

Full Paper

The Effects of Ethosuximide on Amino Acids in Genetic Absence Epilepsy Rat Model

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Abstract. Genetic absence epilepsy rats from Strasbourg (GAERS), a selectively inbred strain of Wistar rats, has been validated as an experimental model for human absence epilepsy. In this model, systemic administration of ethosuximide (ETX) was shown to reduce the spike and wave discharges (SWD). In this study, γ -aminobutyric acid (GABA) and L-glutamic acid levels in response to ETX injections (i.p., 100 mg/kg) were measured in the microdialysis samples collected from the ventrolateral thalamus (VLT) and the primary motor cortex (M1) area of Wistar rats and GAERS by using HPLC with fluorescent detection. Throughout the microdialysis procedure, continuous EEG recording was performed where ETX was shown to suppress the SWD activity. We demonstrated increased basal GABA levels in the M1 and VLT of GAERS, and ETX treatment did not produce any effect on higher GABA levels in the VLT, but suppressed the increased GABA levels significantly in the M1 of GAERS. All these findings denote the importance of corticothalamic circuitry and the role of increased GABA tonus in primary motor cortex and thalamus of GAERS. The primary motor cortex also seems to be involved in the SWD activity and ETX exerts, at least partially, its neurotransmitter effects through it.

Keywords: γ -aminobutyric acid, L-glutamic acid, primary motor cortex, thalamus, absence epilepsy

Introduction

The International League Against Epilepsy (ILAE) defines absence epilepsy as a generalized form of epilepsy that affects 2–10% of children worldwide (1). Absence epilepsy is characterized by a sudden interruption of both physical and mental activity, without major loss of postural tone, coupled to bilateral synchronous spike and wave discharges (SWD) in the electroencephalogram (EEG) (2). The results of many neurophysiological, behavioral, pharmacological, and genetic studies validated “Genetic absence epilepsy rats from Strasbourg” (GAERS) as an experimental model of human absence epilepsy that is a selectively inbred strain of Wistar rats generating SWD spontaneously

through their lifetime (3). The seizures were bilateral and synchronous over the surface of the somatosensory cerebral cortex where they start and end abruptly on a normal EEG background (3). In this model, absences are associated with behavioral immobility and being unresponsive to sensory stimuli, thus reproducing the seizures observed in humans during absence epilepsy. The SWDs generated by the GAERS constitute one-third of a time, that is, they experience a seizure of 20 s within 1 min (3). Ethosuximide (ETX) is a first order anti-absence agent in controlling the SWD of GAERS, so is valproate (4).

The typical absence seizures are generated as a result of a complex interaction between the thalamus and cortical structures (5–7). This thalamocortical circuitry is under control of several specific inhibitory and excitatory systems arising from the forebrain and brainstem. Excess γ -aminobutyric acid (GABA) mediated activity

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has been suggested in the pathogenesis of absence seizures since longstanding hyperpolarization is required to activate low-threshold Ca^{2+} currents (8). The GABA levels were detected to be higher in the ventrolateral thalamus (VLT) of GAERS compared to non-epileptic controls in a previous microdialysis study (9). In a preliminary report, we also demonstrated that GABA levels were increased in the primary motor cortex (M1) area (10). Therefore, the aim of the present study is to demonstrate the changes in GABA and L-glutamic acid levels in the VLT and M1 area of GAERS following systemic ETX treatment using the microdialysis technique.

Materials and Methods

Animals

Four-month-old GAERS (supplied from INSERM Unité, Strasbourg, France) and Wistar rats (supplied from Marmara University, Experimental Research and Animal Laboratory) weighing 250–275 g were used. All experiments were carried out with humane methods and approval of the Marmara University Ethical Committee for Experimental Animals was obtained before the experiments. The animals were kept in a temperature-controlled, 12-h light and dark cycle, and fed with standard animal food and water. Each experimental group consisted of data gathered from 6 rats.

Microdialysis probes

Concentric microdialysis probes were made from 15-mm length 24 G stainless steel tubings (Cooper's Needle Works, Birmingham, UK). The inlet and the outlet tubes were made of fused silica (OD: 0.19 mm, ID: 0.075 mm; SGE, Milton Keynes, UK) connected with polyethylene tubings. The inlet emerges from the tip of the stainless tubing trimmed to a length of 2 mm and the cuprophane dialysis membrane (Gambro Ltd., Gloucester, UK) was passed over it, where all joints were sealed with epoxy resin.

Stereotaxic surgery

The rats were anesthetized with an intraperitoneal ketamine (100 mg/kg) and chlorpromazine (1.0 mg/kg) mixture and placed in a stereotaxic frame (Model 51600; Stoelting, Wood Dale, IL, USA). The scalp skin was incised and the periosteum was separated from the cranium. Four cortical electrodes for EEG recordings were also implanted onto frontoparietal cortices. Screws were also placed for the support of acrylic cement. The microdialysis probe was also covered with dental acrylic cement. The probes were implanted into the right VLT (coordinates: -2.3 mm anteroposterior, -1.7 mm lateral,

6.1 mm ventral to bregma) and into left M1 area (coordinates: $+1.7$ mm anteroposterior, $+3$ mm lateral, 1.8 mm ventral to bregma) according to the Paxinos and Watson rat brain atlas (11). The implantation of 2 microdialysis probes was performed during the same stereotaxic intervention. The collection of intracerebral perfusion samples and simultaneous EEG recording were performed 24 h following the surgery.

Microdialysis procedure and EEG recording

The day after the placement of microdialysis probes, polyethylene tubings were attached to the inlet of the microdialysis probes to collect the samples in the conscious rat model in a plexyglass cage ($42 \times 42 \times 20$ cm). Artificial cerebrospinal fluid (aCSF) was delivered continuously via a 250- μl Hamilton syringe that was connected to a microinfusion pump (KD Scientific, Holliston, MA, USA). The composition of aCSF was 2.5 mM KCl, 125 mM NaCl, 1.26 mM $\text{CaCl}_2(2\text{H}_2\text{O})$, 1.18 mM $\text{MgCl}_2(6\text{H}_2\text{O})$, and 0.2 mM $\text{NaH}_2\text{PO}_4(2\text{H}_2\text{O})$ and the pH was set to 7.0. The aCSF was filtered through 0.4- μm nylon membrane filters.

Two basal samples were collected at 0.5 $\mu\text{l}/\text{min}$ flow rate every 20 min in 0.25-ml Eppendorf tubes from GAERS and Wistar rats after an equilibration period of 1 h. After the collection of basal samples, intraperitoneal physiological saline injection (1 ml/kg) was administered and three more consecutive samples were collected. The same protocol was repeated after a washout period of 2 h, following intraperitoneal ETX treatment at a dose of 100 mg/kg. The dialysates were kept at -80°C until analysis.

Throughout the microdialysis procedure, continuous EEG recording was performed on a 16S Powerlab (AD Instruments, Oxfordshire, UK) connected to bioamplifier (NL 136). All data were stored and analyzed by using software (Chart for Windows, Version 5). The criteria used for identifying the SWD in the EEG recordings were morphology, amplitude, and the frequency. Cumulative duration of SWD generated within 20-min intervals was accepted as "spike and wave discharge incidence". The rats were decapitated upon the completion of the experiments and the brains were cut in 50- μm slices using a microtome (Microm, Walldorf, Germany). The brain slices were stained with thionine dye and the probe placement was confirmed using a rat brain atlas (11). Only histologically verified experiments were included in the data analysis.

Chromatographic system and HPLC analysis of the samples

Chromatographic system consists of a gradient pump (AGILENT 1100; Waldbrom, Germany) with four

solvent bottles, degasser module, C18 reverse phase nucleosil column (15 cm and 3.9 cm length, 4.6 mm diameter and 5 μ m pore size), autosampler unit, fluorescent detector with excitation and emission wave lengths of 360 nm and 410 nm, respectively, and a computer. The mobile phase is composed of 250 mM Na acetate (pH 6.9), deionized HPLC grade water, and methanol, where 0.5%(v/v) tetrahydrofuran was added in all solutions. A gradient flow with an equilibration period of 10 min was delivered at a flow rate of 0.5 ml/min. The temperature of the column is set to 25°C. Pre-column derivatization was performed with *o*-phthalaldehyde (Sigma, St. Louis, MO, USA) and 3-mercaptopropionic acid (Sigma). Injections were given within a volume of 12 μ l with the aid of an autosampler unit using an injection software. The retention times of L-glutamic acid and GABA were 5.96 and 29.94 min, respectively. The chromatographic analysis was carried out with the aid of software (Chemstation).

Data analyses

All data are expressed as means \pm S.E.M. Two-tailed Student's *t*-test for ungrouped data was used to determine the differences between the basal levels of GABA and L-glutamic acid in the M1 area and VLT of Wistar rats and GAERS. The effect of physiological saline or ETX treatment on cumulative SWD duration or amino acids were tested using Two-way Analysis of Variance (ANOVA) followed by Dunn's Multiple Comparison Test as a *post-hoc*. The statistical significance was defined as $P < 0.05$.

Results

Analysis of EEG

All Wistar rats exhibited no SWD, but a normal rat EEG pattern. The mean of cumulative SWD duration recorded during the 20-min interval was 181 \pm 32 s in GAERS (n = 6). Intraperitoneal injection of ETX at 100 mg/kg produced an immediate reduction in the 20-

min cumulative SWD duration compared to the pre-injection 20-min period values ([-20-0] min, $P < 0.05$; Fig. 1). Physiological saline injection produced no significant change in the cumulative SWD duration in GAERS and neither did it produce any effect on the EEG pattern of Wistar rats.

Comparison of extracellular concentrations of L-glutamic acid and GABA in microdialysis samples in Wistar rats and GAERS

In the samples collected within 20-min intervals from the M1 area of GAERS, the L-glutamic acid levels were found to be significantly decreased compared to Wistar rats (Table 1, $P < 0.01$). The comparison of GABA levels detected in the samples of the M1 area demonstrated that GABA was significantly increased in GAERS (Table 1, $P < 0.05$, n = 6).

In the perfusates collected from the VLT, no significant difference was observed in the L-glutamic acid levels ($P = 0.7430$), but the GABA levels were significantly higher in the GAERS compared to that of Wistar rats (Table 1; $P < 0.05$, n = 6).

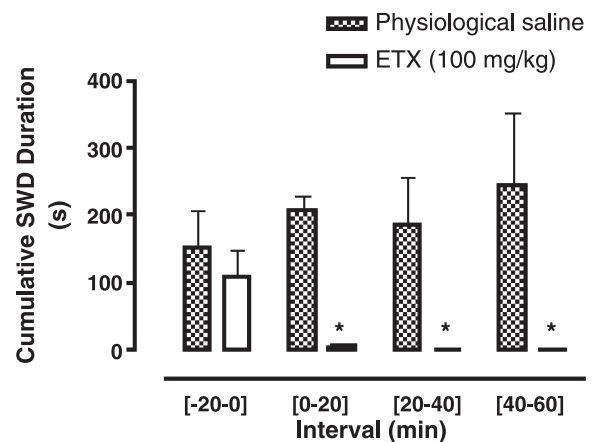


Fig. 1. The effect of intraperitoneal physiological saline or 100 mg/kg ethosuximide injections on cumulative spike and wave discharge duration in GAERS (n = 6). The injections were given at time 0 min. * $P < 0.05$ (compared to the baseline [-20-0] value).

Table 1. The basal extracellular concentrations of L-glutamic acid and GABA in the microdialysis samples collected from primary motor cortex (M1) area and ventrolateral thalamus of Wistar rats and GAERS, at a flow rate of 0.5 μ l/min

Brain region	Amino acid	Concentration (μ M)	
		Wistar (n = 6)	GAERS (n = 6)
M1 area	L-Glutamic acid	1.61 \pm 0.14	0.98 \pm 0.20**
	GABA	0.18 \pm 0.01	0.77 \pm 0.28*
Ventrolateral thalamus	L-Glutamic acid	1.55 \pm 0.22	2.47 \pm 0.40
	GABA	0.22 \pm 0.04	0.74 \pm 0.20*

* $P < 0.05$; ** $P < 0.01$ (compared to that of the Wistar strain).

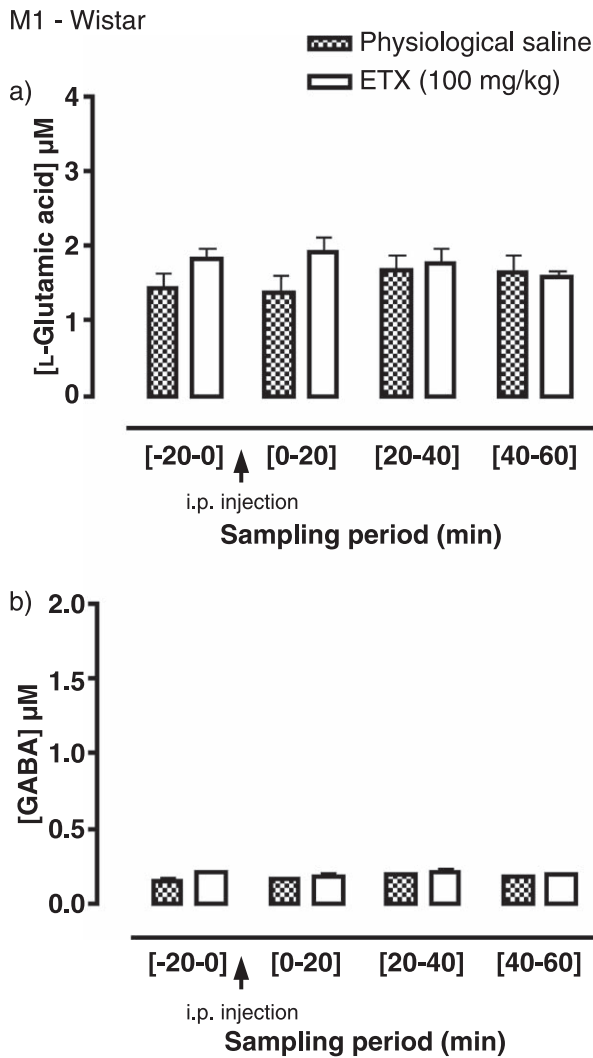


Fig. 2. The effect of physiological saline or ethosuximide injections on L-glutamic acid (a) and GABA (b) levels detected in the microdialysis samples collected from the primary motor cortex of Wistar rats ($n = 6$).

The Effect of ETX injection on neurotransmitter release in the frontal M1 area

Neither physiological saline nor ETX injection produced a change in the L-glutamic acid and GABA levels in Wistar rats (Fig. 2: a and b). Physiological saline injection also produced no significant change in L-glutamic acid and GABA levels of GAERS (Fig. 3: a and b). Extracellular concentration of L-glutamic acid following ETX injection was found to be slightly decreased but this decrease was not found to be significant when analyzed (Fig. 3a). ETX injection produced a significant decrease in the GABA level of the [0-20] min sample collected from the M1 area of GAERS (Fig. 3b; $P < 0.05$, $n = 6$).

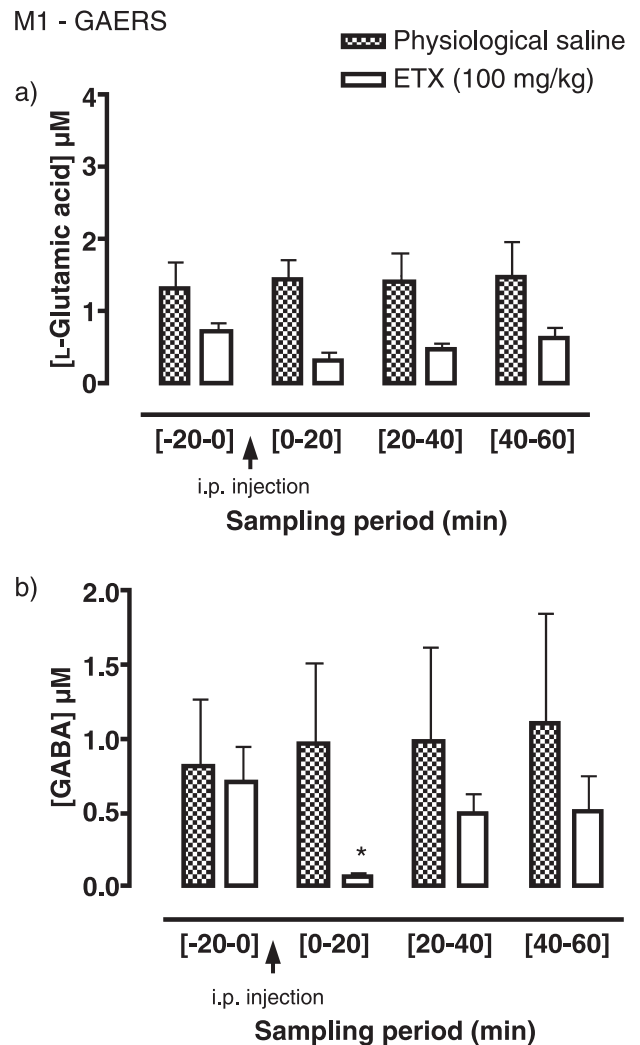


Fig. 3. The effect of physiological saline or ethosuximide injections on L-glutamic acid (a) and GABA (b) levels detected in the microdialysis samples collected from the primary motor cortex of GAERS ($n = 6$). * $P < 0.05$ (ANOVA, Dunn's Multiple Comparison Test)

Effect of systemic administration of ETX on neurotransmitter release in the VLT

Physiological saline injection produced no significant change in both L-glutamic acid and GABA levels in the samples collected from the VLT of Wistar rats (Fig. 4: a and b, $n = 6$). Likewise, physiological saline injection did not produce a significant change in GAERS (Fig. 5: a and b, $n = 6$). Although the extracellular concentration of L-glutamic acid in the VLT of GAERS decreased from $2.35 \pm 0.72 \mu\text{M}$ to $0.56 \pm 0.19 \mu\text{M}$ within 20 min following ETX (100 mg/kg) injection; this decrease was not found to be significant ($P = 0.297$, Fig. 5a). Also, the GABA levels in the dialysates collected from the VLT decreased from $0.55 \pm 0.33 \mu\text{M}$ to $0.31 \pm 0.08 \mu\text{M}$ after ETX injection, but this decrease did not reach statistical

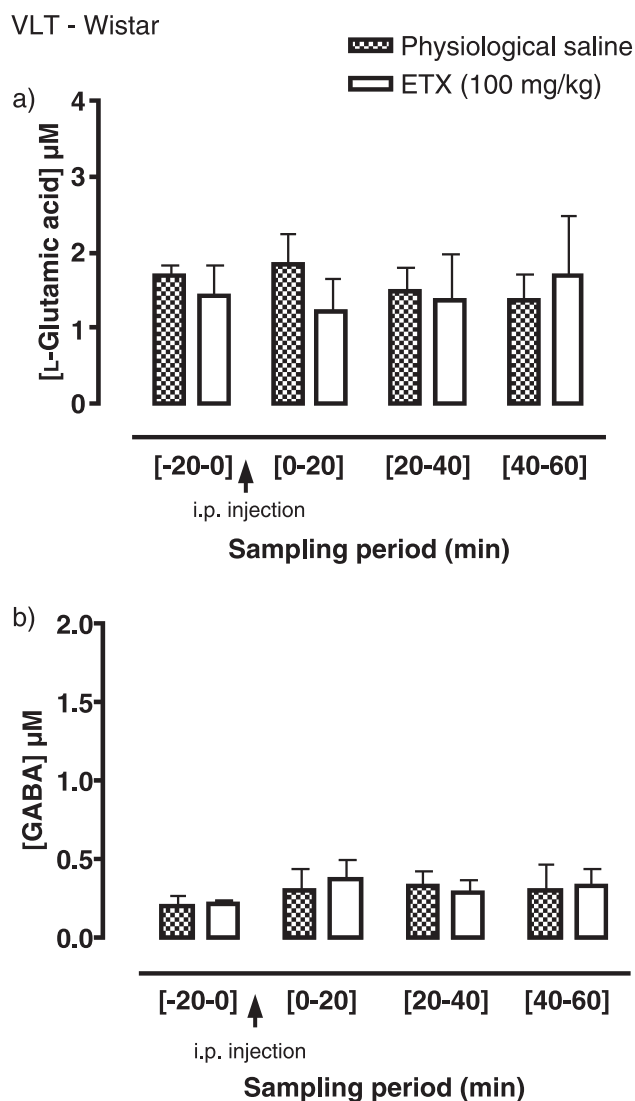


Fig. 4. The effect of physiological saline or ethosuximide injections on L-glutamic acid (a) and GABA levels (b) in the microdialysis samples collected from the ventrolateral thalamus of Wistar rats ($n = 6$).

significance ($P = 0.146$, Fig. 5b).

Discussion

In the present study, we demonstrated that systemic injection of ETX reduced the GABA levels in the M1 area of GAERS. This finding is in agreement with the previous studies showing that the site of action of ETX involves the primary somatosensory cortex. A previous study performed in the WAG/Rij rat model of absence epilepsy by using non-linear association analysis of cortical and thalamic EEG signals during the first 500 ms of SWD showed a seizure initiation site within the peri-oral region of the primary somatosensory cortex

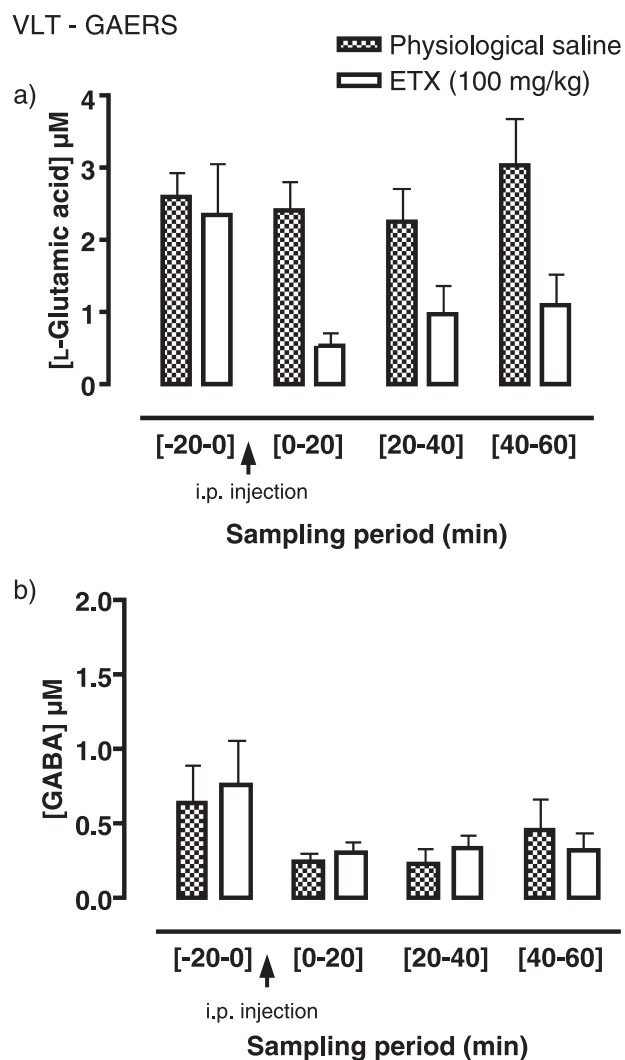


Fig. 5. The effect of physiological saline or ethosuximide injections on L-glutamic acid (a) and GABA levels (b) in the microdialysis samples collected from the ventrolateral thalamus of GAERS ($n = 6$).

rather than other cortical and thalamic areas (7). Moreover, it was demonstrated that microinfusion of ETX into the peri-oral region of the primary somatosensory cortex immediately cleared the SWD activity in the GAERS model (12), whereas the infusion of ETX into the ventrobasal thalamus and reticular thalamic nucleus produced a modest and delayed reduction in the duration of SWD (13). The delayed and weaker response to intrathalamic injection of ETX makes it seem unlikely that the drug preferentially acts at the level of the thalamus. In the present study, we demonstrated that the effect of ETX on SWD activity in GAERS lasted more than 60 min; however, the suppression of GABA level in the M1 area was observed within the first 20 min. Thus,

the discrepancy between time-course suppression of SWD on EEG and GABA release in GAERS treated with ETX suggests that the anti-absence properties of ETX cannot be fully explained by the action on the GABA levels in the M1 area. ETX, a first choice anti-absence drug (14), has been shown to reduce low-threshold T-type Ca^{2+} current in a voltage-dependent manner in the thalamus of rat and guinea pig (15–17). In addition to a direct Ca^{2+} channel-blocking action of ETX, the drug also decreases the persistent Na^+ and Ca^{2+} -activated K^+ currents in the thalamic and layer V cortical pyramidal neurons (18, 19). The blockade of either thalamic low-threshold T-type Ca^{2+} channels or slow inactivated Na^+ currents and Ca^{2+} -activated K^+ currents is commonly believed to be responsible for the action of the drug on SWD. These previous *in vitro* studies showed a 40% reduction in the amplification of low-threshold T-type Ca^{2+} current by therapeutic concentrations of ETX, indicating that the blockade of low threshold Ca^{2+} currents is not entirely responsible for the suppressive effect on SWD in absence epilepsy. Taken together, ETX not only produced antiseizure effects through the reduction in the channel conductances, but also through affecting neurotransmitter release, namely, GABA level as observed in the present study. Additionally it has long been known that systemic administration of ETX produces immediate and marked reduction in SWD in a dose-dependent fashion, that is, 25 mg/kg ETX reduces SWDs by 50% and 50 and 100 mg/kg reduces them over 90% (14). In our preliminary experiments, ETX trials were carried out using different doses and the most effective dose that could suppress the SWD was found to be 100 mg/kg. Therefore, GABA and L-glutamic acid levels using the microdialysis technique were measured in an immediate and almost complete cessation of absence seizure activity in GAERS following systemic ETX treatment.

The involvement of the thalamocortical circuit, particularly the contribution of the ventrobasal thalamus and the reticular thalamic nucleus, in the propagation of absence seizures has been established in several species for several years (20–22). Corticothalamic rhythms are believed to be involved in the generation of SWD. Thalamic neurons have the ability to shift between an oscillatory and burst firing mode (23) where this shift is important in the regulation of external stimuli. Oscillatory neuronal behavior of the circuit is driven by the reticular thalamic nucleus that is situated between the thalamus and the cortex to influence the flow of information (24). The reticular thalamic nucleus comprises mainly of GABA-containing neurons projecting to the thalamic relay nuclei (25). Systemic administration of either GABA_A - or GABA_B -receptor agonists

enhances the duration of SWD in GAERS in a dose-dependent fashion (25). Moreover, local microinjection of γ -vinyl GABA, an irreversible inhibitor of GABA transaminase, into the thalamic relay nuclei has been shown to increase the duration of SWD in GAERS (26). Furthermore, blockade of the GABA_B receptor at the level of the ventrobasal thalamus or reticular thalamic nucleus is known to result in a reduction in seizure activity (13, 27). Collectively, expression of typical absence seizures is known to involve a tight interaction between the thalamic and cortical structures (5, 6). Therefore, the role of the component parts of this circuitry is still a matter of debate. In the present study, we once more demonstrated the increased basal GABA concentrations in the M1 area and partly the VLT of non-treated GAERS compared to those of Wistar rats as in non-epileptic control rats in accordance to the previous findings (9, 10). In view of these informations, we can conclude that there is increased basal GABA level primarily in the M1 area and partly in the VLT of non-treated GAERS, and the pharmacological effects of ETX on the GABA level denoted the importance of corticothalamic circuitry in the genetic model of absence epilepsy.

Besides the changes in the GABAergic system in both the specific areas of the thalamus and the cortex, a decrease in basal glutamate level in the M1 area of GAERS in the present study suggests multiple pathophysiology in the absence epilepsy model. In a previous study, the basal hippocampal extracellular glutamate level in GAERS was found to be increased when compared to that in the non-epileptic control group (28). Moreover, an increase in the density of glutamate immunolabeling within the mossy terminals of the hippocampal CA3 region and a decrease in the dentate gyrus were reported in GAERS at the electron microscopy level (29). These findings suggest that a change in the glutamatergic system might be responsible for the development and/or maintenance of absence seizures in the GAERS model.

In conclusion, our findings imply that cortical structures are important in the generation and the initiation of SWD and GABA-mediated neurotransmission in the frontal cortex is more critical than that of VLT in terms of ETX mediated neurotransmitter effects. Therefore, our data provide the supportive evidence for the multiple sites of action of ETX.

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