ELSEVIER

Brain Research 669 (1995) 67–72

BRAIN RESEARCH

Research report

The significance of extracellular GABA in the substantia nigra of the rat during seizures and anticonvulsant treatments

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Accepted 11 October 1994

Abstract

The effects of the anti-epileptic drugs valproic acid and gamma-vinyl-GABA (vigabatrin) on the extracellular content of GABA was determined by microdialysis. Probes were implanted in the substantia nigra reticulata (SNR) of rats. It was found that gamma-vinyl-GABA (1000 mg/kg) induced a 4–6-fold increase in the extracellular content of GABA. This increase lasted for at least 72 h. PTZ-induced convulsions were partly antagonized by the GVG treatment. The increase of extracellular GABA after gamma-vinyl-GABA was not affected by infusion of tetrodotoxin. In contrast valproic acid (200 mg/kg), although effective in preventing pentylenetetrazol (PTZ)-induced convulsions, did not affect extracellular GABA in the SNR. PTZ-induced convulsions did not modify extracellular GABA, neither in control rats nor in valproic acid or gamma-vinyl-GABA pretreated animals. The results do not support the idea that extracellular GABA in the SNR plays a significant role in anti-convulsive treatment. However, the present data can also be interpreted that extracellular GABA, as sampled by microdialysis, is not a reliable marker for GABA release.

Keywords: GABA; Microdialysis; Anti-epileptic drug; Valproic acid; Gamma-vinyl-GABA; Vigabatrin

1. Introduction

Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the central nervous system. Several studies have demonstrated the importance of GABAergic transmission for the mechanism of action of anticonvulsive treatments [12,13,24]. Administration of drugs that antagonize GABA receptors often results in focal or generalized seizures, whereas drugs that elevate GABA levels in the brain are used to treat various forms of epilepsy [8]. Among the subcortical structures in the brain that are believed to be involved in epilepsy and anticonvulsive treatment, the substantia nigra has received considerable attention [12]. The substantia nigra reticulata (SNR), one of the main output structures of the basal ganglia, contains a high concentration of GABA [9,23,30]. Application of GABA agonists and GABA elevating agents into the SNR has been shown to suppress seizures in different models of epilepsy [5,14,15,19,27–29,36]. It has been hypothesized that GABAergic neurotransmission in the SNR is involved in both tonic and clonic seizures produced in the hindbrain, as well as in clonic seizures originating in the forebrain.

Valproic acid (VPA) and vigabatrin (gamma-vinyl-GABA; GVG) are widely used anti-epileptic drugs. The mechanisms of action of these drugs are often related to their effects on GABA metabolism. The way VPA acts as an anti-epileptic is still unknown. Inhibition of GABA transaminase, which was originally proposed as mechanism of action [20,25], is now generally questioned. There is substantial evidence that VPA increases GABA turnover and thereby potentiates GABAergic functions in some specific brain areas such as the SNR [24]. GVG is a clinically effective anti-epileptic drug that can inhibit various types of induced convulsions [19,21,22,34]. The drug elevates GABA levels in many parts of the brain and this effect is explained by irreversible inhibition of GABA transaminase [10,17]. Both GVG and VPA increase GABA levels in various brain areas and it has been hypoth-

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SSDI 0006-8993(94)01240-7

esized that this will finally lead to enhanced GABAergic neurotransmission. Others have challenged this concept by stating that whole tissue GABA is in general a poor marker of GABAergic transmission and that synaptic concentrations of GABA should be considered [11,18]. In this context the extracellular levels of GABA have not yet been thoroughly studied. We thought it was of interest to investigate whether extracellular GABA concentrations, as sampled by microdialysis, may contribute to our insight in the mechanisms of chemically induced convulsions and anticonvulsive treatments.

In the present study we determined the effects of GVG and VPA on the levels of extracellular GABA in the SNR. In addition we studied the effect of convulsive doses of pentylenetetrazol (PTZ) in control rats and in rats pretreated with GVG or VPA.

2. Materials and methods

2.1. Animals, drug treatment and doses

Male Wistar albino rats (250–320 g) were used for the experiments (C.D.L., Groningen, the Netherlands). The rats were housed in plastic transparent cages (35×35×40 cm) and had free access to food and water; they were kept in a room at 22–24°C, with a 12-h light cycle.

The following drugs were used: pentylenetetrazol (PTZ) (Sigma, St. Louis, MO, USA; 60 mg/kg i.p.), valproic acid, sodium salt (VPA; Research Biochemicals Inc. Natick, USA; 200 mg/kg i.p.), gamma-vinyl-GABA (GVG, Merril Dow Institute, Strasbourg, France; 1000 mg/kg i.p.) and tetrodotoxin (TTX; Sigma, St. Louis, MO, USA; 1 μmol/l applied by infusion through the microdialysis probe).

The experiments were approved by the Animal Care Committee of the Faculty of Mathematics and Natural Science of the University of Groningen.

2.2. Surgery and brain dialysis

Microdialysis perfusions were performed with I-shaped cannulas that were implanted into the substantia nigra reticulata (SNR). The exposed tip of the dialysis membrane was 2 mm. The dialysis tube (i.d.: 0.22 mm; o.d.: 0.31 mm) was prepared from polyacryonitrile/sodium methalylsufonate polymer (AN 69, Hospal, Bologna, Italy). Implantation coordinates were: 5.7 mm posterior to the bregma, 2 mm lateral to the midline and 8.4 mm vertically [32]. The probes were fixed by dental acrylic cement which was fixed by stainless steel screws to the skull. Implantation was carried out under general chloralhydrate anesthesia (400 mg/kg, i.p.) and local lidocaine (6%) anaesthesia. When the experiments were terminated, the rat was given an overdose of chloralhydrate and the brain was fixed with 4% paraformaldehyde via intracardiac perfusion. Coronol sections (40 μm thick) were made, and dialysis probes placement localized according to the atlas of Paxinos and Watson [32].

The perfusion experiments started 24 h after the implantation of the probes. Perfusions were carried out with a Ringer solution at a flow rate of 2.8–3 μl/min (perfusor VI, B. Braun, Melsungen, FRG). The composition of Ringer solution was (in mmol/l) : NaCl, 140.0; KCl, 4.0; $CaCl_2$, 1.2; and $MgCl_2$, 1.0.

2.3. Chemical assay

GABA was quantified with an automated on-line HPLC assay, after automated precolumn derivatization with *o*-phtaldialdehyde, as described previously [39]. In brief, the outlet tube of the microdialysis probe was connected via a T-piece with tubing containing the derivatizing reagent (flow 1 μl/min). The mixture was directly led into a loop (50 μl) of an HPLC valve, in which the derivatization took place. The valve was controlled by an electronic timer that kept it subsequently for 15 min in the load position and 15 s in the inject position.

The derivatized amino acids were separated by an isocratic HPLC separation in conjunction with a fluorimeter. The HPLC system consisted of a S3 ODS2 Spherisorb column (4.2×1000 mm; particle size 3 mm), a Perkin-Elmer Series 10 pump (Norwalk, CT, USA) and a fluorimeter (Autoanalyzer II, Technicon), equipped with a primary interference filter (340 nm; Perkin-Elmer) and a secondary a secondary cut off filter (Kodak Wratten 3-72). The mobile phase consisted of 0.05 mol/l Na_2HPO_4, 0.001 mmol/l Na_2EDTA, 0.6% (v/v) tetrahydrofuran, and 43% (v/v) methanol (adjusted to pH 6.0 with phosphoric acid).

The derivatization reagent was prepared as follows: 5 mg *o*-phtaldialdehyde was dissolved in 50 μl methanol and added up to 5 ml 0.5 mol/l $NaHCO_3$ (pH adjusted to 9.5 with a NaOH solution) containing 10 μl 2-mercaptoethanol. The reagent was freshly prepared daily.

2.4. Assessment of the seizure activity

Seizure stage and seizure latency were the two parameters used to evaluate anti-epileptic activity of the drugs. Full seizure activity began with irritability, hyperexcitation, forelimb cloni, proceeding the generalized convulsions and jumping. Seizure stages were rated according to the following criteria [6,26,32]:

Stage 0: no effect.

Stage I: facial movements; hyperactivity; sniffing; forelimb cloni which are short lasting and not successive.

Stage II: twitches of the face or body muscles; successive forelimb cloni.

Stage III: forelimb and/or hindlimb clonic convulsion with rearing.

Stage IV: generalized tonic-clonic convulsions with rearing and falling down.

Stage V: generalized convulsion with rearing, falling down, jumping and periods of tonus.

The animals that had no seizures within 20 min were considered as protected. Seizure latency was defined as the time that elapsed from the injection of PTZ to the first two myoclonic jerks of the forelimbs. This is considered to be the first sign of the beginning of a seizure activity [33].

2.5. Expression of results and statistics

The average of the last 3 or 4 stable dialysis samples before the drug treatment was considered as the control and defined as 100%. All values given are expressed as percentages of controls. Differences between the average dialysate concentrations of the control and drug treated animals were compared by Kruskal–Wallis analysis of variance by ranks. Comparison of the means (when H value greater than the 95% confidence level) was carried out using Wilcoxon matched pairs signed ranks two-sided test.

Seizure stages have been evaluated by Kruskal–Wallis and Mann–Whitney U-test. Mean seizure latencies were compared with two tailed Student's t-test, after one way analysis of variance (ANOVA) was applied to GVG and control groups (MINITAB PC program/1991).

3. Results

3.1. Basal output

The basal output of GABA expressed as fmol/min ± S.E.M. and not corrected for recovery was 61.3 ± 12.4 (24 h after implantation; $n = 24$) and 37.5 ± 8.7 (48 h after implantation; $n = 12$).

3.2. Effect of i.p. administered GVG on extracellular GABA in the substantia nigra

GVG was administered at a dose of 1000 mg/kg (Fig. 1). During the first 6 h after administration GVG induced an increase in the levels of extracellular GABA to about 300% of the controls. Twenty-four h later GABA dialysate concentration levels were increased to about 450% of controls. To investigate whether the increased GABA release was directly related to nerve impulse activity, TTX (1 μmol/l for 60 min) was added to the perfusion fluid. Fig. 1 shows that TTX was unable to modify the GABA output. 72 h after administration extracellular GABA was still increased 4-fold (data not shown).

3.3. Effect of i.p. administered VPA on extracellular GABA in the substantia nigra

VPA was administered at a dose of 200 mg/kg. Extracellular GABA concentrations were recorded the first hour after the injection of VPA. During this period VPA displayed optimal seizure protection. Fig. 2 shows that extracellular concentrations of GABA in the SNR were not affected by VPA.

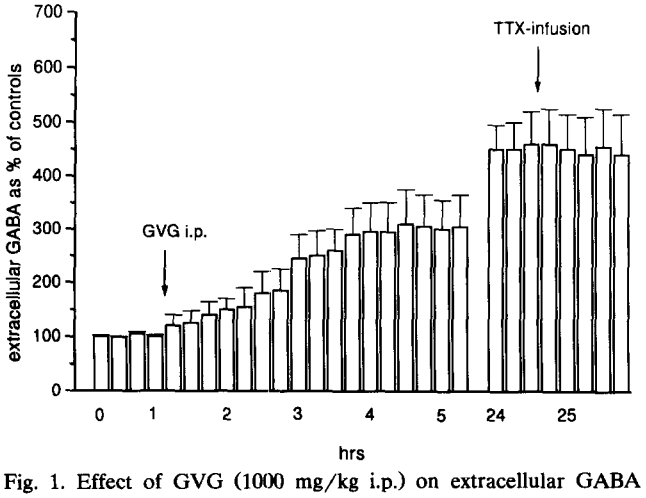

Fig. 1. Effect of GVG (1000 mg/kg i.p.) on extracellular GABA concentrations in the SNR. 24 h after GVG treatment TTX was infused in a concentration of 1 μmol/l. Data are given as % of controls ± S.E.M. ($n = 6$).

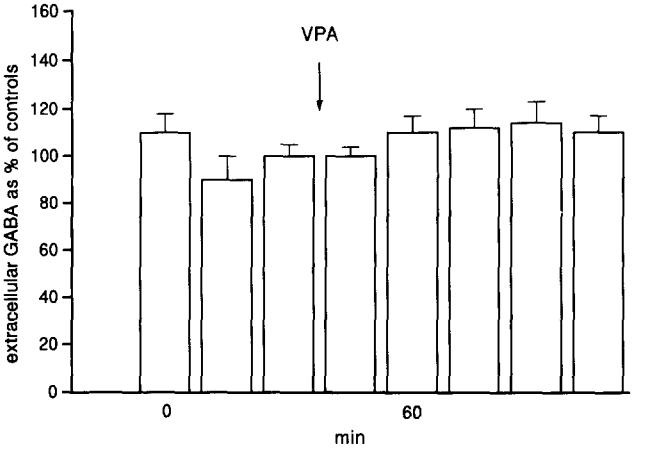

Fig. 2. Effect of VPA (200 mg/kg i.p.) on extracellular GABA concentrations in the SNR. Data are given as % of controls ± S.E.M. ($n = 5$).

3.4. Effect of PTZ on extracellular GABA in the substantia nigra of controls and GVG or VPA treated rats

PTZ was injected i.p. at a convulsive dose of 60 mg/kg. During the convulsions no significant change in the extracellular content of GABA in the SNR was observed (Fig. 3).

Next PTZ was administered to the GVG or VPA treated rats. Extracellular GABA levels in SNR are shown in Figs. 4 and 5. Again no significant change was observed in the levels of extracellular GABA after the injection of PTZ.

3.5. Anticonvulsant effects of GVG and VPA in operated, non operated and control rats

All PTZ injections induced tonic clonic seizures of stage IV or V (Table 1). Seizure stages were compared

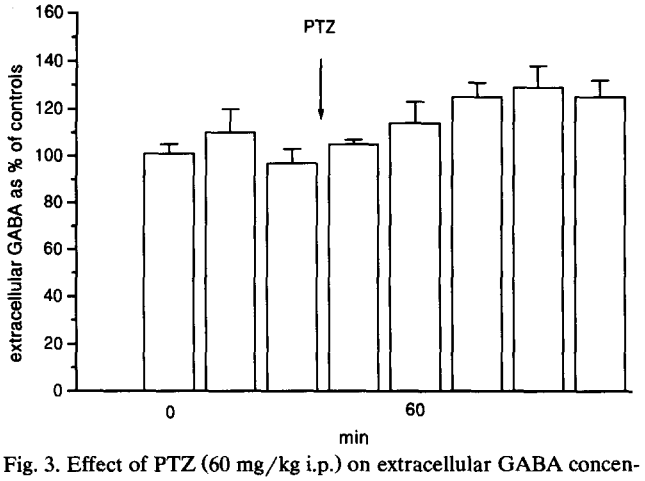

Fig. 3. Effect of PTZ (60 mg/kg i.p.) on extracellular GABA concentrations in the SNR. Data are given as % of controls ± S.E.M. ($n = 4$).

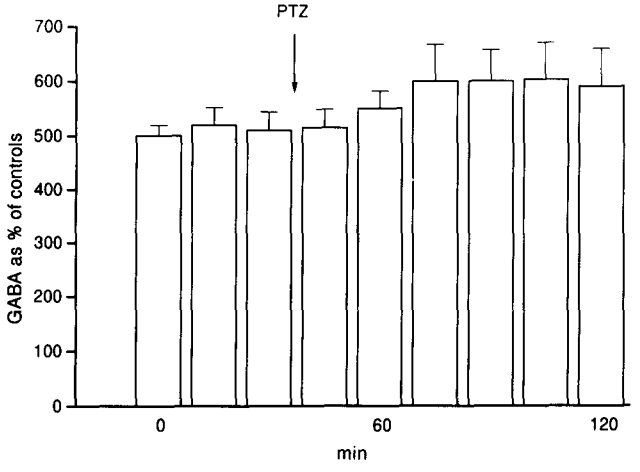

Fig. 4. Effect of PTZ (60 mg/kg) on extracellular GABA concentrations in the SNR of GVG pretreated animals. GVG was administered 24 h earlier. Data are given as % of controls (before GVG treatment) ± S.E.M ($n = 5$).

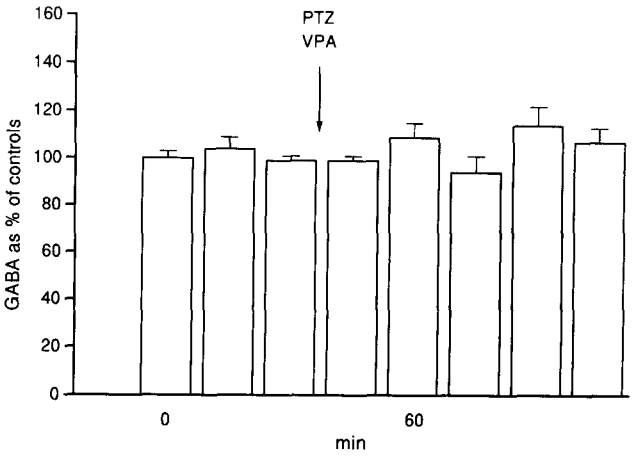

Fig. 5. Effect of combined treatment of VPA and PTZ (60 mg/kg) on extracellular GABA concentrations. Data are given as % of controls ± S.E.M. ($n = 4$).

in all groups by Kruskal–Wallis ($H = 36.3$, $P < 0.05$). Groups were compared according to Mann–Whitney U (Table 1).

The difference between the operated and non-operated control groups did not reach the level of statistical significance.

GVG had only a partial anticonvulsive effect. The seizure stage was not different in GVG rats without probe (group 5), compared to rats without probe (group 2) ($P > 0.05$). However seizure stage was different in GVG rats (group 3), compared to PTZ control rats (group 1) ($P < 0.05$).

VPA was a very potent anti-epileptic drug against PTZ seizures. Groups 4 and 6 had significantly different seizure stages compared to the control groups 1 and 2, respectively ($P < 0.05$).

Seizure latency was assessed only in GVG and control groups, since the VPA treated rats had virtually no seizure latency score. Two tailed t-tests showed that seizure latency was significantly prolonged in GVG treated group ($P < 0.001$)

4. Discussion

The present data show that the anti-epileptic drugs GVG and VPA had very different effects on extracellular GABA levels in the SNR. Although VPA completely blocked the PTZ-induced convulsions 30 min after its administration, it did not affect extracellular GABA in the SNR. These results are in good agreement with earlier studies: a similar dose of VPA was found to be ineffective in modifying GABA levels in a push–pull experiment carried out in the substantia nigra [9] and in a microdialysis study carried out in the hippocampus [2]. GVG only partially suppressed the PTZ-convulsions as reported earlier [29]. However,

Table 1
Seizure stages and latencies in PTZ-treated groups

Group name:	Control with probe	Control without probe	GVG with probe	VPA with probe	GVG without probe	VPA without probe
Group No.:	Group 1	Group 2	Group 3 [b,c]	Group 4 [b,c]	Group 5 [d]	Group 6 [d,e]
No. of Animals:	6	15	7	5	10	10
STAGE 0	–	–	–	60%	–	50%
STAGE I	–	–	–	20%	–	30%
STAGE II	–	–	14%	20%	20%	20%
STAGE III	–	27%	43%	–	40%	–
STAGE IV	34%	60%	43%	–	30%	–
STAGE V	66%	13%	–	–	10%	–
Seizure Latency (s)	135.8 ± 29.5	89.9 ± 7	615 [a] ± 75.4	–	444 [a] ± 34	–

[a] $P < 0.001$ vs. controls (Supplementary Student-test, following ANOVA). [b] Different from control with probe (group 4) $P < 0.05$ (Mann–Whitney U-test). [c] Different from the other antiepileptic drug in rats with probe (GVG or VPA) $P < 0.05$ (Mann–Whitney U-test). [d] Different from the other antiepileptic drug in rats without probe (GVG or VPA) $P < 0.05$ (Mann–Whitney U-test). [e] Different from the control group without probe $P < 0.05$ (Mann–Whitney U-test).

GVG induced a strong increase in extracellular GABA, that lasted for at least 72 h. The time course of the GVG effects on the GABAergic system has been reported in earlier papers e.g. [12,13,16]. Tissue levels of GABA increased several fold after a single injection of GVG; maximal concentrations were reached 60 h after administration of the drug and this increase paralleled the development of anticonvulsive effects [12,13]. The results of the present study are in good agreement with these tissue data.

Gale [13] hypothesized that the increase in tissue GABA after GVG treatment represents two GABA pools: a storage compartment and a neurotransmission compartment. In her view the neurotransmission pool becomes first evident 24–72 h after administration of GVG, when the anti-convulsive efficacy of the drug is optimal. To test this hypothesis we further investigated the nature of the GVG-induced rise in extracellular GABA. During microdialysis experiments the neuronal origin of transmitter concentrations can be demonstrated by infusion of the sodium channel blocker tetrodotoxin (TTX) via the microdialysis probe [38]. We found that 24 h after administration of GVG the increased GABA levels were clearly not affected by TTX-infusion. A similar experiment carried out 48 h after GVG-administration was also without effect (data not shown). These results do not support the assumption that the GVG-induced increase in extracellular GABA – as detected by microdialysis – is directly derived from GABAergic neurotransmission.

A significant finding of the present study is that PTZ, although it induced pronounced and long-lasting convulsions, did not cause statistical significant changes in extracellular GABA in the SNR. PTZ treatment also failed to affect extracellular GABA in the GVG- and VPA-pretreated rats. In this respect extracellular GABA differs from other neurotransmitters as convulsions induced during microdialysis experiments were reported to induce marked increases in extracellular levels of dopamine, serotonin and acetylcholine [40].

When summarizing our results it is apparent that VPA was able to abolish PTZ-induced seizures without elevating the GABA levels in SNR. In contrast, GVG, although only partly effective in preventing seizures after PTZ, increased extracellular GABA in SNR several fold during various days. At first glance these data strongly challenge the assumption that an increase in extracellular GABA in the SNR is per se anticonvulsive. However, these negative findings should also raise the question whether extracellular GABA, as sampled by microdialysis, represents neuronal GABA release. Doubt has been expressed as to the sources of basal GABA levels in brain dialysates [35,37,39]. Various authors have demonstrated that TTX is without effect on extracellular GABA levels in microdialysates [4,7,35,39]. Others have found that only a minor part (maximally 40%) responded to TTX [3,31]. Similar data have been reported with regard to the calcium-dependency of GABA in dialysates [3,4,31,39]. We have hypothesized that GABA in dialysates is derived from both a neuronal and a non-neuronal origin [35]. Others have postulated that reversal of the uptake carrier may induce a TTX- and calcium-insensitive release of GABA [1]. However it has to be established whether the latter type of release is a physiological process. At present we conclude that, at least in the SNR, non-neuronal extracellular GABA dominates over the GABA that is derived from neurotransmission. The present results should not be interpreted as excluding the possibility that the neuronal release of GABA is not modified during convulsions or anti-epileptic treatment. They only suggest that it is difficult to demonstrate such effect by microdialysis.

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