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Parallel changes in the promoter methylation of *voltage-gated T-type calcium channel alpha 1 subunit G* and histone deacetylase activity in the WAG/Rij model of absence epilepsy

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ABSTRACT

Objective: In the last two decades, research on epigenetic mechanisms has expanded dramatically. Recent studies demonstrated that epigenetic mechanisms regulate epilepsy and epileptogenic pathologies. In this study, we aimed to investigate changes in the promoter methylation status of the *voltage-gated T-type calcium channel alpha 1 subunit G* (*CACNA1G*) gene and total histone deacetylase activity in Wistar Albino Glaxo/Rijswijk (WAG/Rij) rats which is one of the commonly used genetic absence rat models of epilepsy in the three different age groups (3, 6, and 9 months old) on both sexes.

Material and Method: Evaluation of changes in the spike-wave discharges (SWDs) was performed with electrocorticography (ECoG). The promoter methylation status of the *CACNA1G* gene was determined by methylation-specific PCR (MSP), and histone deacetylase (HDAC) activity was determined spectrophotometrically.

Results: Our results demonstrated that the number of SWDs increased age-dependent in WAG/Rij. Additionally, it was observed that *CACNA1G* promoter methylation decreased, and total HDAC activity increased with age in both sexes.

Conclusion: Our results provide further support for epigenetic regulation in the absence epilepsy phenotype and suggest that the underlying mechanism behind the increase in the number of SWDs with age in the WAG/Rij animals might be regulated by *CACNA1G* promoter methylation or HDAC activity.

Keywords: Epilepsy, voltage-gated T-type calcium channel, histone deacetylation, methylation, electrocorticography.

INTRODUCTION

Characterized by abrupt, comparatively brief lapses of consciousness, unusual electrographic spike-wave discharges (SWD) at 2,5-4 Hz, and involuntary movements, absence epilepsy is described as the generalized non-convulsive polygenic type of epilepsy (1). Psychiatric comorbidities and cognitive and mood impairments also accompany the clinical picture. Because the high incidence of pharmacoresistant, unresponsive patience and persistent comorbidities even following complete control of seizures indicate the unmet need for novel therapeutic interventions (2). Finding or identifying a novel approach is challenging, taking into consideration the large spectrum of absence seizure semiology and EEG features. Hopefully, the deficiency of biomarkers and high incidence around the world also motivate several groups to cope with this challenge. Recent decades also allowed improving experimental models that better mimic disease pathology and progression. Wistar Albino Glaxo/Rijswijk (WAG/Rij) is one of the strains that showed the crucial role of the localized cortex region in initiating the absence seizures (3). Several studies demonstrated that WAG/Rij is an appropriate model for absence seizures seen in humans (4). However, the molecular basis of absence epilepsy is not entirely understood (5).

Voltage-gated calcium channels manage the calcium entry into the cell in response to changes in membrane

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potential in the heart, brain, and nervous system. As calcium has a crucial role in cell signaling, these channels are critical in regulating mechanisms such as muscle contraction, hormone release, synaptic transmission, and gene expression (6). As a member of the voltage-gated calcium channels family, the T-type calcium channels are low-voltage-activated ones and have a crucial role in the functioning of the nervous system. Therefore, research on whether T-type calcium channels can be drug targets has gained attention (7). In many studies, dysfunctions of these ion channels have been associated with many neurological diseases, but the most well-studied pathological implication is the absence epilepsy. Moreover, these channels have been demonstrated to be associated with peripheral neuropathic pain. More recently, T-type calcium channels have been revealed to take part in an important role in mitochondrial stress and apoptosis in dopaminergic neurons in Parkinson's disease (6).

In humans, T-type calcium channel isoforms are encoded by the *CACNA1H*, *CACNA1G*, and *CACNA1I* genes which express the Cav3.1, Cav3.2, and Cav3.3 isoforms. In addition to these isoforms, many splice variants have been identified for T-type calcium channel isoforms, enriching the functional and molecular diversity of the channels (7). In patients with the absence of epilepsy, genetic analyses indicated the *CACNA1G* gene, which is located on chromosome 19 and encodes the pore-forming α1A subunit of Cav2.1 channels, linked to the epileptic phenotype (8). Functional expression studies on *CACNA1G* demonstrated that the E147K mutation impairs calcium channel function and is related to abnormal neuronal firing (9). Further studies revealed that Cav2.1 channels are expressed in the cerebellar Purkinje and granule cells at high levels, directly related to the initiation point of absence seizures (10). However, another animal strain commonly used for absence seizure studies, Genetıc Absence Epileptic Rats From Strasbourg (GAERS), has *CACNA1G* mutation as a causative factor for phenotype of absence epilepsy; the WAG/Rij strain showed a polygenic background (11). Additionally, information about the possible role of *CACNA1G* channels in the phenotype of absence epilepsy in the WAG/Rij strain is limited. Although epileptic phenotype is mainly related to P-Q-type calcium channels in WAG/Rij rats, the other role of *CACNA1G*, another P-Q-type calcium channel, is still missing. In addition, the increase in seizures and SWDs also exacerbates in the aging WAG/Rij rats (12). Nevertheless, the possible mechanism behind that increase is also another gap now. Therefore, studies that investigate both genetic factors and cellular mechanisms opened new avenues for the further investigation of epigenetics to understand the possible relationships.

Studies in the recent decade also demonstrated that the absence epilepsy is affected by epigenetic mechanisms as well in humans and animals. The epigenetic mechanisms, such as DNA methylation, histone modifications, and microRNAs (miRNAs), establish the distinctive chromatin structure and modify the gene transcription without making alterations to the DNA sequence. DNA methylation, in mammals, usually takes place in clustered CpG dinucleotides located especially in the promoter regions of genes. The hypermethylation of the promoter blocks the binding of transcription factors to DNA and results in the silencing of gene expression, whereas in the hypomethylated state transcription factors can bind to DNA and activate the transcription (13). Additionally, covalent histone modifications, including acetylation and methylation, occur at histone tails as post-translational and control the chromatin state and gene transcription. Of these modifications, acetylation and methylation of histones have been the most widely studied. Histone acetyltransferases (HATs) transfer the acetyl groups to lysine residues and result in gene activation. On the other hand, histone deacetylases (HDACs) remove acetyl groups from histone tails and result in gene silencing (14). Especially methylation and histone acetylation, which have a strong effect on gene regulation, are shown to affect several causative genes related to epileptic phenotype. Thus, several studies investigated the impact of epigenetic modifications and tried to demonstrate possible interactions with neuronal excitability, which abnormally occurs in the absence of epilepsy.

In this study, we investigated possible epigenetic changes on *CACNA1G* in WAG/Rij rats. We focused on *CACNA1G* methylation status due to its relationship with the absence seizures seen in humans and looked for the total HDAC activity in the different age groups. However, most studies about the absence epilepsy in WAG/Rij studies conducted in male rats to avoid estrogen or other female sex-dependent effects; we investigated changes in methylation profile and total HDAC activity in both sexes.

MATERIAL AND METHOD

Animals

Experiments were conducted with eighteen female and eighteen male WAG/Rij rats. Ethical approval was obtained from the Sivas Cumhuriyet University Experimental Animals Local Ethics Committee (Date; 28.07.2022, Decision No: 540/2022). Animals were maintained in optimal laboratory conditions and fed ad libitum. All experimental procedures were performed following the principles of the Guide for the Care and Use of Laboratory Animals, and the Turkish legislation acts concerning animal experiments, according to the European Union Directive (2010/63/EU), protecting animal rights. All endeavors were done to minimize animal suffering, and experiments were conducted and recorded according to

the ARRIVE guidelines (15). Power analysis was carried out with G-power to establish the number of animals to examine the effects with 95% power.

Experimental Design and Animal Surgeries

Animals were selected according to their precise birthdate. In both sexes, animals that were 12 weeks old are included in 3-month groups, 24 weeks old are included in 6-month groups, and 36 weeks old are included in 9-month groups. After a one-week acclimation, animals were anesthetized with ketamine: xylazine (80:10 mg/kg, i.p.) and positioned in the stereotaxic apparatus. Hair above the skull was shaved, and a small incision was made to visualize the cranium under the subcutaneous tissue. Three burr holes were drilled carefully without disturbing the dura mater. Three screw electrodes were positioned according to the rat brain atlas (2 mm anterior, 3.5 mm right lateral for the first electrode, 4 mm right lateral, and 6 mm posterior, and for the second electrode) the earth electrode was positioned on the cerebellum) (16). Screw electrodes immersed and adjusted to the skull with dental cement. Animals were maintained in separate cages for at least three days for recovery. Following the recovery period, animals were placed in a plexiglass apparatus for observation and ECoG recordings (PowerLab, 16/SP, AD Instruments, Australia). Baseline electrocorticography (ECoG) recordings from each animal were collected for 3 hours at the same time (11:00 AM). The number of SWDs and the mean duration and amplitudes of SWDs were evaluated and determined every 20 minutes by LabChart software (v7 Pro, AD Instruments, Australia). ECoG recordings were performed after determining the estrous cycle as previously described to avoid the possible effects of sexual hormones (17).

DNA and Total Protein Isolation

Twenty-four hours after EcoG recordings, animals were anesthetized. Afterward, the animals were transcardially perfused with ice-cold phosphate-buffered saline, and then decapitated. Total brain tissues from each animal were carefully dissected, and total brain tissue was homogenized with liquid nitrogen. 50 mg of liquid nitrogen powdered brain tissues were suspended in RIPA (Radio-immunoassay precipitation buffer) solution for protein analysis and HDAC activity assay. Total protein amounts in the samples were determined by BCA assay. For DNA isolation, 50 mg of powdered brain tissues were resuspended in the 100 µl ice-cold PBS, and then DNAs were isolated with Pure Link® Genomic DNA Mini Kit (Invitrogen, USA), according to the manufacturer's protocols. The purity and concentration of DNA samples are measured by nanodrop (Jenway Genova Nano, England).

Determination of Total HDAC Activity and Methylation-specific PCR (MSP) Analysis

Total histone deacetylase (HDAC) activity was evaluated by a HDAC activity assay kit (#GTX85529, Genetex, California, USA) colorimetrically. Equal amounts of protein samples were treated with HDAC substrate (Boc-Lys(Ac)-pNA) assay buffer for 30 min at 37°C. Thereafter, the reaction was then completed with a lysine developer. Subsequently, optical densities were measured at 405 nm by a microplate reader (Tecan AG, Austria).

Methylation-specific PCR (MS-PCR) experiments were performed after bisulfite modification of all the DNA samples. The bisulfite modification was exerted by EpiJET Bisulfite Conversion Kit (#K1461, Thermo Fisher Scientific, USA), strictly following the manufacturer's instructions. Methylation-specific PCR was performed for *CACNA1G* promoter regions with methylation and unmethylation-specific primer pairs, designed via http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi, and sequences and annealing temperatures of primers were provided in **Table**. The MSP was carried out in a thermal cycler with DreamTaq™ Hot Start DNA Polymerase (#EP1702, Thermo Fisher Scientific, Lithuania) and methylation specific-primer pairs in a final volume of 25 µl at the following cycling condition for *CACNA1G*: initial denaturation at 95°C for 3 min, followed by 40 cycles at 95°C for the 40s; 50 °C for 40s and 72°C for 70s. Afterward, the final extension step was carried out at 72°C for 7 min. Then, maintained at 4°C. The promoter methylation status of *CACNA1G* was analyzed with agarose gel electrophoresis and UV transilluminator after the MSP. A DNA Ladder (#SM1193, Thermo Fisher Scientific, Lithuania) was used as a size marker. Then, the agarose gel results were analyzed using ImageJ software.

Table. The sequence of methylation-specific primers in this study.

Primer Name	Sequences	Tm (°C)
CACNA1G MSP-F	5'-AGATGTTAAATATTTTGATTTTTCGAG-3'	49
CACNA1G MSP-R	5'-AAAACCACAACTAAAATCCGATTC-3'	50
CACNA1G USP-F	5'-ATTAGATGTTAAATATTTTGATTTTTTGAG-3'	49
CACNA1G USP-R	5'-AAAACCACAACTAAAATCCAATTC-3'	49

Abbreviation(s): MSP, methylation-specific primer; USP, unmethylation-specific primer; F, forward; R, reverse; Tm, melting temperature.

Statistical Analysis

All the experimental data were recorded and then analyzed by GraphPad Prism (v9.0, USA). Data distribution was evaluated with Shapiro-Wilk's test. As the data were not normally distributed, statistical analyses were performed by non-parametric tests. The differences between male and female groups were assessed via the Mann-Whitney U test. For comparison among the different age groups, the Kruskal-Wallis test was used. P values less than 0.05 were taken into account as significant.

RESULTS

Total SWDs Increase with Age in Both Sexes

ECoG analysis was carried out to assess the seizure status of animals in all experimental groups (**Figure 1**). Our results demonstrated that the number of SWDs, which indicates the intensity of seizure, significantly increased in 6 (77.3±22.41) and 9 (154.25±34.89) months old male rats compared to 3-month-old males (30.50±13.30, p=0.07, p<0.001, respectively, **Figure 1**). Moreover, that rising at nine months is also more potent in the 9-month males than at six months (p<0.001, **Figure 1**). In parallel, numbers of SWDs also significantly increased in 6 (99,40±6,95) and 9 (116.20±24.99) months females compared to 3-month-old ones (59.66±7.57, p=0.046, p=0.004, respectively, **Figure 1**). In contrast to the difference between 6- and 9-month males, there were no significant differences between 6- and 9-month females (p>0.05, **Figure 1**).

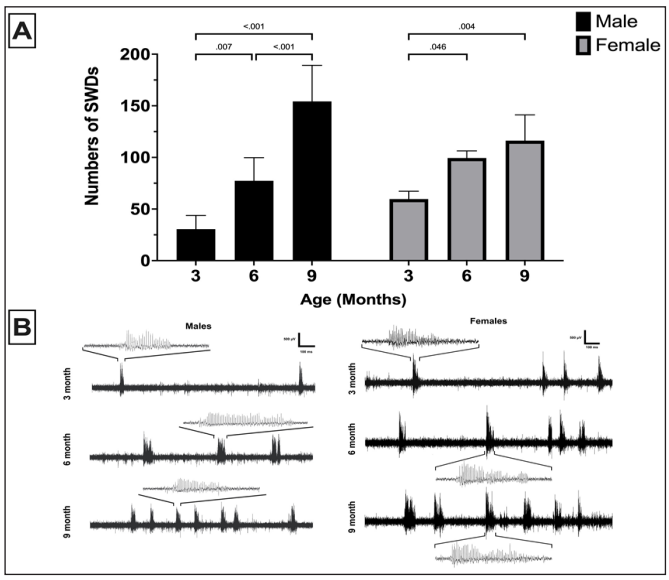

Figure 1. The number of SWDs was determined by ECoG in 3,6- and 9-month groups in both sexes. Significant increase in 6 and 9 months compared to 3-month-old seen in both experimental groups (A). ECoG recordings stand for six biological replicates for all groups at the 80th minute (B). All data were expressed as mean±SD.

The Promoter Methylation of *CACNA1G* Showed an Age-dependent Decreasing Profile in Both sexes

The MSP was carried out to the investigated promoter methylation status of the *CACNA1G* gene. In both sexes, the methylation percentage of the treatment groups was found to be decreased depending on the age (Fig 2). In males, although there was no remarkable difference in methylation profile between 3 (59.50±8.82) and 6 (56.16±6.55, p>0.05, **Figure 2**) months old animals, there was a significant decrease in the 9 (41.16±8.63, p<0.001, **Figure 2**) months old animals compared to the 3-month group. Moreover, that decrease was also significant when six-month and 9-month males were compared (p=0.007, **Figure 2**). In female animals, methylation of *CACNA1G* significantly decreased 6 (47.16±9.01) and 9 (39.66±4.63) month old animals, compared to the 3 (58.83±8.54, p=0.040, p<0.001, respectively, **Figure 2**A) months old females.

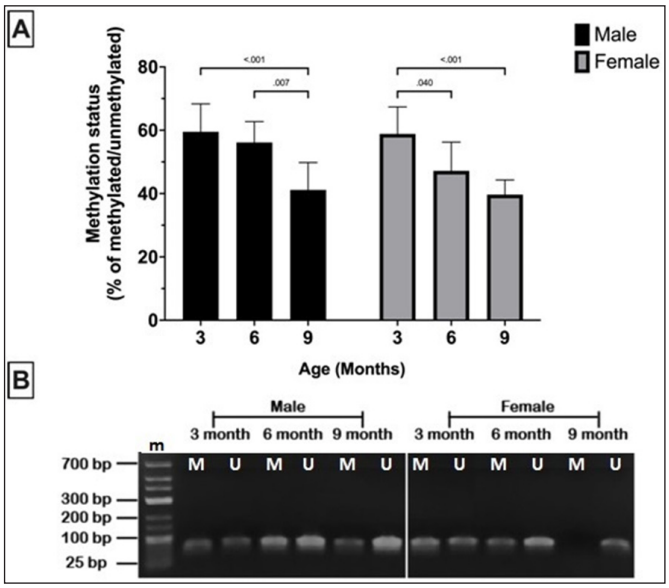

Figure 2. The MSP analysis of *CACNA1G* promoter region. Methylation status decreased with age in both sexes (A). The Gel picture stands as the representative of all groups (B). The methylated PCR product is 98 bp, and the unmethylated PCR product is 100 bp. Abbreviation(s): bp, base pairs; M, methylated; U, unmethylated; m, size marker.

HDAC Activity Increased in Both Sexes

Total HDAC activity was assessed in all experimental animals (**Figure 3**). In males, HDAC activity was significantly increased in 6 (7.81±2.81, **Figure 3**) and 9 (9.85±4.24) month old animals, compared to 3 months (2.03±0.68, p=0.005, p<0.001, respectively, **Figure 3**). There was no remarkable difference among 6- and 9-month-old males (p>0.05, **Figure 3**). In females, although both 6 (8.41±2.01) and 9 (7.51±3.79) months old animals showed increased HDAC activity, only 6-month animals were able to show a significant increase compared to the 3-month females (3.76±2.41, p=0.028, p>0.05, respectively, **Figure 3**).

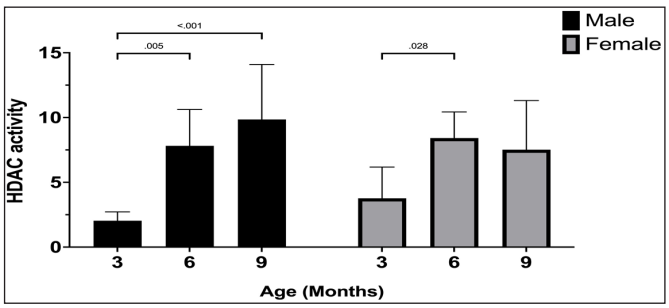

Figure 3. Total HDAC activity was assessed in all experimental groups. HDAC activity significantly increases in 6- and 9-month males. Although HDAC activity increased in 6-month females, there was no significant difference in 9-month females. All data were represented as mean±SD.

DISCUSSION

In this study, we have investigated possible changes in the promoter methylation profile of the *CACNA1G* gene, which is strongly related to the absence epileptic phenotype, in age and sex-dependent manner. Our results demonstrated that the number of SWDs increases in both sexes ages dependently. Also, the methylation status of *CACNA1G* in brain tissues was age dependently decreased in both sexes. Additionally, total HDAC activity also decreased time-dependent in male and female WAG/Rij rats.

Given the effects of T-type calcium channels on the function of the nervous system, it is not surprising that these ion channels are associated with numerous neurological diseases (7). Ion channel dysfunction is one of the accepted mechanisms underlying the mechanism of impaired synchronized circuits in the epileptic brain (18). In these circuits, P/Q-type calcium channels and *CACNA1* regulate membrane excitability in dendrites, dynamic oscillation balance in somas, and neurotransmitter recycling in the presynaptic terminals (19). It has already been shown that loss of function mutations reduces neurotransmitter recycle rates and causes an imbalance in the PQ-mediated exocytosis, leading to the absence of epilepsy in both humans and rodents (20). Additionally, with the increased use of sequencing technologies, studies demonstrated that inherited or childhood epilepsies also regulated epigenetic mechanisms (21). Because this P/Q alpha and regulatory subunit mutations demonstrate downstream rising in thalamic T-type calcium currents and disturb stable connection between brain areas, which is essential for expressing spike-wave rhythmicity, we selected WAG/Rij strain to investigate possible changes with epigenetic modification of *CACNA1G*. Recent studies demonstrated that WAG/Rij rats show age-dependent exacerbation of SWD profile (12). However, several studies were investigated to understand the age-dependent increase in SWD profile, and possible mechanisms remain elusive. We first examined the SWD profile in WAG/Rij rats in both sexes with that goal. Our results were in parallel with the previous studies, the number of SWDs were increased. The only difference was that females also had the same pattern as males, even though there was a conflict between males and females due to different brain metabolism.

Epigenetic mechanisms are well-known to sustain long-lasting gene expression patterns and show tissue-specific differences (22). These mechanisms comprise diverse levels of regulation and affect different transcript regulators such as repressors and enhancers and the transcription machinery to manage the expression of specific genes. Recent knowledge supports the hypothesis that distinctive DNA methylation patterns can establish in response to environmental changes after birth and are long-lasting in rodents and humans (23). Additionally, excitatory neurotransmission in the hippocampus was demonstrated to be affected by DNA methyltransferases (24). Valproic acid, a commonly used antiepileptic drug, is also a histone deacetylase inhibitor and inducer of DNA demethylation in vitro, supporting the epigenetic mechanism hypothesis (25). Sarkisova and Gabova (3) showed that WAG/Rij rats with a high level of maternal care exhibit reduced depression-like comorbidity and less and shorter SWDs in adulthood by comparison with in adulthood by comparison with the WAG/Rij offspring with less maternal care. This study also reveals possible epigenetic changes affecting the epileptic phenotype of WAG/Rij rats. We demonstrated that *CACNA1G* promoter methylation decreased, which means an increase in *CACNA1G* signaling and indicates changes in the expression profile of the channels. Previous studies demonstrated that the *CACNA1* promoter is regulated by methylation in different pathologies (26-28). Therefore, our results indicate that methylation of the *CACNA1G* promoter might be another mechanism underlying age-dependent changes in the WAG/Rij animals.

Furthermore, our results also demonstrated that HDAC activity increased by age in both sexes. Several studies investigated the antiepileptic and antidepressant effects of histone deacetylase inhibitors. Additionally, a recent study demonstrated diminished acetylation of histone H3 and histone H4 protein in WAG/Rij rats at seven months of age than WAG/Rij before seizure onset (30 days old). Also, VPA and sodium butyrate (NaB), another histone deacetylase inhibitor, markedly increase these acetylation levels during co-administration (29,30). Therefore, our results also prove that increased HDAC activity may be another epigenetic mechanism behind the age-dependent deterioration of SWDs in WAG/Rij animals.

However, our study has two significant limitations. First, although we investigate the methylation status of *CACNA1G*, mRNA and membrane expression levels should also be demonstrated to confirm age-dependent decreased expression. Second, histone deacetylase activity is regulated by several histone deacetylase enzymes, which consist of four different classes. Therefore, possible changes in the activity of these enzymes also need to be demonstrated to provide further proof for our results. Besides, our study demonstrated that *CACNA1G* promoter methylation status and HDAC activity change with age in both sexes of WAG/Rij animals.

ETHICAL DECLARATIONS

Ethics Committee Approval: The study was carried out with the permission of the Sivas Cumhuriyet University Experimental Animals Local Ethics Committee (Date; 28.07.2022, Decision No: 540/2022).

Referee Evaluation Process: Externally peer-reviewed.

Conflict of Interest Statement: The authors have no conflicts of interest to declare.

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Author Contributions: All of the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version.

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