DOI:10.4274/tnd.08058
Turk J Neurol 2018;24:313-322

Original Article / Özgün Araştırma

Evaluation of Behavior, Learning, Memory Along with Apoptosis, Neuronal Damage, and GABA-A Alpha-1 Receptor Level After Status Epilepticus in Immature and Mature Rats

İmmatür ve Matür Sıçanlarda Status Epileptikus Sonrası Apoptoz, Nöronal Hasar, GABA-A Alfa-1 Reseptör Miktarı ile Davranış, Öğrenme ve Hafızanın Değerlendirilmesi

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Abstract

Objective: In this study, we aimed to evaluate age-dependent cognitive and behavioral changes, neuronal damage, and the amount of gamma-aminobutyric acid-A (GABA-A) alpha 1 in immature, mature, and adolescant rats after status epilepticus (SE).

Materials and Methods: SE was induced in immature (17 days), adolescant (45 days), and mature (150 days) rats using pentylenetetrazole (PTZ). After SE, adolescant and mature rats underwent open field test and Morris water maze test. After behavioral tests, the animals were sacrificed and we performed histologic investigations on the immature, adolescant, and mature rats to assess neuronal cell damage (caspase and calpain activity) and amount of GABA-A alpha 1 receptor and compared them with a control group.

Results: There were no statistically significant differences between the control and experimental groups in behavioral tests in the early stage after SE. Calpain-mediated neuronal damage was observed in mature rats with necrotic morphology after SE, but this was not observed in adolescant and immature rats. Caspase-mediated neuronal damage was observed in immature rats with apoptotic morphology after SE. The amount of GABA-A alpha 1 receptor was decreased in the three experimental groups compared with the control groups. The decrease in GABA-A alpha 1 receptor amount was highest in the mature experimental group. The amount of GABA-A alpha 1 receptor in the hippocampus decreased to level higher than in the cortex.

Conclusion: This study show that there is no negative impact on learning and behavioral functions in the early stage after PTZ-induced SE, but histologically led to necrosis dominant calpain and caspase-mediated neuronal damage, calpain-mediated cell necrosis is seen particularly in the mature group and caspase-dependent apoptotic morphology observed in immature rats.The decreased of GABA-A alpha 1 receptor is highest in the adults. Our study supports that SE-induced cell damage is more pronounced with increased age, and calpain-mediated cell damage that can clearly be observed in the mature group. Long-term follow-up studies are needed to understand the long-term effect of SE-dependent neuronal damage on cognition and behavior.

Keywords: Memory, calpain, caspase, GABA receptor, status epilepticus, mature and immature rat

Abstract

Amaç: Çalışmada status epileptikus (SE) sonrası immatür, matür ve adölesan sıçanlarda kognitif ve davranış değişiklikleri, nöronal hasar, gamma-amino bütirik asit-A (GABA-A) alfa 1 reseptör miktarının yaş bağımlı olarak değerlendirilmesi amaçlanmıştır.

Gereç ve Yöntem: İmmatür (17 gün), adölesan (45 gün) ve matür (150 gün) sıçanlarda pentilentetrazol (PTZ) ile SE oluşturuldu. Adölesan ve matür sıçanlara açık alan ve Morris su labirenti testi uygulandıktan sonra adölesan, matür ve immatür sıçanlarda histolojik incelemeler yapılarak SE sonrası nöronal hücre hasarını değerlendirmek amacıyla kaspaz ve kalpain aktivitesi, GABA-A alfa 1 reseptör miktarı değerlendirilerek kontrol gruplarıyla karşılaştırıldı.

Bulgular: SE sonrası erken dönemde yapılan hafıza, öğrenme ve belleği değerlendiren davranışsal testlerde gerek adölesan gerek matür deney grubunda kontrol grubuna nazaran istatistiksel anlamda farklılık izlenmedi. SE sonrası matür sıçanlarda nekrotik morfolojide kalpain aracılı nöronal hasar gözlenirken, adölesan ve yavru sıçanlarda kalpain aktivitesine rastlanmadı. İmmatür sıçanlarda SE sonrası apoptotik morfolojide kaspaz aracılı nöronal hasar bulguları izlendi. SE sonrası üç deney grubunda GABA-A alfa 1 reseptör sayısı kontrol grubuna nazaran azalmış olup, azalma en fazla matür deney grubunda izlendi. GABA-A reseptör miktarı hippokampusta, kortekse nazaran daha fazla oranda azalmış olarak saptandı.

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Received/Geliş Tarihi: 09.01.2018 **Accepted/Kabul Tarihi:** 06.04.2018

Öz

Sonuç: Bu çalışma ile elde edilen veriler; PTZ ile indüklenen SE sonrasında erken dönemde öğrenme ve davranış fonksiyonları üzerine olumsuz bir etki görülmezken histolojik olarak nekrozun hakim olduğu kaspaz ve kalpain aracılı nöronal hasara yol açmakta olduğunu, kalpain aracılı hücre nekrozunun özellikle matür grupta görüldüğü ve kaspaz bağımlı apoptotik morfolojilerin immatür sıçanlarda gözlendiğini ve GABA-A alfa 1 reseptör sayısında yol açtığı azalmanın ise erişkinde belirgin olduğunu göstermektedir. SE'ye bağlı hücre hasarının yaşın artmasıyla daha belirgin olduğu ve kalpain aracılı hücre hasarının yine matür grupta belirgin olarak gözlendiği ortaya koymaktadır. SE'ye bağlı hücresel etkilenmenin kognisyon ve davranışa ait uzun dönemdeki etkilerinin anlaşılması için uzun süreli izlem çalışmalarına gereksinim vardır.

Anahtar Kelimeler: Bellek, kalpain, kaspaz, GABA reseptörü, status epileptikus, matür ve immatür sıçan

Introduction

The epileptic seizure is defined as "transient, the self-limiting occurrence of signs and symptoms due to abnormal, excessive and/or hypersynchronous activity in a group of neurons in the brain" (1). Status epilepticus (SE) is mentioned in the presence of a single epileptic seizure lasting more than 30 minutes (min) or intermittent seizures lasting more than 30 minutes without the person returning to normal between them. SE has high mortality and morbidity (2).

The underlying mechanism for SE being different from and longer than short seizures is still not fully understood. However, in a few generalizations, the pathophysiology of SE was attempted to be explained, and the hippocampus was found to be continuously active during SE. Suppression of the gamma-amino butyric acid (GABA)-mediated inhibitor system in the hippocampus has a critical prognosis for the emergence of SE. Glutamatergic excitatory synaptic transmission plays an important role in the continuation of SE (3). SE seizures can lead to memory, learning, and behavioral disorders. SE animal models are used to assess memory, learning, behavior, and neuronal cell death and cell damage mechanisms after SE because anatomic and physiologic changes observed in SE in animal models show similar characteristics to humans (4).

The immature brain exhibits differences from the mature brain regarding the development and spread of seizures, electroencephalographic (EEG) features, behavioral characteristics, and consequences of seizures (5). Animal experiments show that immature brains have more seizure tendencies than mature brains (6). The possible reason for this is that the balance between the inhibitor and the excitatory structures cannot reach the required level (7).

GABA-A receptors are areas where many antiepileptic drugs act during seizures. Regarding this information, GABA-A receptors in the hippocampus can be considered to have varying properties and functions during SE. Understanding the functional properties of GABA-A receptors and the effects of SE on the properties of these receptors are guiding in the pathogenesis and treatment of SE (3).

Experimental models and clinical studies have shown that prolonged seizures or SE may lead to neuronal cell death in the brain and neuronal damage secondary to brain injury, and neuronal death mechanisms have been described as associated with apoptosis, which is part of programmed cell death (8). Apoptosis is a physiologic process for cell death in the normal developmental process of multicellular organisms (9). In ultrastructural studies with electron microscopy, apoptosis is defined as typical well-defined morphologic changes that occur in the cell, together with a decrease in growth factor activation after some triggering conditions. Well-defined key features include aggregation of chromatin threads, preservation of intracellular organelle integrity, and formation of membrane-enclosed cell contents called apoptotic bodies (10). Apoptosis occurs in the presence of a regular molecular cascade. It is typically energy dependent and requires new gene transcription. There are two major gene regulatory families for apoptosis, caspase, and Bcl-2 (10). Caspase-3 is the most studied determinant among the apoptosis regulators in seizure-triggered neuronal damage and neuronal cell death. After epileptic seizures, caspase-3 mRNA and protein synthesis are induced in the hippocampus and extra-hippocampal areas (11). In many studies, there were findings suggestive of an increase in caspase-3 activity after seizure; however, an increase in caspase-3 activity after seizures has not been shown in some studies (12). In previous studies, it has been observed that the apoptotic pathway continues to be active in the epileptogenesis process after the acute phase of events that trigger neuronal cell damage such as SE, that the activity of caspase-3 and caspase-6 is prominent, and that caspase-3, in particular, is active until after one week from the onset of neuronal damage (13).

"Necrotic cell death" or "necrosis" is morphologically characterized by increased cell volume, swelling of organelles, and rupture of the plasma membrane and subsequent loss of intracellular content. Biochemically, necroptosis is defined as a spectrum between uncontrolled random cell death and programmed cell death (13,14,15).

Substances that cause necrosis have not yet been fully clarified. Nonetheless, this definition includes unpaired reactive oxygen species, nitrotoxicity, oxidative stress, lysosomal changes, and mitochondrial changes such as mitochondrial membrane permeability (16). Their presence increases the concentration of cytosolic calcium and leads to the activation of non-caspase proteases such as calpain and cathepsin due to mitochondrial overloading (16,17). Overall, although not completely, serine / threonine kinase has been shown to have the main role in most cases of necrotic cell death (18).

In SE models, apoptosis is the main mechanism of death in immature neurons, and necrosis is the main mechanism of death in mature neurons (19,20). This indicates that the maturation level is an important parameter in determining the type of cell death triggered by seizures. With a classic view, necrosis is believed to be a passive process, and a systematic cell death mechanism is not required. For this reason, experimental models of SE will lead us to determine whether there are some active forms of necrosis (19).

In this study, behavior, learning, and memory were assessed using the open field test and the Morris water maze in immature and mature rats after pentylenetetrazole (PTZ), a GABA inhibitor, -induced SE, as well as immunohistochemical expression of caspase-3, which is a cellular damage mechanism. We also investigated changes in the number of GABA-A alpha 1-positive neurons, which is a subunit of the main inhibitor neurotransmitter GABA.

Materials and Methods

Experimental Animals

In this study, the experiment was conducted after receiving approval of Erciyes University Animal Experiments Local Ethics Committee (HADYEK) (decision: 13/36; date: 13.02.2013). In the study, 20 mature male Wistar rats of 150 days, 20 adolescent male Wistar rats of 45 days, and 12 immature male Wistar rats of 17 days were used. All rats were obtained from Erciyes University Hakan Çetinsaya Experimental and Clinical Research Center and maintained on a 12h:12h light/dark cycle with free access to food and water. All experiments were done at the same time in the afternoon so that the rats would not be affected by circadian rhythm changes.

In order to assess behavior, learning, and memory in immature and mature animals after SE, the rats were initially divided into two groups as control and SE groups. The control and SE groups were subdivided into subgroups as mature control (n=10) and adolescent control (n=10) groups, and mature SE (n=10) and adolescent SE (n=10) groups. The disappearance of caspase activity after seven days from the initiation of SE was taken into account (21). Twelve immature rats, an experimental group (n=6) and control group (n=6) were also included in the study to assess caspase-3 activity 24 hours after SE because learning and behavioral tests applied to experimental and control groups persisted for seven days, and because disappearance of caspase activity seven days after SE has been demonstrated in previous studies (21). Immature rats were compared with adolescent and mature rats for GABA-A alpha 1 level and calpain activity.

Induction of Status Epilepticus

To induce SE, PTZ was injected intraperitoneally (IP) to each rat in the experimental group, and physiologic saline (PS) was injected IP to each rat in the control group.

Seizures were induced by repetitive injections of PTZ given as the first dose of 35 mg/kg, followed by additional injections of PTZ 10 mg/kg until the onset of SE. The number of injections is shown in Table 1. The time interval between the first dose and the second dose was 10 min, and the time interval between the subsequent doses was 5 min. After PTZ injection, animals were placed in plastic cages of 50x20x25 cm in size and seizure scoring was performed during the 30-min observation as follows (22):

Stage 0: No response.

Stage 1: Ear and facial twitching.

Stage 2: Myoclonic jerks without an upright position.

Stage 3: Myoclonic jerks, upright position with bilateral forelimb clonus.

Stage 4: Clonic seizures with loss of postural control.

Stage 5: Recurrent severe tonic-clonic or fatal seizures.

Seizures that lasted 30 minutes after the onset of stage 4 or stage 5 seizures or myoclonic or clonic seizures with a maximum of 5 minute-intervals that lasted at least 30 minutes after the onset of stage 4 or stage 5 seizures were accepted as SE.

Behavioral Tests

Morris Water Maze

The Morris water maze is the preferred apparatus for assessing locomotion (spatial learning), especially in rodents such as rats and mice. In order to test spatial learning in rats, a Morris water maze with a diameter of 131 cm and a depth of 44 cm was used. For the experiments, the water in the maze was colored with universal concentrated color paste (DINCKIM). The water temperature was set at 26±2 °C. In the training period of learning, a cylindrical platform 10 cm in diameter was placed 10 cm from the poolside under one centimeter of water. Staying in the field of view of the rat on the water surface, the white, red, yellow geometric-patterned panels that contrasted with the black and black panels were hung on the back and sidewalls of the quadrant. The water tank was divided into four imaginary quadrants, and the rats were left in quadrants where the platform was not present. In the first trials, the rat was allowed to swim for 1 min, expecting it to find the platform. The rats that failed to find the platform were guided to the platform. The animal was allowed to sit on the platform for 20 seconds. All subjects were taken to the Morris water maze between 2 and 5 pm. They were trained to swim 4 times a day with 20 min-intervals between each quadrant. After 4 consecutive days, the platform was removed on the 5th day, which was the learning period. Facing the wall of the pool, the rat was placed in the water just across the quadrant in which the platform is located. For the evaluation, the time it took for the animal to reach the platform, the time spent in the target quadrant, the average swimming speeds, and the path traveled were recorded using video recording system and EthoVision software (Noldus Information Technology), and the output values of the analysis were evaluated statistically as percentages.

Open Field Area

The rats were always left in the middle of a square-shaped apparatus made of Plexiglas (100x100x30 cm) with its floor divided into 16 equal squares. For the evaluation, the number

Table 1. The number of injections and seizure latency

		Groups			
		Mature status epilepticus	Adolescent status epilepticus	Immature status epilepticus	p value
Variables	The number of injections	1 (1-4)	1.7 (1-3)	1.5 (1-3)	>0.05
	Seizure latency (minute)	7.5 (3-22)	11 (6-20)	10.5 (4-15)	>0.05

of rearing, grooming, freezing, defecation and line-crossing movement during the 5-minute test period were noted and the time spent in the periphery and in the center of the platform was calculated using the video recordings. After each trial, the area was cleaned with 70% alcohol.

Behavioral Criteria in Open Field Test

Freezing: No movement except breathing for at least 8 seconds.

Rearing: Standing on the hind legs for at least 3 seconds.

Grooming: Licking or scratching itself for at least 10 seconds.

Preparation and Evaluation of Tissue Sections

Under general anesthesia (80-100 mg/kg ketamine and 10-12.5 mg/kg xylazine, IP), after the thorax was opened and the heart was isolated, a cannula was placed in the left ventricle and the right atrium was cut at 24 hours post-injection in immature rats in the experimental and control groups without applying neurophysiologic tests and after behavioral tests in adolescent and adult rats. The brain tissue was perfused first with 50 mL of SF, then with 50 mL of 10% formalin solution under constant pressure through the cannula placed in the left ventricle. As soon as the perfusion process finished, the brain tissue was removed entirely, put into a 10% formaldehyde solution, and switched to an immersion technique. Brain tissues were trimmed by taking coronal sections from the bregma, and the hippocampus region and cortex of the brain were examined. Tissue pieces from each animal were placed in trays and fixed in 10% formaldehyde solution (this solution was renewed daily) for up to 5 days. At the end of the fifth day, the tissues were washed under running water for 24 hours. The tissues were dehydrated in 70%, 80%, 96%, 100% alcohol, respectively, and then placed in methyl benzoate for 2 days. At the end of this period, the tissues that passed through the benzoles were embedded in the paraplast. The blocks containing the hippocampus and cortex were sectioned to lysine-coated slides at 5-micron thickness with regular spaces (50-100 µm). The Kluver-Barrera method was used in a series of sections to determine the general structure, and the streptavidin biotin complex (Strept-ABC) immunoperoxidase technique was applied to the other series. For each parameter, a series of 12 specimens in 4 slides were prepared, with 3 specimens per slide. For the determination of the general structure and 3 kinds of antibodies (calpain 1, caspase-3 and GABA-A receptor alpha 1), 16 serial preparations (960 sections in total) were prepared from each tissue.

Sections prepared for immunohistochemical studies were washed in phosphate buffer solution (phosphate- buffered saline, PBS) for 5 min following deparaffinization and rehydration, and then treated with 3% hydrogen peroxide (H_2O_2) prepared in methanol for 15 min to block the endogenous peroxidase activity. The sections were washed twice in the PBS for 5 min, boiled in citrate buffer (pH: 6.0) at 80 °C for 30 min, and left to cool in the same solution for 20 min to recover the tissue antigen. Following washing with PBS 4 times for 5 min, sections were incubated in a humid chamber with blocking solution (Ultra V block, Thermo Fisher Scientific Lab Vision Corporation, Freemont, USA; TA-125UB) for 5 min to prevent non-specific binding. Subsequently, primer antibodies (Table 2) prepared at appropriate dilutions were added onto the sections and incubated in the humid chamber at room temperature for 1 hour or at 4 °C overnight relative to the antibody to be used. Tissue samples taken as negative controls were treated with either primary non-antibody PBS or non-immunized rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, sc-2027). Following incubation, the sections were washed 3 times in PBS, and then incubated with biotinylated secondary antibody (Ultravision Detection System/HRP, Thermo Fisher Scientific Lab Vision TR-125-HL) for 20 min at room temperature. The sections were then washed 3 times with PBS and treated with enzyme-conjugated streptavidin (Ultravision Detection System/HRP, Thermo Fisher Scientific Lab Vision TR-125-HL) for 20 min at room temperature. After washing 3 times with PBS, they were incubated in the chromogen solution (DAB, Thermo Fisher

Table 2. The antibodies used in the immunohistochemical analysis

Antibody	Host	Immunogen	Company/ Catalog number	Dilution ratio	Species reactivity
Primary antibodies					
Calpain 1 (H-65) polyclonal antibody	Rabbit	The epitope corresponding to the 608-672 amino acid sequence near the C-terminus of the human origin calpain 1	Santa Cruz Biotechnology, sc-13990	1:100	Rat, mouse, human, dog, cattle, pig, horse
Caspase-3 (CPP32) Ab-4 polyclonal antibody	Rabbit	Human caspase-3 recombinant protein	Thermo Scientific, RB-1197	1:100	Rat, mouse, human, monkey, rabbit, hamster, dog, cattle, pig, sheep
GABA-A receptor alpha 1 polyclonal antibody	Rabbit	The KLH-conjugated linear peptide corresponding to the topologic domain of rat GABA-A receptor alpha 1	Millipore 06-868	1:100	Rat, mouse
Secondary antibody					
Anti-rabbit IgG	Goat	Ultravision Detection System/HRP, Thermo Fisher Scientific Lab Vision TR-125-HL			

GABA-A: Gamma-aminobutyric acid-A, IgG: Immunoglobulin G

Scientific Lab, Vision Corporation, Freemont, USA) for 5-20 min. They were stained in Gill's hematoxylin for 3 min and then washed in tap water until they turned blue. Sections were passed through alcohol and xylol series, and then adhesive (Entellan®) was dropped, and lamella was applied. The observation of brown precipitation was evaluated as a positive reaction, and it was photographed under a light microscope (Olympus BX51, Japan). In the evaluation of the preparations, the number of positive-stained cells in the hippocampus CA1, CA2, CA3, and dentate gyrus regions and cortex were calculated. For the calculation, the number of cells stained positive with the relevant antibody was recorded under a light microscope with x20 magnification using 5 sections for the hippocampus CA1 region, 2 sections for the CA2 region, 3 sections for the CA3 region, 4 sections for the dentate gyrus, and 4 sections for the cortex. The number of cells stained with the respective antibodies indicated in this study represents the numerical sum of cells calculated per section by magnification at x20 under a light microscope.

Statistical Analysis

Statistical analysis was performed using the SPSS for Windows (v.15.0) package program. In the evaluation of open field test parameters, One-Way analysis of variance (ANOVA) was used to compare the groups. In assessing the Morris water maze test parameters, One-Way ANOVA was used for intergroup comparison, and repeated-measures ANOVA was used for intragroup comparisons. Regarding the GABA-A alpha 1 and caspase antibody group comparison, the independent samples t-test was used for normally distributed data, and the Mann-Whitney U test was used for non-normally distributed data. The normality of the tests was evaluated using the Shapiro-Wilk test. The Kruskal-Wallis test was used in multiple group comparisons while assessing calpain antibody levels. A value of $p<0.05$ was considered statistically significant.

Results

Assessment of Seizure Latency and Number of Injections

In the study, mature, adolescent, and immature study groups were compared with each other in terms of time to SE onset (latency) and the number of injections (Table 1). There was no statistically significant difference between the groups ($p>0.05$). SE lasted for 30 min to 3 hours in all groups ($p>0.05$), and there was no attempt to stop the seizure. During SE, 3 mature, 3 immature, and 3 adolescents were excluded from the study due to death.

Evaluation of Behavioral Parameters

The number of lines crossed on the platform, freezing and grooming, defecation and rearing, and the time spent (seconds) in the center and the periphery of the control and experimental groups in the open field test is given in Table 3. No statistically significant difference was found between the mature and adolescent experimental groups and the control groups in the behavioral tests in any of the parameters ($p>0.05$).

Evaluation of Memory and Spatial Learning Performance

In the Morris water maze test, which is used for spatial learning and memory assessment, the latency to find the target zone (sec), the total time (sec) spent on the target quadrant, swimming speeds, and total swimming distance were evaluated on a daily basis.

Regarding latency to find the target zone, there was no statistically significant difference between the groups on any of the trial days ($p>0.05$). When latencies to find the target zone were compared according to days, a statistically significant shortening was observed in all groups day after day ($p<0.05$). When all groups were evaluated according to daily swimming speeds, there was a statistically significant difference only in the mature experimental group ($p<0.05$). When the 1st day and the 4th day were compared in the mature group, swimming speed on day 1 was statistically significantly lower than day 4 ($p=0.001$). When the daily swimming distance was compared in all groups, it was found that the swimming distance on the 1st day was significantly longer than on the 4th day in all groups except in the mature experimental group ($p=0.001$). When swimming distance was evaluated according to the groups, a statistically significant difference was detected between the groups only on the 2nd trial day ($p=0.027$). The swimming distance on the 2nd day in the mature control group and the adolescent experimental group was statistically longer than the mature experimental group and the adolescent control group ($p<0.05$). Regarding the time spent on the target quadrant in the learning period of the Morris water maze test, no statistically significant difference was observed between the mature control and experimental groups and between the adolescent control and experimental groups ($p>0.05$).

Table 3. Open field test parameters of control and experimental groups

Groups	Defecation (n)	Rearing (n)	Freezing (n)	Grooming (n)	line crossing (n/5 min)	Time spent in the center (sec)	Time spent in the periphery (sec)
Mature experimental	3.7	6.6	0.5 (0-11)	5.2	18.5 (5-26)	3.5 (2-26)	296.5 (274-298)
Mature control	2.8	2.8	1 (0-2)	4.3	10.5 (5-31)	1 (0-24)	299 (276-300)
p value	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05
Adolescent experimental	4	6	0 (0-2)	2 (1-5)	53 (26-75)	0 (0-10)	300 (290-300)
Adolescent control	3.8	6.5	0 (0-1)	2 (1-3)	28.5 (20-72)	0 (0-10)	300 (290-300)
p value	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05

sec: Second, min: Minute

Assessment of Caspase-3, Calpain 1, and GABA-A Alpha 1 Activities

Assessment of Caspase-3-positive Staining Cells

The number of caspase-3-stained cells that were examined in order to investigate the role of caspase-3 in neuronal cell damage due to SE is given in Table 4.

The number of caspase-3-positive cells was significantly higher in the experimental group compared with the control group (p=0.002). The caspase-3-positive cell counts in the hippocampus and cortex in the experimental group are shown in Table 5. The caspase-3-positive cell counts in the cortex were found to be significantly higher than in the hippocampus (p<0.001). The caspase-3 activity in the 17-day immature control and experimental groups is shown in Figure 1.

Assessment of Calpain 1-positive Staining Cells

Table 6 shows the number of cells stained positive for calpain 1 in order to investigate the role of calpain 1, which plays a role in caspase-independent cell damage, on neuronal cell damage due to SE. The numbers of cells stained positive for calpain 1 were as follows: 2.72 (2.51-3.05) in the mature experimental group, 0.08 (0.02-0.11) in the mature control group, 0 in the adolescent experimental group, 0.02 (0-0.08) in the adolescent control group, 0.03 (0-0.06) in the immature experimental group, and 0.03 (0-0.06) in the immature control group. Regarding intergroup comparisons, only the number of calpain 1-positive cells was significantly higher in the mature experimental group than in the other groups (p<0.05) (Figures 2, 3). The number of cells stained with calpain in the hippocampus and cortex in the entire experimental group is shown in Table 5. There was no statistically significant difference between calpain 1-positive staining cells in the cortex and hippocampus (p=0.533).

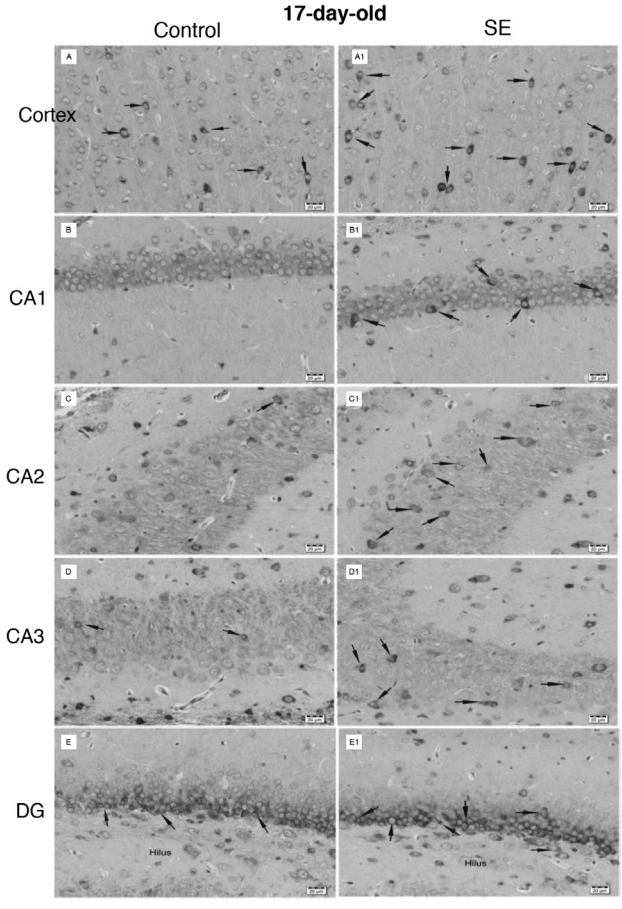

Figure 1. Caspase activity in the control and experimental groups in immature rats

Assessment of GABA-A Alpha 1 Levels

The effect of SE on the level of GABA subunit GABA-A alpha 1 levels, which play a major role in the inhibition of the central nervous system, was assessed (Figures 4, 5). The numbers of cells stained positive with GABA-A alpha 1 antibody were as follows: 2.183±0.562 in the mature experimental group, 3.132±0.066 in the adolescent experimental group, 2.450±1.36 in the immature experimental group, 3.812±0.222 in the mature control group, 3.324±0.106 in the adolescent control group, and 3.401±1.70 in the immature control group.

Regarding intragroup comparisons of mature, adolescent, and immature experimental groups, the number of cells stained positive with GABA-A alpha 1 antibody was statistically significantly lower in the experimental groups (p<0.001, p=0.004, and p<0.001, respectively) (Figure 4).

The GABA-A alpha 1-positive cell count was significantly lower (p=0.009) in the mature experimental group than in the adolescent experimental group, and significantly higher in the mature control group than in the adolescent control group

Table 4. Number of cells stained positive with caspase antibody (number/x20 magnification)

Groups	Control	Experimental	p value
Immature	2.150 (1.69-2.23)	3.72 (2.92-3.58)	0.002

Table 5. Number of cells in the cortex and hippocampus that stained positive with caspase or calpain

Groups	Cortex	Hippocampus	p value
Experimental group (caspase)*	5.683±0.53	2.357±0.14	**<0.001**
Experimental group (calpain)	0 (0-1.17)	0 (0-6.56)	>0.05

*Only caspase groups are present in immature rats (caspase activity disappears after 7 days after the status epilepticus has occurred)

Table 6. Number of cells stained positive with calpain

Groups	The amount of calpain (median)
Mature experimental	2.72 (2.51-3.05)
Mature control	0.08 (0.02-0.11)
Adolescent experimental	0
Adolescent control	0.02 (0-0.08)
Immature experimental	0.03 (0-0.06)
Immature control	0.03 (0-0.06)

(p=0.002) (Figure 4). When the GABA-A alpha 1-positive cells in the hippocampus and the cortex were compared, it was found that the number of GABA-A alpha 1-positive cells in the hippocampus was significantly higher than GABA-A alpha 1-positive cells in the cortex in the mature, adolescent, and immature experimental groups (p<0.001, p<0.001, and p<0.001, respectively).

Discussion

Animal models are used to assess post-SE memory, learning, behavior, and neuronal cell death and cell damage mechanisms because memory, learning, and behavioral disturbances can occur in SE and neuronal cell death and cell damage mechanisms are similar to animal models (4). The development and spread of seizures, EEG features, behavioral characteristics, and consequences of seizures are related to brain maturation (5). In this study, cellular damage mechanism was evaluated immunohistochemically as well as through evaluation of behavior, learning, and memory in immature and mature rats after PTZ-induced SE. We also investigated changes in the number of GABA-A alpha 1-positive neurons, a subunit of the main inhibitor neurotransmitter GABA.

In a study evaluating the effects of SE on behavior, memory, and learning in immature and mature rats, it was shown that cognitive damage occurs in rats after SE, but that cognitive impairment in immature rats is not as severe as in mature groups (23). In another study, it was reported that immature rats had transient behavioral changes following PTZ-induced SE and had no problems in emotional memory or learning (24). In a study assessing immature rats 60 days after lithium-pilocarpine-induced SE, impaired cognition and reduced anxiety were observed, and it has been reported that seizures during early life may lead to cognitive impairment and behavioral disturbance in the long term (25). In another short-and long-term study of memory, learning, and behavioral functions, it was reported that 50-60-day-old rats had an anxiety-related increase in the open field test in the early post-SE period and that the experimental group had impairment in memory associated with unconditional fear. However, when the tests were repeated 6 months later, no significant difference was reported between the groups (26).

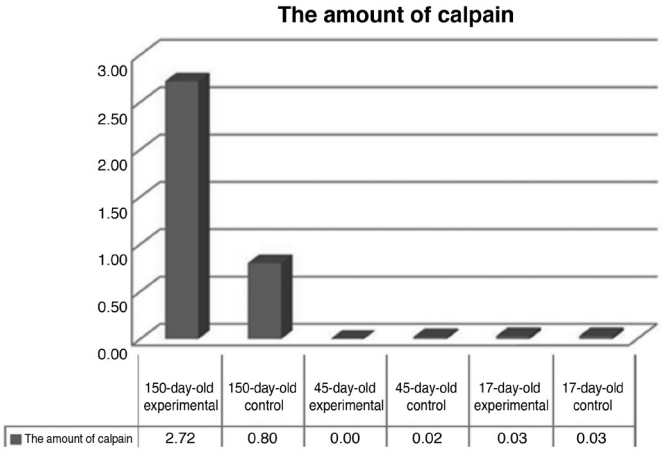

Figure 2. Distribution of the amount of calpain by groups

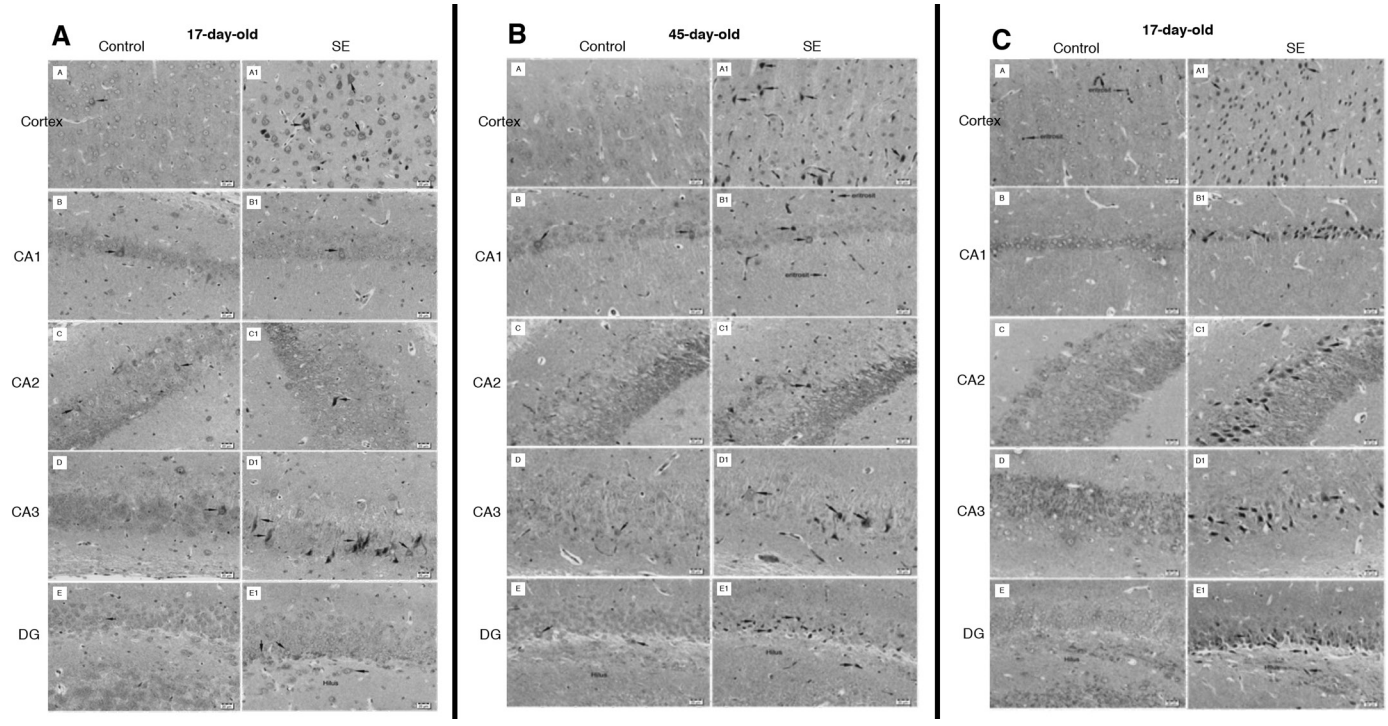

Figure 3. Calpain activity in the control and experimental groups. A) Calpain activity in the control and experimental groups in immature rats. B) Calpain activity in the control and experimental groups in adolescent rats. C) Calpain activity in the control and experimental groups in mature rats

In our study, the results of the open field and Morris water maze test for evaluating post-SE behavior, memory, and learning in 45-day-old adolescent and 150-day-old mature rats were similar in both experimental groups compared with the control groups, and this finding was in accordance with the literature. In the Morris water maze test, it was found that all animals found the platform more easily as the number of trials increased, but there was no significant difference between the groups. This suggests that the spatial learning processes were not affected in the experimental groups.

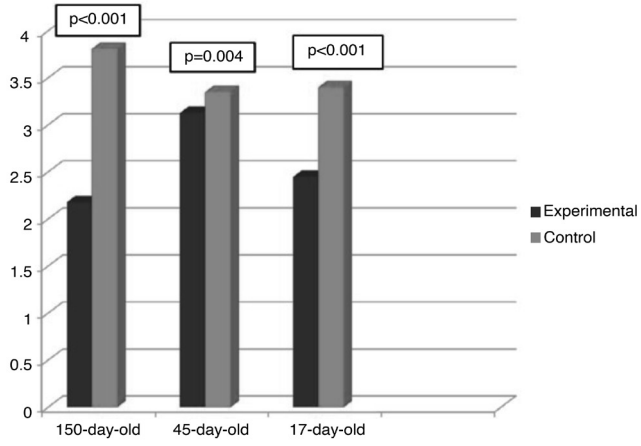

Figure 4. Comparison of gamma-aminobutyric acid-A alpha 1 receptor numbers between groups

Although seizures have been shown to lead to neuronal cell death in many studies, the underlying mechanisms are not fully understood. Although the main mechanism of neuronal death due to seizures is necrosis in the adult brain, apoptotic morphology has been described in several SE models, and the role of apoptotic mechanisms has been demonstrated to be irrefutable (27,28,29). Post-injury apoptosis was found to be more probable than necrosis in the immature brain than in adults (30), suggesting that age-related apoptotic death factors are rarely present (31). In studies, both apoptotic and necrotic morphologies were detected at light and electron microscopic levels in the immature brain after SE, and apoptosis was reported to be observed in the inner granular cells of the dentate gyrus in the hippocampus in 14-day-old rats (32,33). It was also reported that SE-induced neuronal damage was widely observed in the CA1 pyramidal cell layer of the hippocampus and that necrotic morphology was found in 47 of 50 cells in CA1 24 hours after SE and in all 50 cells after 72 hours. Based on this, it is suggested that the main type of neuronal cell damage after SE in the immature brain is necrosis (32,33).

Among apoptosis regulators, caspase-3 is the most studied marker of neuronal damage and neuronal cell death triggered by seizures (11,34). Caspase-3 is known to play an active role until 1 week after the onset of neuronal damage (13,21). Although an increase in caspase-3 activity after seizures has been demonstrated in many studies, it has not been shown in others (12,35). To investigate the role of SE-associated neuronal cell damage in our study, we found that the number of caspase-3 antibody-positive

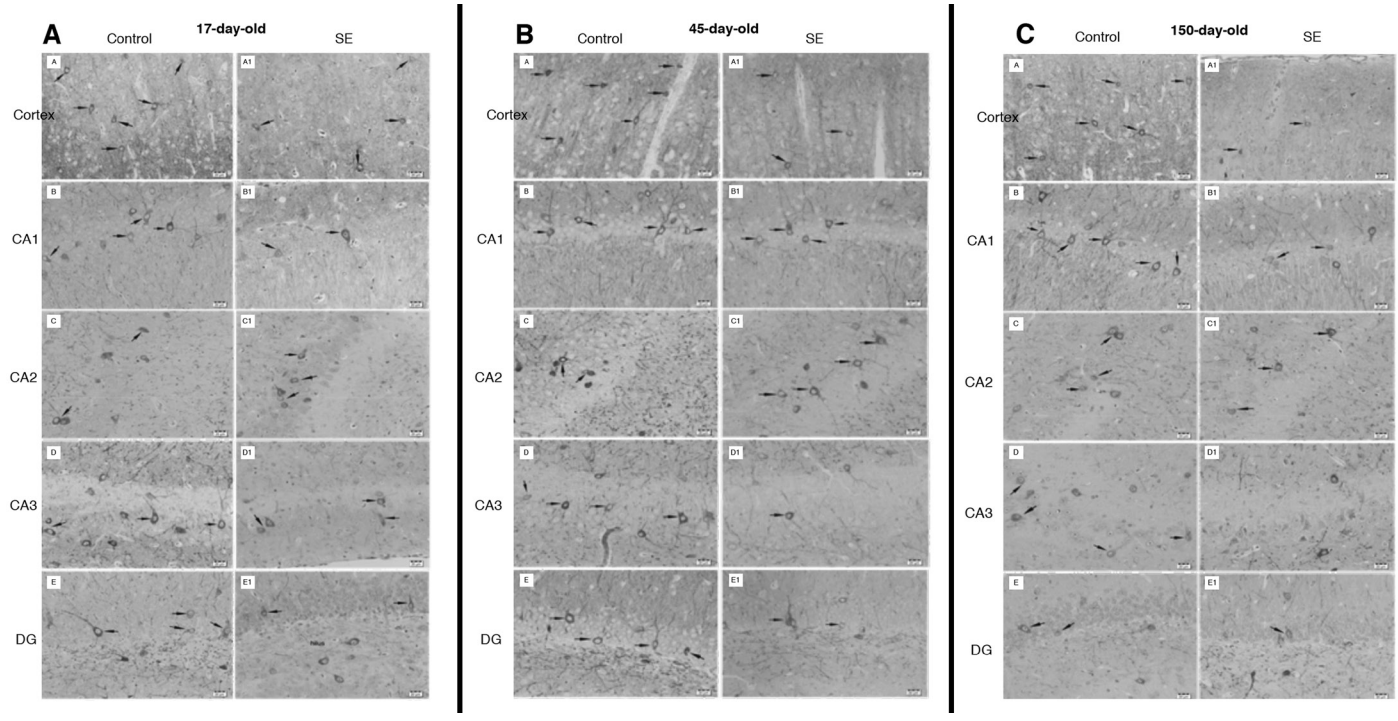

Figure 5. Gamma-aminobutyric acid-A (GABA-A) alpha 1 receptors in the control and experimental groups. A) GABA-A alpha 1 receptors in the control and experimental groups in immature rats. B) GABA-A alpha 1 receptors in the control and experimental groups in adolescent rats. C) GABA-A alpha 1 receptors in the control and experimental groups in mature rats

staining cells 20 h after SE was found to be significantly higher in the experimental group compared with the control group (p=0.002) and caspase-3-positive staining cells were found to show apoptotic morphology.

In a study conducted in rats, it was shown that calpain activity was dominant on days 1-3 and that caspase-3 activity was dominant on days 5-7 in the hippocampus after lithium-pilocarpine-induced SE. Calpain-mediated cell damage in the rat hippocampus was reported to decrease after the injection of MDL-28170, an inhibitor of calpain (36). In another study, immunohistochemical evaluation was performed 24 hours after kainic acid-induced epileptic seizure to assess caspase and calpain activation, it was reported that caspase activation was not found in the acute phase of SE, but that calpain activation was found and that calpain, a calcium-dependent protease, had a role in the early period of cell damage after SE (37).

In our study, in order to investigate the role of calpain, which plays a role in caspase-independent cell damage, on the SE-induced neuronal cell damage, when all groups were compared with each other in terms of number of calpain 1 positive staining cells, the results were statistically significantly higher only in the mature experimental group than in the other groups ($p<0.05$). No significant calpain activity was observed in the immature experimental group or in the adolescent experimental group. Studies in the literature generally evaluate calpain activity comparing according to the changing processes after SE. Post-SE activation of calpain was demonstrated in mature and adolescent rats, but there were no age-dependent studies evaluating calpain activity. The detection of calpain-mediated neuronal damage only in the mature group suggests that SE, which causes neuronal damage, has more destructive effects when exposed to adult age groups. It is possible that the brain will be less affected in the long term because SE encountered during the early stages of life or in young adulthood may lead to less cell damage.

It is known that SE restricts the expression of GABA-A receptor subtypes in rat hippocampus in normal development and inhibits the physiologic alterations of alpha 1 and alpha 2 subtypes. The expression of the alpha 1 subtype plays a role in the resistance of inhibitor synapses. Decreased expression of the alpha 2 subtype is necessary for healthy functioning of the inhibitor system. A decrease in the amount of alpha 1 subunit in SE leads to impairment in the development of dendritic synapses (38).

In our study, when numbers of cells stained positive with GABA-A alpha 1 antibody in mature, adolescent, and immature experimental groups and control groups were compared amongst themselves, it was found that the numbers of GABA-A alpha 1-positive cells in the experimental groups were significantly lower ($p<0.001$, $p=0.004$, $p<0.001$, respectively) (Figure 4). The amount of GABA-A alpha 1-positive cells was significantly lower in the mature experimental group than in the adolescent experimental group ($p=0.009$) and higher in the mature control group than in the adolescent control group ($p=0.002$) (Figure 4). Comparing GABA-A alpha 1-positive cell counts in the hippocampus and cortex, the number of GABA-A alpha 1-positive cells in the hippocampus was higher than in the cortex in the mature, adolescent, and immature experimental groups ($p <0.001$, $p<0.001$, $p <0.001$, respectively).

In our study, the results of GABA-A alpha 1 were similar to those in the literature (32,39,40,41), suggesting that SE in the mature period leads to more GABA-A alpha 1 receptor destruction than during the immature or adolescent periods and predisposes to epileptogenesis. It also suggests that SE exposure during the mature period reduces the seizure threshold by leading to more GABA inhibition. Until now, studies have been made with lithium-pilocarpine and kainic acid SE models, which represent the temporal lobe epilepsy model. When reviewing the literature, it is seen that the PTZ-induced epilepsy model represents idiopathic generalized epilepsy and that the PTZ SE model is not used frequently. For this reason, our study method differs from other studies. Previous studies have evaluated caspase, calpain activity, and the number of GABA-A alpha 1 receptors separately after SE-mediated neuronal injury. In addition, there is no study evaluating age-related calpain-mediated cell damage in the literature. In our study, they were evaluated together and compared by age.

Conclusion

The data obtained in this study suggest that PTZ-induced SE does not negatively affect learning and behavioral functions in the early period. Immunohistochemical findings show that SE leads to caspase-3 and calpain 1-mediated neuronal damage, that SE-mediated cell damage increases with increasing age and that caspase-dependent apoptotic morphology is more evident in immature rats and calpain-1 mediated cell necrosis in mature groups. Furthermore, the findings indicate that SE causes a geater decrease in the number of GABA-A alpha 1 receptor-positive neurons in mature and adolescent rats. Long-term follow-up studies are needed to understand the long-term effects of these changes on the cellular level of cognition and behavior.

Ethics

Ethics Committee Approval: In this study, the experiment was conducted after receiving approval of Erciyes University Animal Experiments Local Ethics Committee (HADYEK) (decision: 13/36; date: 13.02.2013).

Informed Consent: Experimental study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: A.S., N.L., Ali.S., Concept: F.F.E., A.S., N.L., Ali.S., Design: F.F.E., N.L., Data Collection or Processing: Ali.S., M.F.G., F.F.E., Analysis or Interpretation: Ali.S., F.F.E., M.F.G., Literature Search: Ali.S., M.F.G., Writing: M.F.G., F.F.E.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: This project is supported by Scientific Research Projects Coordination Unit of Erciyes University with TDK-2013-4529 code.

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