THE EFFECTS OF PARTIAL BILATERAL LESIONING OF SUBSTANTIA NIGRA IN A GENETIC ABSENCE EPILEPSY RAT MODEL

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ABSTRACT

Objective: "Genetic Absence Epilepsy Rats from Strasbourg" (GAERS), an inbred Wistar strain, serve as an experimental venue. These rats generate spontaneous spike-and-wave discharges (SWD) and have increased γ-aminobutyric acid (GABA) levels in the ventrolateral thalamus (VLT). Recently, substantia nigra pars reticulata (SNpr) was reported to act as an endogeneous inhibitory mechanism in the generation, onset and maintenance of various types of seizures. The presence of tonic control exerted by SNpr in absence seizures should also be tested in GAERS.

Methods: In this current study, GABA and L-glutamic acid release in VLT of GAERS with partial bilateral electrolytic lesions of SNpr was evaluated by using microdialysis technique with fluorescent detection.

Results: GABA levels in VLT were 0.12±0.04 μM and 0.24±0.08 μM in sham-lesioned and SNpr-lesioned GAERS, respectively. L-glutamic acid level was found to be 0.415±0.150 μM in sham-lesioned group and 0.324±0.094 μM in SNpr-lesioned GAERS. Statistical analysis revealed no significant difference between sham-lesioned and SNpr-lesioned rats. The number and the duration of SWD were also similar in two groups.

Conclusion: These findings show that SNpr does not exert a tonic control in GAERS and we assume that intact SNpr acts as a site that may exert an inhibition on target structures when activated in GAERS.

Key Words: GAERS (Genetic Absence Epilepsy Rats from Strasbourg), GABA (γ-aminobutyric acid), Substantia nigra, Ventrolateral thalamus, Absence epilepsy, Microdialysis, Nigral control of epilepsy.

INTRODUCTION

Existence of an endogeneous inhibitory mechanism in the seizure control was reported in different experimental models of generalized and partial epilepsy (1-6). Pharmacological studies showed that the brain regions involved in this inhibitory mechanism are not the structures primarily involved in the seizure generation. The inhibitory mechanism was first proposed by Gale (2) and also regarded as "nigral control of epilepsy"(4). Basal ganglia subserves an integrative role in the manifestation of motor

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behaviours as an interface between limbic and motor systems (7). It was reported that bilateral intranigral injections of γ-aminobutyric acid (GABA) agonists in kindling and genetic absence models of epilepsy produced suppression of seizures (5,6). Substantia nigra pars reticulata (SNpr) is the region, responsible in generating antiseizure effects (2,4). The exact mechanism of the antiepileptic effect is not yet known but striatonigral pathways seem to trigger the nigral control of epilepsy. The direct and indirect pathways of SNpr with striatum exert the antiseizure effects. The neuroanatomical studies showed that indirect pathway is comprised of relay structures reaching the superior colliculus (8,9). The mechanism of the antiseizure effect has been proposed to be a result of the tonic disinhibition of neurons in the superior colliculus(4).

Genetic Absence Epilepsy Rats from Strasbourg (GAERS), a selective inbred Wistar strain generating spike-and-wave discharges (SWD), were regarded as a valid experimental model for the absence seizures (10, 11). Increased GABA-mediated neurotransmission is an important feature of absence seizures (12). Previously, it was reported that the basal level of GABA in ventrolateral thalamus (VLT) of GAERS is higher than that of the non-epileptic Wistar control strain (13). This important finding is one of the most verified neurochemical data of experimental genetic absence seizures.

In this current paper, we aimed to demonstrate tonic functions of SNpr in GAERS. For this purpose, we performed bilateral electrolytic ablation of SNpr and examined the GABA and L-glutamic acid levels of VLT by using intracerebral microdialysis technique in conscious GAERS with simultaneous EEG recordings.

MATERIALS AND METHODS

GAERS used in this study were donated by INSERM Unité, 398, Strasbourg, France. The rats weighed 200-250 g and the mean age was approximately 4-6 months. GAERS were kept in a temperature controlled (21±3°C) room with 12 hour light and dark cycle where standard rat food and water were given. All of the experiments were performed after approval was obtained from "Marmara University Medical School Animal Care and Use Committee".

Stereotaxic surgery

GAERS were anesthetized with intraperitoneal ketamine (100 mg/kg) and chlorpromazine (1.0 mg/kg) mixture. Following anesthesia, the rats were placed in a stereotaxic frame (Stoelting, Model 51600, USA). GAERS were divided into 2 groups; sham-lesioned GAERS (n=6) and lesioned GAERS (n=6). The scalp skin was incised and the periosteum was separated from the cranium. Porcelain-covered, stainless steel electrodes were used for electrolytic ablation of SNpr. The SNpr co-ordinates were 5.2 mm anteroposterior, 2.2 mm lateral (right and left) from bregma and 8.4 mm ventral from the skull surface (14). Anodal direct current of an amplitude of 3 mA was applied for 20 seconds. After completion of bilateral electrolytic ablation of the SNpr, the holes were filled with bone-wax. The scalp was cleaned and screw electrodes for electroencephalography (EEG) recordings were placed onto frontal and parietal regions of the scalp on both sides. The screw electrodes were then connected to a female socket. The screw electrodes and the socket were sealed with dental acrylic cement by preserving the bregma and the right parietal region. The rats were left to recover in separate cages. Sham lesions were also performed with the same procedure but anodal electrical current was not applied. After 7 days, concentric microdialysis probe was inserted into the right ventrolateral thalamus (the co-ordinates: 2.8 mm anteroposterior and 1.2 mm right from bregma; 8 mm ventral from the skull surface (14) at a second stereotaxic intervention. The microdialysis probe was also covered with dental acrylic cement. Microdialysis samples were collected the day after the implantation of microdialysis probes.

Microdialysis probe

Concentric microdialysis probes were made from 15 mm length 24 G stainless steel tubings (Cooper's Needle Works, UK) as described previously (15). Inlet and outlet tubes were made of fused silica (outer diameter: 0.19 mm; inner diameter: 0.075 mm) (SGE, UK). The fused silica tubes were inserted into the steel tubings under surgical microscope and the inlet which emerges from the tip of the tubing was trimmed to a length of 2 mm. Cuprophan dialysis membrane (outer

diameter: 0.216 mm; inner diameter: 0.2 mm) (Gambro Ltd, UK) was passed over the inlet silica tubing. All of the joints of the probes were sealed with epoxy resin.

Collection of microdialysis samples and simultaneous EEG recordings

On the day of the experiment, polyethylene tubing was attached to the inlet of the microdialysis probe to collect the samples in conscious rat model. Artificial cerebrospinal fluid (aCSF) was perfused continuously via a 500 µl Hamilton syringe connected to a microinfusion pump (KDS Scientific, USA). The composition of aCSF was as follows (mM): KCl: 2.5; NaCl: 125; $CaCL_2$: 1.26; $MgCL_2$: 1.18; NaH_2PO_4: 0.2. The pH of aCSF was 7.0 and filtered through 0.4 µm nylon membrane filters. Samples were collected after a 2 to 3 hour equilibration period. The perfusion was first started at a rate of 1 µl/minute and then the samples were collected at 0.5 µl/minute perfusion rate every 20 minutes. The dialysates were kept at -70°C until analysis. EEG was recorded using Grass polygraph (Model 7; USA) with a Grass AC/DC strain gage amplifier (Model P122; USA). The chart speed was set at 10 mm/sec.

Histological verification

Following collection of the samples, the GAERS were anesthetized with ether and decapitated after the parenchymal dye was passed through the probe. The brains were stored in formol-sucrose solution and 40 µm coronal sections were taken by a cryostat at -20°C (Microm, Germany). The slices were then stained by thionine and checked for lesion sites and probe placements. Improper placements and lesion sites were not included in the data analysis. The coronal sections at the lesion sites and the exact probe placements are schematically presented in Fig 1.

Chromatographic system and HPLC analysis

Chromatographic system consists of a pump (Jasco PU-980, Japan) with a 100 µl loop and a rheodyne valve, C18 reverse phase nucleosil working and guard columns (15 cm and 2.5 cm in length, 4.6 µm in diameter, 5 µm pore size; Macherey Nagel, Germany), fluorescent detector (excitation and emission wavelengths, 360 nm and 495 nm respectively; Waters Model 420, USA) and a computer. The area under curve in the chromotograms was analyzed by Borwin Chromotography software (Version 1.20,

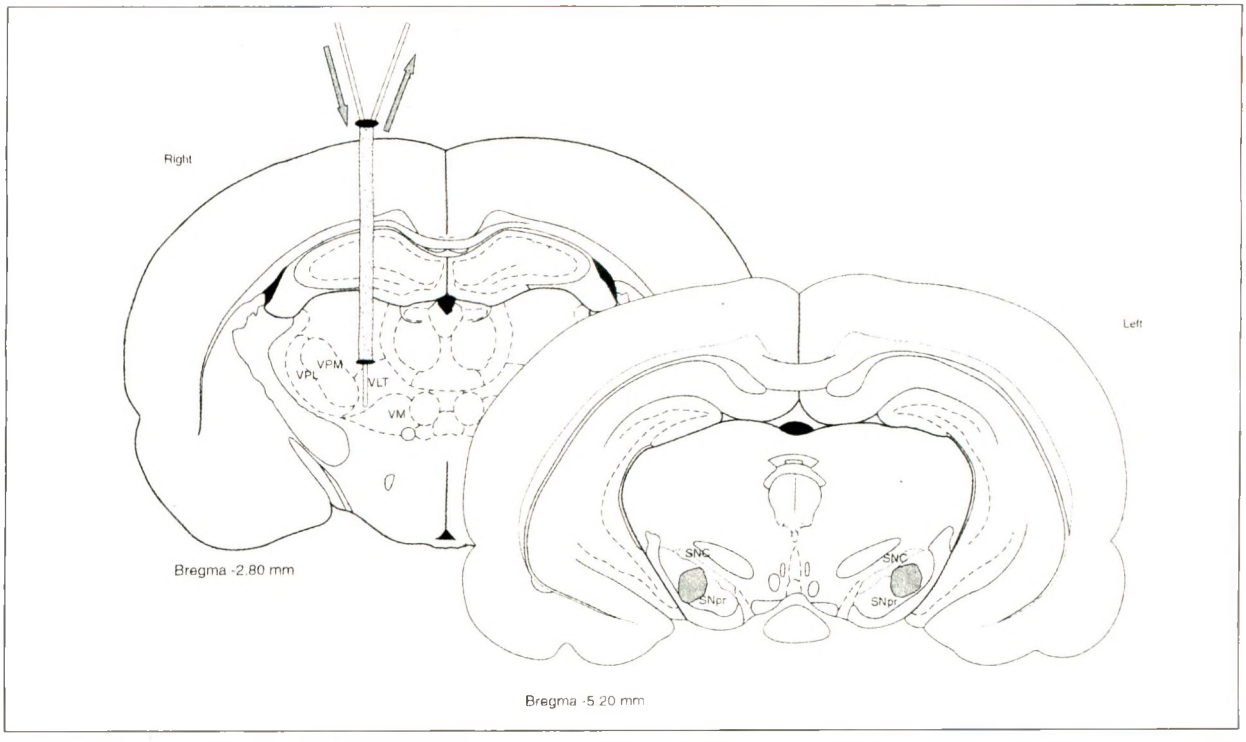

Fig. 1 : The schematic presentation of bilateral electrolytic lesions of SNpr and microdialysis probe placement in VLT (VLT: ventrolateral thalamus; VPL: ventroposterolateral thalamus; VM: ventromedial thalamus; SNpr: substantia nigra pars reticulata; SNC: substantia nigra pars compacta; Simplified from Paxinos and Watson (14).

France). The HPLC method was adapted from the methods described by early papers (16,17).

The mobile phase was a mixture of 250 mM Na acetate buffer (pH: 6.9), MilliQ water and HPLC grade methanol (Labscan, Ireland) at a ratio of 5:3:2, filtered through 0.40 µm nylon membrane (Sigma, USA) and degassed in an ultrasonic bath.

GABA (Sigma, USA) and L-glutamic acid (Sigma, USA) were dissolved in 0.1 M HCl in a plastic beaker and aliquots were stored at –20°C and fresh dilutions were prepared daily. Precolumn derivatization was performed by mixing 10 µl of either external standards or the samples with 3 µl derivatizing reagent. The mixture was left to derivatize for 2 minutes in the dark at room temperature. The working derivatization reagent was prepared by mixing 1/10 dilution of 3-mercaptropropionic acid (3-MPA) in methanol (v/v) and 1 mg/ml o-phytaldialdehyde solution (OPA; Sigma, USA) at a final ratio of 1:40 (v/v). Twelve µl of the samples or standards were injected into the system and the flow rate of the pump was set to 0.50- 0.65 ml/minute.

Data analysis

The mean of 3 or 4 samples were evaluated as the basal levels of each rat. Two-tailed Student's t-test for ungrouped data was used to determine the difference between the basal levels of GABA and glutamic acid.

RESULTS

The retention times of GABA and L-glutamic acid injected into the system were 11.8±0.56 and 2.67±0.06 min, respectively. Neither the peaks of GABA nor L-glutamic acid overlapped with the standard injections of several other amino acids and catecholamines. The peaks were recognized as GABA or L-glutamic acid at a peak window of ±5 % of the peak width (Fig.2). The lowest detectable amount for GABA and L-glutamic acid on the column were 43 fmol and 25 fmol respectively. In vitro regeneration for GABA and L-glutamic acid through the dialysis membrane at 0.5 µl/minute flow rate was found to be 31.5 % and 29.3 % respectively.

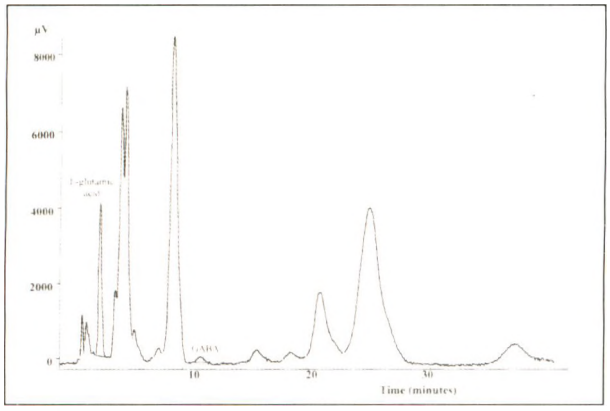

Fig.2 : The detector response of a 10 µl dialysate sample collected within a 20 minute period indicating L-glutamic acid and GABA peaks. The HPLC injections were given in 12 µl volume after pre-column derivatization.

The mean of basal levels of GABA in VLT in SNpr-lesioned GAERS was found to be 0.24±0.08 µM, whereas the mean of basal levels of GABA in VLT was 0.12±0.04 µM in the sham-lesioned group. The statistical comparison of the two groups did not show any significant difference (n=6 per group). L-glutamic acid levels in the same region were 0.32±0.09 µM and 0.41±0.15 µM in SNpr-lesioned and sham-lesioned GAERS respectively. L-glutamic acid levels in both groups were not found to be significantly different (n=6 per group).

The cumulative duration of spike-and-wave discharges (SWD) and the number in SNpr-lesioned and sham-lesioned GAERS did not produce a statistical significant change (Table I).

The GAERS that were subjected to electrolytic lesions of SNpr exhibited circling behavior when handled, so precise care was given while the rats were being perfused in the plexiglass cage.

Table I: The cumulative number and duration of spike-and-wave discharges (mean ± s.e.m) observed within 900 seconds in sham and substantia nigra pars reticulata electrolytic lesions of GAERS

GROUPS	Cumulative spike-and-wave discharge duration (sec)	The number of spike-and-wave discharges
Sham lesion	235 ± 33	38 ± 1
SNpr lesion	231 ± 40	27 ± 10

DISCUSSION

Although the exact mechanism of nigral control of epilepsy is still unknown, it has been postulated that complex neuronal interactions of the SNpr control the seizures (3). Substantia nigra is located in the ventral midbrain and considered to be the main output of the basal ganglia (18,19). Substantia nigra has another subnucleus called as substantia nigra pars compacta (SNpc), located above SNpr. SNpc projects to striatum and contains dopaminergic neurons where SNpr is rich in GABAergic neurons (20,21). The projections of SNpr are ventromedial nucleus of thalamus, intermediate and deep layers of superior colliculus, the pedunculopontine and laterodorsal nuclei of ventral tegmentum (20). The projections to the target structures constitute the elements that produce the inhibition of epileptic activity. The GABAergic nigrocollicular pathway shown previously is the most probable mechanism in the generation of antiseizure effects (3,8,9). Previously, it was shown in a microdialysis study that activation of direct-striatonigral pathway released GABA in the SNpr (22). Neuroanatomical investigation showed that superior colliculus was under tonic inhibition of this nucleus and the decrease in SNpr activity produced disinhibiton of the collicular neurons (8).

The rationale of our study was to examine the extracellular GABA and L-glutamic acid levels in VLT of GAERS following bilateral destruction of SNpr. It was shown that SNpr has projections to ventromedial nucleus of thalamus (20) but VLT, a structure close to the ventromedial thalamus has neurochemical changes in GAERS (13). Although we expected GABA levels to be increased in SNpr-lesioned GAERS, we observed no statistically significant difference. Collectively, neither the duration nor the number of SWD were altered with ablation of SNpr. Earlier studies demonstrated that the lesions of substantia nigra fascilitated kindling development (23). Our results indicate that SNpr may be considered as a site which is not tonically involved in generating antiseizure effects. The antiseizure effect may emerge in genetic absence epilepsy when the intact nucleus is stimulated as it was shown previously (6).

The most accepted hypothesis explaining the etiology of the absence seizures is increased GABAergic transmission (11). GAERS as a model for absence epilepsy displays increased GABAergic levels in VLT (12). This increased extracellular GABA level may be regarded to be specific only to the regions involved in the generation of seizures. Frontocortex F1 region, which is another brain region where SWD can be recorded, also had increased GABA levels in GAERS, when compared to non-epileptic Wistar controls (24). The hippocampus is silent during the seizures and it was reported that there was no change between the epileptic and non-epileptic strains, in terms of basal GABA levels in CA1 region of the hippocampus (24). However, these microdialysis experiments also showed that (-) baclofen treatment did not alter GABA levels in VLT as it produced exacerbation of SWD (13,24). We still do not know whether increased GABA levels in VLT cause seizures or increased thalamic GABA level is a natural consequence of seizures. Unlike GABA, the glutamic acid levels in VLT is not a neurochemical sign of GAERS as it was reported (13). We also measured extracellular L-glutamic acid levels and we observed no difference in terms of L-glutamic acid levels in VLT.

It is worth noting the limitations of our experiments, such as the extent of electrolytic lesions. As demonstrated in Fig.1, the lesions produced by applying anodal current were partial lesions of SNpr. This might be the reason why we could not demonstrate a statistically significant change in GABA and L-glutamic acid release in VLT. Electrolytic lesions performed in the experiments not only damaged the cell bodies but also other structures, axons and dendrites as well. Specific lesions could also be produced by using various neurotoxins (25). We chose electrical ablation as a suitable method to inhibit the tonic effects of SNpr because we wanted to discard all probable effects exerted by SNpr. However, specific lesions of the cell bodies produced by chemical toxins in the SNpr remain to be investigated in further studies. The effect of SNpr lesions on GABA levels in other regions such as F1 region of frontocortex can also be studied.

Twenty-min period of collecting samples is quite long for detecting the neurochemical changes during seizures, since GAERS can generate SWD in about one third of a minute (10). In the

interpretation of our results, we have noticed that GABA levels had a trend to increase, but statistical comparison yielded no difference. In the future, the advent of newer probes and analytical systems showing on-line changes may show instantaneous changes and minimize the variations. This would enable us to detect definite levels of neurochemicals while SWD are being generated.

With the above explanations our data may indicate that partial bilateral SNpr lesioning failed to affect the most verified neurochemical sign of genetic model of absence epilepsy, the increased GABA levels in VLT. We may regard intact SNpr, as a site exerting an inhibition at the target structures when activated.

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