the time course of the induced elevation of electroconvulsive threshold and the time course of the increase in whole brain GABA concentrations has recently been demonstrated [19]. Furthermore, at the time of maximum anticonvulsant effect of the respective drugs, a close relationship was observed between the changes in GABA content of the whole brain and brain nerve endings (synaptosomes) [20].

A further explanation for decreasing anticonvulsant activity in spite of high GABA concentrations in the brain may lie in the reduction of GAD activity observed, i.e. the enzyme which catalyses the formation of GABA. GAD is thought to exert direct control over the GABA pool involved in neurotransmission and inhibition of this enzyme might actually lower the amounts of GABA available for release into the synaptic cleft even though overall brain GABA content is elevated [21]. Especially in the case of EOS and γ-vinyl GABA, which, contrary to AOAA and γ-acetylenic GABA, do not inhibit GAD *in vitro* [22, 23], one may attribute the decrease in GAD activity during chronic medication to a feedback effect of high brain GABA levels, most probably repression of GAD synthesis as outlined previously [24]. Some degree of reduction of GAD activity may therefore be an inevitable consequence of chronically elevating brain GABA concentrations and this is supported by recent studies [7, 24–26]. Decrease of GAD activity either by a feedback mechanism or by the drug itself would also explain the narrow anticonvulsant dose–response of GABA-T inhibitors during long-term medication as found in the present experiments. Evidence for feedback repression of GAD synthesis by high GABA levels has also been provided by acute experiments with GABA-T inhibitors, in which the decline in GAD activity was associated with a decrease of anticonvulsant effect [19].

Besides possible feedback effects of highly elevated GABA levels on GAD there is evidence for another compensatory mechanism in that GABA release can be inhibited via autoreceptors [27]. This would also limit the efficacy of drugs which exert their pharmacological effects through pronounced elevations of GABA concentrations. Furthermore, recent neurophysiological studies suggest that following direct microiontophoretic application of GABA to neurons, a loss of efficacy even occurs after the first administration and is probably caused by desensitization at the receptor level [28]. In this respect, the "acute tolerance" reported for the antiepileptic effects of benzodiazepines, drugs which are also thought to act via GABA-ergic mechanisms [29], seems of interest.

With VPA, in contrast to the inhibitors of GABA-T tested, no tendency towards a reduced anticonvulsant effectiveness during treatment was noted. However, whole brain GABA levels were only non-significantly increased by VPA though GAD activity was significantly increased. It has been shown recently that following single intraperitoneal doses VPA increased central GABA concentrations predominantly in nerve terminals [30, 31] and this effect could be attributed to an increase in GABA synthesis relative to degradation [31]. Therefore, the increment of whole brain GAD activity observed during subchronic treatment may point to an elevation of synaptosomal GABA levels. Since VPA is eliminated with a half-life of less than 1 hr in mice [32] it was rather unexpected to find that anticonvulsant and biochemical effects were maintained during continued treatment with this drug. Moreover, after cessation of treatment the seizure threshold was still significantly elevated 2 days later. Similar carryover effects in terms of the anticonvulsant activity of VPA have been reported for monkeys and man [33, 34] and may point to the existence of active metabolites which accumulate during treatment. In fact, first pharmacological studies on several VPA metabolites have shown that some of these compounds exert anticonvulsant activity and raise GABA concentrations in the brain and brain nerve endings [35, 36].

Chronic administration of GABA-T inhibitors and VPA was found not to produce any changes in general behaviour of the animals except in the experiments with γ-vinyl GABA and γ-acetylenic GABA in which the daily fluid consumption was greatly reduced. High, acute doses of GABA-T inhibitors produce a characteristic syndrome the major symptoms of which are general sedation, ataxia, impaired motor coordination, hunched posture and hypothermia [9, 37]. Furthermore, AOAA and γ-acetylenic GABA may cause excitation and convulsions during acute and chronic administration [9, 37, 38]. Most of these symptoms have been ascribed to the increase

of GABA levels to values in excess of those attained by our chronic treatment.

To summarize, brain GABA levels can be elevated substantially by continued oral administration of relatively low, untoxic doses of GABA-T inhibitors. However, the anticonvulsant efficacy of such treatment is uncertain and seems to be limited by the development of compensatory mechanisms within the GABA system. Drugs such as VPA which cause only moderate changes in GABA metabolism seem superior in this respect and this is supported by a recent comparison on the acute anticonvulsant effects of GABA-T inhibitors and VPA [9].

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