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Glutamic Acid Decarboxylase Immunoreactivity in the Mossy Fiber Terminals of the Hippocampus of Genetic Absence Epileptic Rats

Genetik Absans Epilepsili Sıçan Hipokampusu Mossy Lif Terminallerinde Glutamik Asit Dekarboksilaz İmmünreaktivitesi

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ABSTRACT

AIM: Genetic absence epilepsy rats from Strasbourg (GAERS) provide a model of absence epilepsy. Although excessive GABA mediation within the thalamo-cortico-thalamic circuit has been shown to play a role in absence epilepsy, neuronal networks of hippocampus have recently received attention. Glutamic acid decarboxylase (GAD) was previously shown to be increased after convulsive seizures in the mossy fiber terminals (MFTs) of hippocampus. The aim of the present study was to investigate whether the change in the level of this enzyme in convulsive seizures is also observed in rats having genetic absence epilepsy.

MATERIAL and METHODS: Hippocampal CA3 and dentate regions were processed for transmission electron microscopic evaluations. Thin sections were incubated with anti-GAD65/67 antibody. The NIH Image Analysis program was used for the quantitative analysis.

RESULTS: It was observed that GAD65/67 immunoreactivity was positive in CA3 and dentate gyrus MFTs of both groups and the difference in the density of immunolabeling between the groups was not statistically significant.

CONCLUSION: The present study demonstrated that GABA synthesizing enzyme, GAD, is found in MFTs of Wistar and GAERS hippocampus and this enzyme does not show an increase in these terminals in absence epilepsy, in contrast to convulsive seizures.

KEYWORDS: Genetic absence epilepsy rats from Strasbourg, Glutamic acid decarboxylase, Hippocampus, Immunocytochemistry, Mossy terminal

ÖZ

AMAÇ: Genetik absans epilepsili sıçanlar (GAERS, genetic absence epilepsy rats from Strasbourg) absans epilepsi modeli olarak deneysel çalışmalarda kullanılmaktadır. Talamo-kortiko-talamik döngüde artmış GABA aracılı mekanizmaların absans epilepside rol oynadığı gösterilmiş olmasına rağmen, son zamanlarda yapılan çalışmalarda hipokampustaki nöronal ağ dikkati çekmektedir. Yapılan çalışmalarda hipokampus mossy lif terminallerindeki (MLT) glutamik asit dekarboksilazın (GAD) konvülfif nöbetlerden sonra artış gösterdiği saptanmıştır. Bu çalışmanın amacı, konvülfif nöbetlerde görülen GAD enzimi seviyesindeki değişikliklerin genetik absans epilepsili sıçanlarda da görülüp görülmediğini araştırmaktır.

YÖNTEM ve GEREÇLER: Hipokampal CA3 ve dentat girus (DG) bölgelerinden alınan dokular elektron mikroskopik işlemler için hazırlandı. İnce kesitler anti-GAD 65/67 primer antikoruna ile inkübe edildi. Niceliksel analiz için "NIH Image Analysis" görüntü analiz programı kullanıldı.

BULGULAR: Her iki grup hipokampusunda CA3 ve DG MLT'de GAD 65/67 immünreaktivitesinin pozitif olduğu gözlemlendi. Kontrol ve GAERS hipokampusunun her iki bölgesinde veziküler GAD 65/67 immün-işaretlenme yoğunlukları arasında istatistiksel olarak anlamlı fark bulunmadı.

SONUÇ: Bu çalışmada GAERS ve Wistar MLT'de GABA sentezleyen enzim olan GAD'ın varlığı gösterilmiş ve konvülfif nöbetlerden farklı olarak, MLT'deki bu enzimin absans epilepside artış göstermediği sonucuna varılmıştır.

ANAHTAR SÖZCÜKLER: Genetik absans epilepsili sıçanlar (Strasbourg), Glutamik asit dekarboksilaz, Hipokampus, İmmünsitokimya, Mossy terminali

INTRODUCTION

Genetic absence epilepsy rats from Strasbourg (GAERS), a strain of Wistar rats with genetically determined seizures, has been used as an animal model of human absence epilepsy since it was introduced by Vergnes et al. in the early 1980s (30,31). Although an excessive GABAergic mediation in thalamo-cortico-thalamic circuits has been shown to play a role in absence epilepsy, recent studies have drawn attention to the limbic structures including the hippocampal neuronal network (17,18,21). In a study in which GAERS were stimulated for the process of limbic kindling, only Wistar control animals were reported to reach stage 5 generalized convulsive seizure state, whereas GAERS failed to progress beyond stage 2 seizure state, even after 30 kindling stimulations (5). An oppositional mechanism in conversion of limbic seizure to motor seizure in GAERS and different pathophysiological mechanisms in the neuronal network of absence epilepsy and secondarily generalized convulsions were suggested.

Glutamic acid decarboxylase (GAD) synthesizes GABA from glutamate in the brain. It has 2 isoforms as GAD65 and GAD67 and both isoforms have the capability to synthesize GABA (2). GAD65 is predominantly found in the axon terminals, whereas GAD67 is found predominantly in the cell body, as well as in axon terminals (4,7,9,11). Previous studies used peroxidase-antiperoxidase and immunogold methods to show the localization of GAD in different parts of the body (6,10,29).

Light microscopic studies in 1980s suggested that the MFTs might have been the source of GABA immunoreactivity observed in CA3 stratum lucidum of the rat hippocampus (19,27). Another study reported the presence of GAD immunoreactivity in the same region (32). Later studies showed an increase in GABA, GAD and GAD mRNA in MFTs after seizures, epilepsy, or after an increase in the synaptic strength (3,8,13-15,20,23,26,28). Also, previous studies showed the presence of both GABA and glutamate immunoreactivity in the MFTs of GAERS and non-epileptic control rat hippocampus, using the immunocytochemical techniques at the ultrastructural level (24,25). In the present study, we aimed to investigate the presence of GAD in MFTs of GAERS hippocampus and whether the change in the level of this enzyme in convulsive seizures is also observed in rats having genetic absence epilepsy.

MATERIAL and METHODS

Animals

Adult male (250-300 g, 7 months old), non-epileptic control Wistar albino rats ($n = 6$) and GAERS ($n = 6$) having absence seizures on cortical EEG were used in the study. The animals were housed in a temperature-controlled room ($20 \pm 3^\circ\text{C}$) with a 12 h light / dark cycle and fed a standard diet. Full approval of the experimental procedures was obtained from the Animal Care and Use Committee of Marmara University (50.2002.mar).

Immunocytochemistry

For the ultrastructural observation and immunocytochemistry, the animals were deeply anesthetized with ketamine (100 mg/kg) and xylazine hydrochloride (20 mg/kg) and then sacrificed by transaortic perfusion with a fixative solution containing 2.5% glutaraldehyde, 0.5% paraformaldehyde and 0.1% picric acid in 0.1 M HEPES, pH 7.3. Following fixation, the animals were decapitated and the entire brain left overnight in the same fixative at 4°C .

The following day, the brains were washed several times in 0.1 M HEPES, pH 7.3, and cut into 300 μm slices using a Leica VT 1000S vibratome (Wetzlar, Hesse, Germany). The CA3 and dentate regions of the hippocampus were dissected and incubated in 1% osmium tetroxide / 1.5% potassium ferricyanide for 30 min at room temperature. The samples were then washed several times in deionized water and stained *en bloc* with aqueous 0.5% uranyl acetate for 30 min at room temperature. The tissue was then dehydrated in graded series of ethanol, cleared in propylene oxide, and embedded in Epon 812 for 24 h at 60°C . Semi-thin sections (1 μm) were cut on a Leica Ultracut R ultramicrotome and stained with toluidine blue and viewed with the light microscope to ensure proper orientation. The tissue was then thin sectioned, collected on 200 mesh nickel grids coated with a Coat-Quick "G" pen (Daido Sangyo Co., Ltd. Japan), and air dried for 3-4 h.

The grids containing thin sections were washed in TRIS-buffered saline, pH 7.6, containing 0.1% Triton X-100 (TBST 7.6) and incubated in the primary antibody overnight in a moist chamber at room temperature. The anti-GAD65/67 antibody (Sigma-Aldrich G5163, St. Louis, MO, USA) was diluted 1:2000 in TBST 7.6. The samples were washed several times in TBST 7.6 and TBST 8.2 and incubated for 90 min in goat anti-rabbit IgG (Sigma-Aldrich G7402, St. Louis, Missouri, USA) conjugated to 10 nm gold, which was diluted 1:50 in TBST 8.2. The samples were then washed in TBST 7.6 and deionized water. The sections were counterstained with uranyl acetate and lead citrate before being viewed and photographed on the JEOL 1200 EX II transmission electron microscope (Tokyo, Japan) by an individual blinded to the groups. Negative control experiments were carried out by omitting the primary antibody from the procedure. All of the samples were processed simultaneously with the same antibody solutions to avoid day-to-day differences. Ten photographs from each grid were taken randomly within the same region of the stratum lucidum layer of the CA3 subfield and within the hilar region of dentate gyrus. The photomicrographs were analyzed for the density of immunogold labeling within MFTs using the program NIH Image Analysis (Image J). Gold particles within pre-synaptic terminals were counted and the area of nerve terminals was determined. The density of GAD immunoreactivity was determined by dividing the number of 10 nm gold particles by the area of the MFT (number of particles/ μm^2). From each section, we photographed 5 vessel lumens containing gold particles, the mean density of which we considered background labeling. For each grid, the average

background labeling was calculated and subtracted from the density of each terminal in that section. Only the nerve terminals with a density greater than the mean background labeling were included in the study.

Statistical analysis

The mean density of GAD65/67 immunolabeling was calculated for each animal for both regions and the groups were compared using an unpaired Student's t parametric test. The results of the density of GAD65/67 and the area of MFTs were expressed as "mean ± S.E.M." and $p < 0.05$ was considered as significant.

RESULTS

CA3 region

GAD65/67 immunoreactivity was associated with the spherical and dense core vesicles (DCVs), and the mitochondria in the MFTs in CA3 region of both groups (Figure 1A,B). The negative control experiments resulted in lack of immunolabeling.

There was no difference in the areas (control: $4.63 \pm 0.44 \mu\text{m}^2$ [n=6, a total of 60 terminals], GAERS: $5.81 \pm 0.41 \mu\text{m}^2$ [n=6, a total of 60 terminals]) of MFTs (Figure 2) and in the density (control: $8.95 \pm 2.98 \text{ particles}/\mu\text{m}^2$, GAERS: $5.66 \pm 1.76 \text{ particles}/\mu\text{m}^2$) of GAD65/67 immunolabeling (Figure 3) between Wistar and GAERS groups in CA3 region.

Dentate gyrus region

GAD65/67 immunoreactivity was associated with the spherical and DCVs, and the mitochondria in mossy fiber collateral terminals in dentate region of both groups (Figure 1C,D). The negative control experiments resulted in lack of immunogold labeling.

As in CA3 region, there was no difference in the areas (control: $4.42 \pm 0.67 \mu\text{m}^2$ [n=6, a total of 60 terminals]; GAERS: $4.06 \pm 0.28 \mu\text{m}^2$ [n=6, a total of 60 terminals]) of mossy fiber collateral terminals (Figure 2) and in the density (control: $11.99 \pm 3.76 \text{ particles}/\mu\text{m}^2$, GAERS: $7.50 \pm 2.62 \text{ particles}/\mu\text{m}^2$) of GAD65/67 immunolabeling (Figure 3) between the control and GAERS groups in the dentate region.

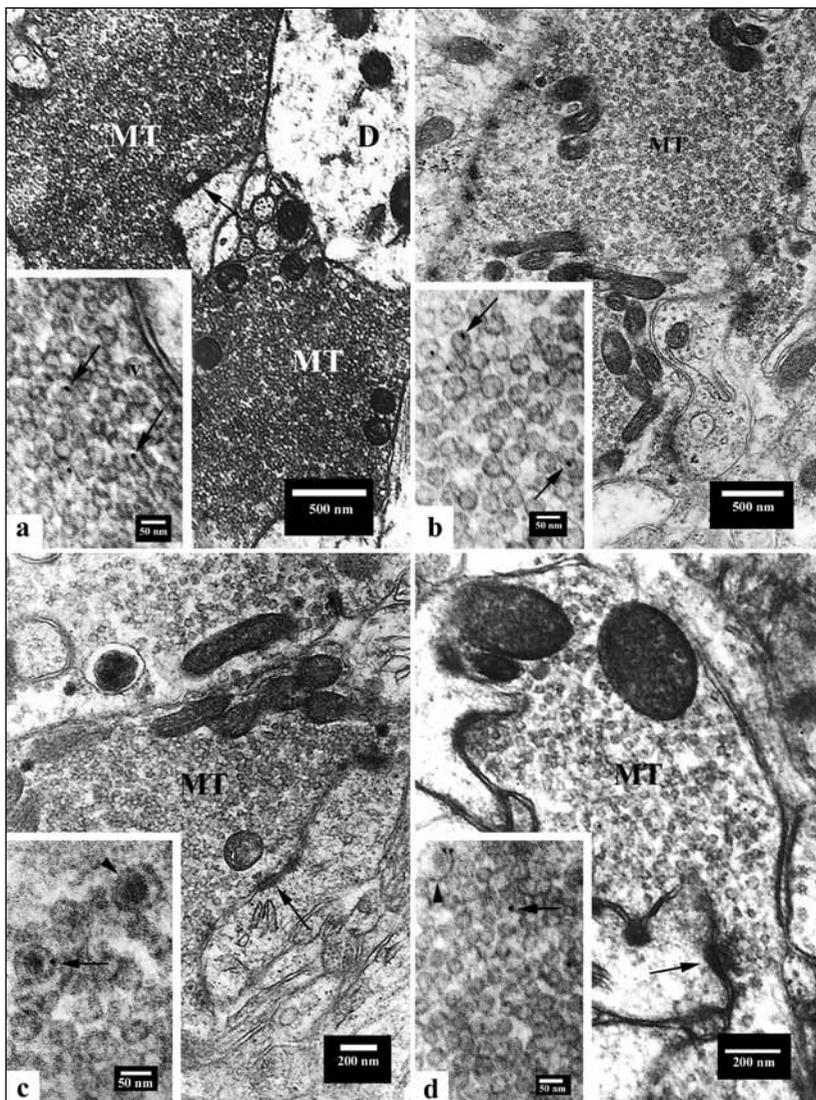


Figure 1: GAD65/67 immunoreactivity in MFTs. **A)** MFTs (MT) making asymmetrical synapse (arrow) with dendritic spines in CA3 region in control hippocampus. D: Dendritic shaft. **Inset:** GAD 65/67 immunoreactivity (arrow) associated with the spherical vesicles (v). **B)** A MFT (MT) in CA3 region in GAERS hippocampus. **Inset:** GAD 65/67 immunoreactivity (arrow) associated with the spherical vesicles. **C)** A mossy fiber collateral terminal (MT) making asymmetrical synapse (arrow) with a dendritic spine in dentate gyrus region in control hippocampus. **Inset:** GAD 65/67 immunoreactivity (arrow) associated with the DCVs (arrowhead). **D)** A MFT (MT) in dentate gyrus of GAERS hippocampus. **Inset:** GAD 65/67 immunoreactivity (arrow) associated with the spherical and DCVs (arrowhead).

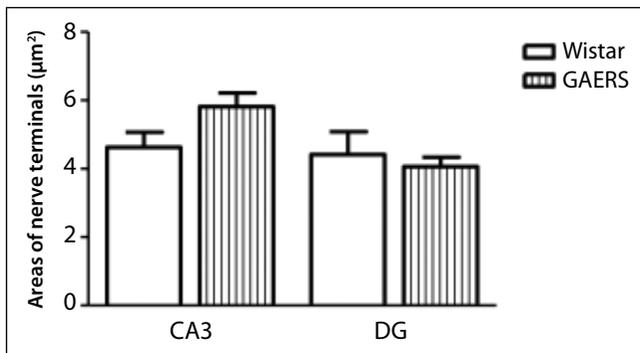


Figure 2: Area of mossy fiber terminals in CA3 and dentate gyrus regions.

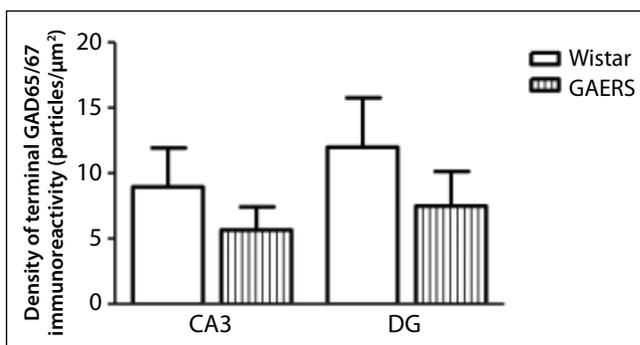


Figure 3: Density of GAD65/67 immunoreactivity per μm^2 of mossy fiber terminals in CA3 and dentate gyrus regions.

DISCUSSION

The present study shows for the first time the localization of GAD in MFTs of GAERS hippocampus by immunogold techniques at the ultrastructural level. There was no significant difference in the mean area of mossy terminals and in the GAD density in MFTs between Wistar and GAERS groups both in CA3 and dentate regions. Similarly, in previous studies, no significant difference in the areas of mossy terminals between GAERS and their non-epileptic control groups was found (24,25).

Mossy fibers originating from dentate granule cells and synapsing with CA3 pyramidal cells are excitatory (12). Sandler and Smith are the first researchers reporting that the mossy fiber terminals contain both glutamate and GABA (22). It was previously demonstrated that MFTs were GABA immunopositive in GAERS and their non-epileptic control hippocampus (24,25). In an *in situ* study by Bergersen et al., both GABA and glutamate were shown to be in relation with the synaptic vesicles in the same MFT (1). Sloviter et al. were first to report that the source of GABA in mossy fiber terminals was GAD (26). They showed that GABA and GAD67 were normally present in MFTs of rat, monkey and human hippocampus and seizures upregulated GABA and both isoforms of GAD. GAD65 and GAD67 mRNA upregulation in granule cells were also proved in other animal studies using electrical kindling or chemical convulsants (3,13,20,14,23,28). These studies concluded that GABA in MFTs was synthesized

by GAD and the increase in GABA levels after seizures may be due to an effort to compensate for the hyperexcitability. The quantitative results of the present study showed that GAD65/67 immunoreactivity was present in MFTs of both Wistar and GAERS hippocampus but the difference in its density between the groups was not statistically significant, suggesting that it was not modified in absence seizures in contrast with convulsive seizures.

The two isoforms of GAD were proved to show different cellular distribution, suggesting different functions in neurons (4). GAD65 is found in synaptic terminals and GAD67 in terminals, somata and dendrites (11). This localization suggests that GAD67 in the soma synthesizes metabolic GABA pool and GAD65 the releasable pool. However, previous studies manifested that the GABA released from mossy fibers was synthesized from 67kDa isoform (15,20,23,26). A mild immunostaining of GAD67 and GABA was previously reported in DG of control preparations (23,26). In a study by Maqueda et al., GAD67 was constitutively shown to be expressed in some granule cells of adult control rats (15). In kindled epileptic rats, a marked expression of GAD67 in granule cells and mossy fibers was observed. They also showed that despite the lack of GAD65, its mRNA was found in adult control and epileptic rats. They concluded that GAD65 mRNA in granule cells was neither regulated developmentally nor over-expressed by seizures, despite the down-regulation of GAD67 after the completion of the development and its up-regulation in an activity-dependent manner. Recently, colocalization of both GAD65 and GAD67 with vesicular GABA transporter (VGAT) in hippocampal mossy fiber terminals in adult Wistar rats was demonstrated by ultrastructural immunocytochemical methods (33). Similarly, GAD65/67 immunoreactivity was found to be positive in MFTs of non-epileptic control Wistar rats in the present study.

In conclusion, the mossy terminals of GAERS contain GAD65/67 and this enzyme probably synthesizes GABA that was previously demonstrated in the hippocampus of this strain (24,25). In contrast to GAD increase in the hippocampus of convulsive epilepsy models, the present study revealed that the density of GAD immunoreactivity was not altered in rats with genetic absence epilepsy. This points out different mechanisms underlying two particular epilepsy types.

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