

Investigation of anti-epileptic mechanisms of 5HT_{1A} receptor with pentylenetetrazole induced epilepsy model in rats

Sıçanlarda pentilentetrazol ile oluşturulan epilepsi modelinde 5HT_{1A} reseptörünün antiepileptik etki mekanizmalarının araştırılması

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SUMMARY






Objective: According to current neurophysiological evidence, the role of 5-hydroxytryptamine (5-HT) receptors in epileptic seizure formation is still not fully elucidated. The aim of this study was to investigate the effects of 5-HT_{1A} receptor on epileptic seizure with pentylenetetrazole induced epilepsy in rats.

Method: In this study, 28 male Wistar Albino rats weighing 240-260 g were used. Pentylenetetrazole (PTZ, 35 mg/kg, i.p.) was injected to the rats to induce epilepsy, and seizure stages were determined according to the Racine scale. Electrodes were placed in the skulls of the animals under stereotaxis for ECoG recording. All the experimental animals were sacrificed by decapitation after ECoG and video recordings. GABA level was measured using the Elisa kit from brain tissues, and c-Fos expression was shown immunohistochemically.

Results: According to the results, it was shown that 8-OH-DPAT increased the time of the initial myoclonic jerk (FMJ ($p<0.05$)). However, the number of epileptic spikes was reduced by 8-OH-DPAT ($p<0.05$). GABA levels decreased in PTZ group ($p<0.05$). 8-OH-DPAT and WAY-100135 decreased c-Fos expression in all hippocampal areas ($p<0.05$).

Conclusions: In conclusion, 5-HT_{1A} receptor agonist 8-OH-DPAT, showed an anti-epileptic effect. The anti-epileptic effects of 5-HT_{1A} receptor were found to be inconsistent with changes in GABA level. the c-Fos expression is a marker of neuronal activation and may be related to the anti-convulsive effect of 5-HT_{1A} receptor.

Keywords: Epilepsy, pentylenetetrazole, serotonin, GABA, c-Fos, serotonin receptor agonist

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ÖZET

Amaç: Güncel nörofizyolojik kanıtlara göre, epileptik nöbet oluşumunda 5-hidroksitriptamin (5-HT) reseptörlerin rolü hala tam olarak aydınlatılmıř deęildir. Bu arařtırmada amacımız, pentilentetrazol (PTZ) ile epilepsi oluřturulmuř sıçanlarda 5-HT₁ reseptörün epileptik nöbet üzerine etkilerini arařtırmaktır.

Yöntem: Çalışmada 28 adet 240-260 g aęırlığında erkek Wistar Albino sıçan kullanıldı. Epilepsi oluřturmak için sıçanlara pentilentetrazol (PTZ, 35 mg/kg, i.p.) enjekte edildi ve nöbet evreleri Racine skalasına göre belirlendi. Elektrokortigram (ECoG) kaydı için hayvanların kafataslarına sterotaksi altında elektrotlar yerleřtirildi. EcoG ve video kayıtları alındıktan sonra tüm deney hayvanları dekapitasyon ile sakrifiye edildi. Beyin dokularından GABA düzeyi Elisa kit ile ölçüldü ve c-Fos ekspresyonu immünohistokimyasal olarak gösterildi.

Bulgular: Sonuçlara göre, 8-OH-DPAT'ın ilk myoklonik jerk (İMJ) başlama zamanını arttırdığı gözlemlendi ($p < 0,05$). Bununla birlikte epileptik spike sayısını 8-OH-DPAT azalttı ($p < 0,05$). GABA seviyeleri PTZ grubunda azalma gösterdi ($p < 0,05$). 8-OH-DPAT ve WAY-100135 c-Fos ekspresyonunu hipokampusün tüm alanlarında azalttı ($p < 0,05$).

Sonuç: Sonuç olarak, 5-HT₁ reseptör agonisti 8-OH-DPAT anti-epileptik etki göstermiřtir. 5-HT₁ reseptörünün anti-epileptik etkileri GABA düzeyi deęişiklikleri ile uyumlu bulunmamıřtır. Nöronal aktivasyon belirteci olan c-Fos ekspresyon sonuçları, 5-HT₁ reseptörünün anti-konvülfif etkisi ile iliřkili olabileceğini göstermektedir.

Anahtar sözcükler: Epilepsi, pentilentetrazol, serotonin, GABA, c-Fos, serotonin reseptör agonist

INTRODUCTION

Epilepsy is a chronic brain disease characterized by sudden, recurrent epileptic seizures caused by abnormal and excessive electrical discharge in cortical neurons¹. Epileptic seizures are caused by disruption of the balance between excitatory and inhibitory systems in the central nervous system. Animal models for epilepsy and epileptic seizures are vital for understanding the underlying mechanisms involved in epileptogenesis and for developing more effective antiepileptic treatments². The kindling model created by pentylenetetrazole (PTZ) injection is one of the most famous models of epilepsy used to investigate neurochemical processes and long-term structural changes in the brain and is used as a fundamental animal model for temporal lobe epilepsy (TLE) and complex partial epilepsy³.

Serotonin (5-HT) plays an essential role in many physiological (body temperature regulation, sleep, appetite), behavioral (aggression, mood) and cognitive (learning, memory) functions. Serotonin-containing neuronal cell bodies are confined to discrete clusters or cell groups along the midline of the brainstem. However, the axons can stimulate almost every region of the central nervous system (4). The serotonergic receptors are divided into seven different groups as 5-HT₁, 5-HT₂, 5-HT₃, 5-HT₄, 5-HT₅, 5-HT₆, 5-HT₇ and 14 different subgroups according to their structure, function, and location. They can have a wide range of effects due to their different distribution in neuronal tissues, vascular structures and smooth muscle cells⁵. The 5-HT_{1A} receptor subtype is one of the most widely expressed 5-HT receptors in the brain, and therefore the most studied^{6; 7; 8}. 5-HT_{1A} receptors are sub-classified into two groups:

presynaptic autoreceptors located on the dendrites and cell body of 5-HT neurons in the raphe nuclei; and postsynaptic heteroreceptors, which exist on the dendrites and cell body of target non-5-HT neurons in 5-HT projecting areas^{9; 10; 11}. Investigation of the role of the 5-HT₁ receptor in epileptic seizures has been achieved by the use of specific agonists and antagonists of the receptor or by genetical deletion of the receptor. Despite some exceptions, stimulation of 5-HT_{1A} receptors has protective effects against convulsive epilepsies while lowering the seizure threshold of absence epilepsies^{12; 13; 14}. Although there are studies on 5-HT₁ receptors in different experimental epilepsy models in the literature, no reviews have been found on the effects of ptz-induced kindling model and gamma-aminobutyric acid (GABA) levels. In this study, we aimed to show the effects of 5-HT_{1A} receptor on epileptic seizure activity in PTZ kindling model epilepsy.

MATERIAL AND METHODS

Animals

Twenty-eight adult male Wistar Albino rats (weighing 220-260 g) were used in this study. Rats were obtained from the Experimental Animals Laboratory of Sivas Cumhuriyet University. The animals were kept at 22 ± 1 °C room temperature for 12 hours in a light / dark cycle, in a sound-insulated room and containing $55 \pm 6\%$ humidity and fed in an appropriate ratio. Experimental studies were carried out between 09.00-12.00 every day in accordance with the circadian rhythm changes. In addition, the light and sound levels of the test medium were kept under constant control. Prior to the study, permission was obtained from Cumhuriyet University Animal Experiments Local Ethics Committee (No: 2016/96).

Epilepsy model

PTZ 35 mg/kg was administered intraperitoneally to animals on every Monday, Wednesday, and Friday up to 15 injections. After each injection, the animals were observed for 20 minutes, and the seizure stages were determined according to the Racine scale (RS). RS categorizes five stages of intensity, and it is based on the behavioral repertoire of the animals during a seizure, including “mouth and facial movements” (intensity stage 1); “head-nodding” (stage 2); “forelimb clonus” (stage 3); seizures characterized by rearing, (stage 4) and seizures characterized by rearing and falling (stage 5)¹⁵. The animals that had three subsequent five-stage seizures three times were accepted kindled. Then electrodes were placed these animals' skulls under stereotaxy to receive EEG recordings.

Drugs

Animals were separated into four groups: 1. Control (1 ml/kg saline; n = 7), 2. PTZ (35 mg/kg, i.p, Sigma-Aldrich, n=7)3. 8-hidroksi(OH)-dipropilaminotetralin (DPAT) (5-HT_{1A} agonist, 0,3 mg/kg, i.p, Sigma-Aldrich, n=7), 4. WAY-100135 (5-HT_{1A} antagonist, 1 mg/kg, i.p, Sigma-Aldrich, n=7). PTZ dissolved in saline. 8-OH-DPAT and WAY-100135 first dissolved in 1% lactate solution and then diluted with saline. All agents were administered intraperitoneally.

Electrocorticography (ECoG) Recordings

Before anesthesia, ketamine (90 mg / kg, i.p.) and xylazine (10 mg / kg, i.p.) was injected to the animals. The depth of anesthesia was controlled by corneal and paw reflexes. The locations of the screws to which the electrode will be placed were calculated using the rat brain atlas of Paxinos and Watson (1998), with reference to bregma and designated as the “0” point. With reference to bregma three holes were opened with a drill (OmniDrill35, WPI, Korea) and two screws were placed over the left somatomotor cortex (positive coordinates: AP: +4.0 mm, LL: 3.0 mm; negative coordinates: AP: -4.0 mm, LL: 3.0 mm), and a reference screw was attached to right hemisphere (coordinates: AP: -4.0 mm, RL: 3.0 mm). In these holes, stainless steel screws were placed in contact with the membranes for electrocorticogram (ECoG) recording. Socket electrode was placed on three screws. Electrodes were fixed to the skull using two layers of dental acrylic. Electrical activity with electrodes were amplified at the BioAmp (AD Instruments, Australia) interface and connected to the PowerLab 4 / SP (AD Instruments, Australia) ECoG recording system. The frequency and amplitude of the ECoG activity

were measured off-line. ECoG recordings were filtered between 1-34 Hz to eliminate ambient noise signals using PowerLab 4 / SP. After 30 minutes of administration of drugs, 35 mg/kg PTZ was given to induce seizures. ECoG and video recordings of animals were taken simultaneously for thirty minutes. In the evaluation of the video and ECoG recordings, the seizure stages of animals, the first myoclonic jerk time (FMJ), the number of spike-wave discharge per minute (SWDs) and the percentage of spike-wave discharge time (% SWDs) were calculated. The seizure times corresponding to epileptic spikes for 30 minutes were calculated using the program and converted to percentages, and referred to as the percentage of epileptic spikes. ($[\text{Seizure time} * 100] / 1800$; 1800 = seconds corresponding to 30 minutes).

GABA level measurement

For homogenization, phosphate-buffered saline (PBS, pH: 7.4) was used. The brain tissues were weighed and transferred to glass tubes following the cold chain, and then five mL of PBS buffer was added. The tissues in glass tubes were placed in plastic containers and homogenized at 4000 rpm for 10 minutes. Buffer was added such that the final volume was five times the tissue weight. The homogenates were numbered and placed in Eppendorf tubes without increasing the temperature. Brain GABA levels from the obtained homogenates were measured using a rat GABA ELISA kit (Sunred Biological Technology, Shanghai, China) according to the manufacturer's instructions.

Immunohistochemical Staining

Left cerebral hemispheres of control and study groups were detected in 10% buffered neutral formalin for 30-36 hours. Tissues were raised to + 4 °C after dehydration, clearing and paraffin embedding. A few hours before the sectioning with a microtome (Leica Germany), the blocks were taken to - 20 °C and serial sections containing five mm hippocampus area were taken. Each 12th section of serial sections was taken on a poly-L-lysine coated slide for evaluation, and five sections for each rat were evaluated for c-Fos immunoreactivity. The dentat gyrus (DG), CA1, CA2, and CA3 regions of the hippocampus of the control and study groups were examined separately by Olympus BX51 (Tokyo, Japan) microscope at 400X magnification. The c-fos positive cells in the DG, CA1, CA2 and CA3 regions of the hippocampus were quantitatively analyzed by counting using ImageJ (National Institutes of Health, Bethesda, MD, USA) software.

Statistical analysis

After the electrophysiological recordings were converted to numerical data, Statistical comparisons were made using SPSS software (Windows vers. 25.0). All experimental results were expressed as Mean \pm SEM (standard error of the mean). One-way analysis of variance (ANOVA) was used to compare the data between the groups and Tukey post hoc tests were performed for multiple comparisons. For all statistical tests, $p < 0.05$ was considered statistically significant.

RESULTS

The seizure stages were determined according to the Racine scale by observing the behavior of the animals for 30 min after PTZ injection into experimental groups. The highest seizure stages were scored as five in all experimental groups. In

the 8-OH-DPAT WAY-100135 groups, mean \pm SEM value was measured as 4.66 ± 0.21 ; 4.83 ± 0.16 respectively. No significant difference was found between groups in terms of seizure stages.

8-OH-DPAT significantly increased FMJ duration compared to the PTZ group ($p = 0.047$). Although WAY-100135 group decreased FMJ duration, the difference was not statistically significant ($p = 0.150$) (Fig.1). Epileptic spikes were found significantly lower in the 8-OH-DPAT group than in the PTZ group ($p = 0.008$). However, although there was a decrease in the number of epileptic spikes when the WAY-100135 group and PTZ group were compared, the difference was not statistically significant ($p = 0.599$) (Fig.2). 8-OH-DPAT showed anticonvulsant effect by reducing the percentage of epileptic spike time compared to the control group ($p < 0.05$) (Fig.3).

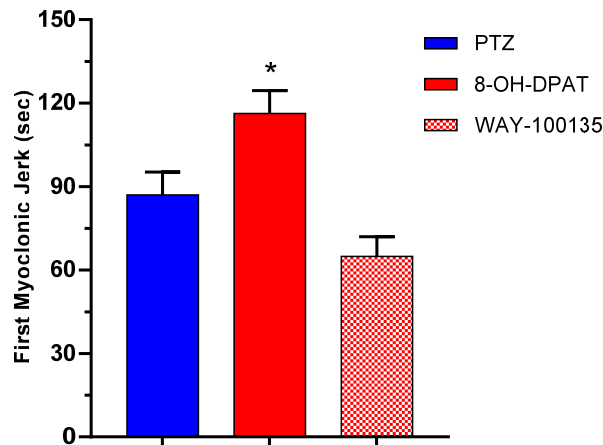


Figure 1: First myoclonic jerk (FMJ) duration mean (\pm SEM) values. * $p < 0.05$, compared with the PTZ group.

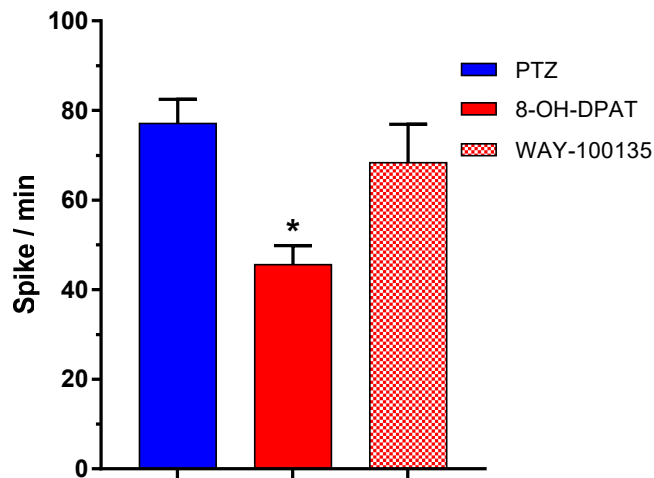


Figure 2: Mean values (\pm SEM) of epileptic spikes per minute. * $p < 0.05$, compared with the PTZ group.

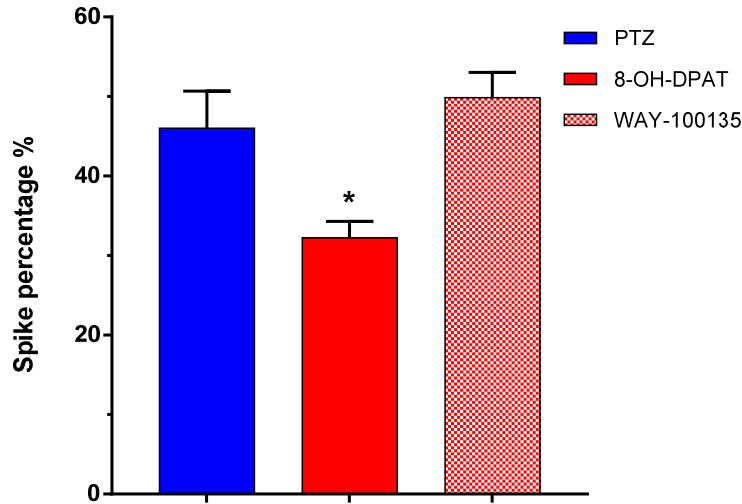


Figure 3: Mean (± SEM) values of epileptic spike time percentage. * p < 0.05, compared with the PTZ group.

The mean ± SD values of GABA levels were 139.94 ± 4.57 in the control group and 98.97 ± 6.99 in the PTZ group and were statistically significant

(p < 0.05). On the other hand, no significant difference was observed in the 8-OH-DPAT and WAY-100135 groups (p > 0.05) (Fig.4).

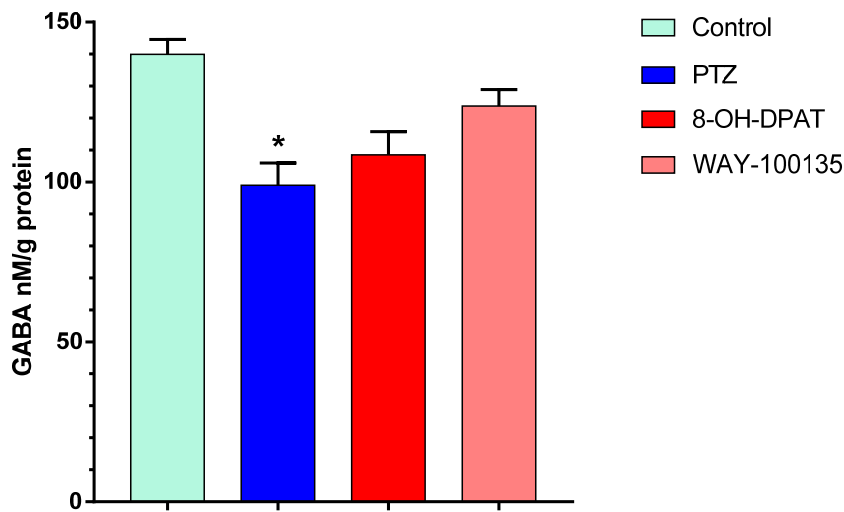


Figure 4: Mean (± SEM) values of GABA levels in groups. * p < 0.05, compared with the control group.

Immunohistochemical Findings

Figure 5A shows light microscopic (a, b) and c-Fos immunohistochemistry (c, d, and e) images of the control group. c-Fos immunoreactivity in the hippocampal DG region is indicated by yellow arrows (Figure 5B). When the 5-HT₁ receptor

groups were compared with the PTZ group, c-Fos immunoreactive cells were significantly decreased in both 8-OH-DPAT and WAY-100135 groups (p < 0.05) (Fig.5C). In the hippocampal CA1, CA2, CA3 regions, 8-OH-DPAT, and WAY-100135 were observed to reduce c-Fos immunoreactivity (p < 0.05) (Fig.6-8).

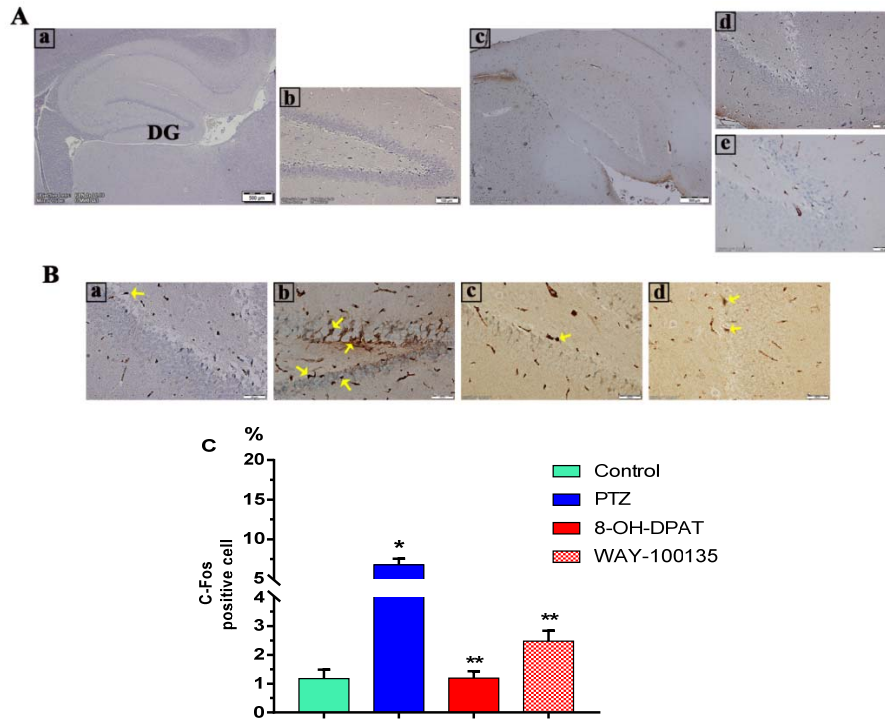


Figure 5: C-Fos Expression in the DG region. **A)** Light microscopic (a and b) and c-Fos immunohistochemistry (c, d, and e) images of the control group. A-X40 magnification, B-DG region X100 magnification, C-X40, D-DG region X100 magnification and, E-DG region X100 magnification negative control. **B)** c-Fos immunoreactivity in the DG region (X400). α -Control group, b-PTZ, c-8-OH-DPAT, d-WAY-100135 Yellow arrow; c-Fos positive neurons. **C)** c-Fos positive neuron numbers. * $p < 0.05$, compared to the control group; ** $p < 0.001$, compared with the PTZ group.

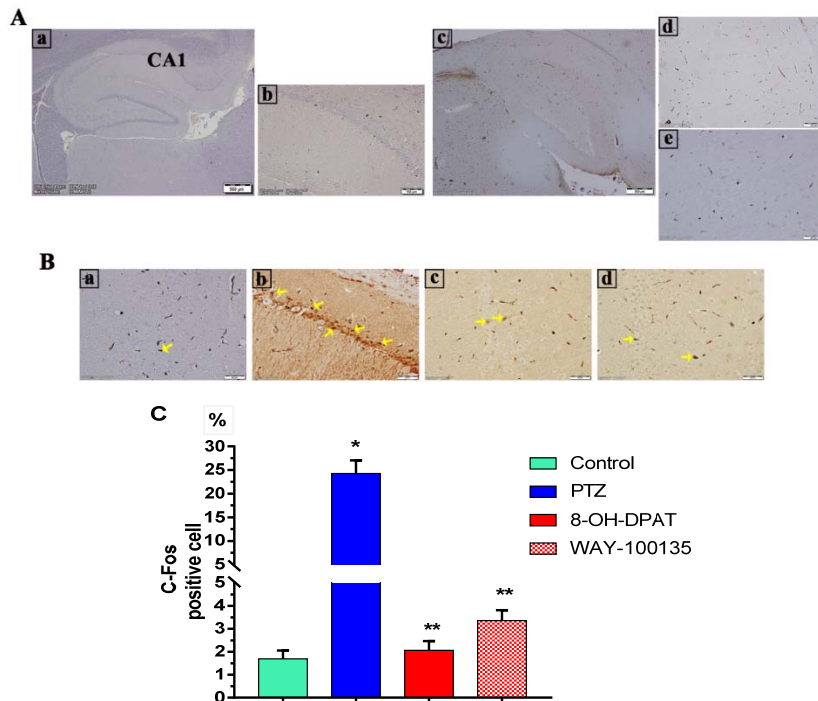


Figure 6: c-Fos expression in the CA1 region. **A)** Light microscopic (a, b) and c-Fos immunohistochemistry (c, d, and e) images of the control group. A-X40 magnification, B-CA1 region X100 magnification, C-X40, D-CA1 region X100 magnification and, E-CA1 region X100 magnification negative control. **B)** c-Fos immunoreactivity in the CA1 region (X400). α -Control group, b-PTZ, c-8-OH-DPAT, d-WAY-100135 yellow arrow; c-Fos positive neurons. **C)** c-Fos positive neuron numbers. * $p < 0.05$, compared to the control group; ** $p < 0.001$, compared with the PTZ group.

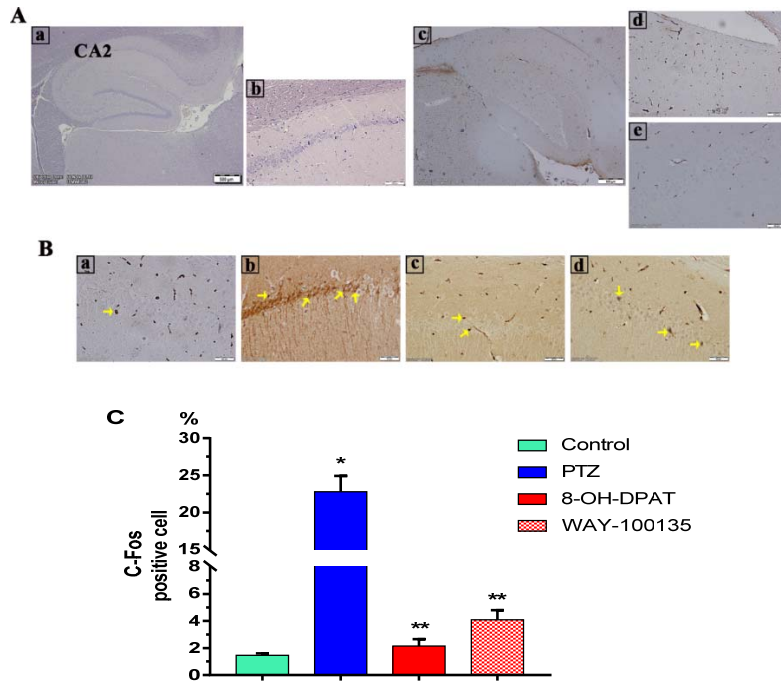


Figure 7: C-Fos Expression in CA2 Region. **A)** Light microscopic (a and b) and c-Fos immunohistochemistry (c, d, and e) images of the control group. A-X40 magnification, B-CA2 region X100 magnification, C-X40, D-CA2 region X100 magnification and, E-CA2 region X100 magnification negative control. **B)** c-Fos immunoreactivity in the CA2 region (X400). α -Control group, b-PTZ, c-8-OH-DPAT, d-WAY-100135 Yellow arrow; c-Fos positive neurons. **C)** c-Fos positive neuron numbers. * $p < 0.05$, compared to the control group; ** $p < 0.001$, compared with the PTZ group.

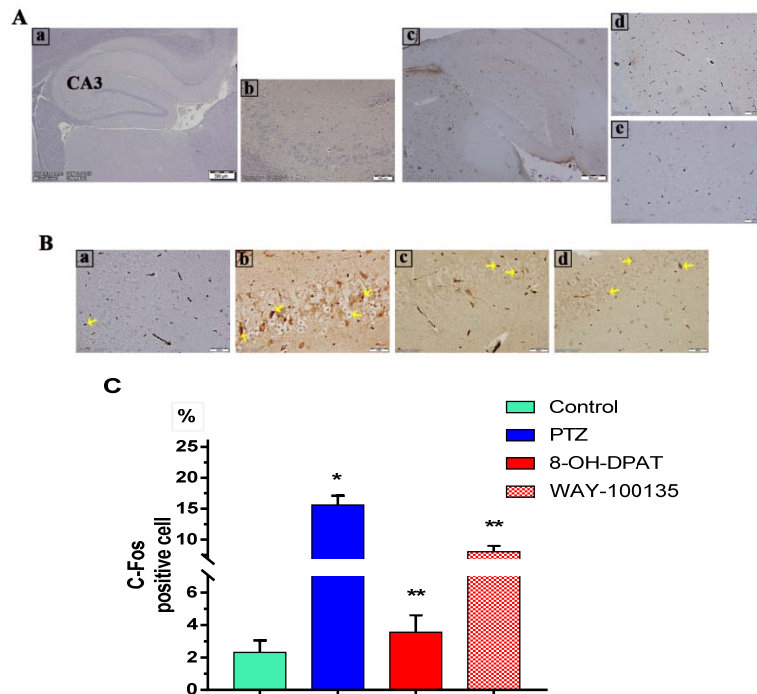


Figure 8: C-Fos Expression in CA3 Region. **A)** Light microscopic (a and b) and c-Fos immunohistochemistry (c, d, and e) images of the control group. A-X40 magnification, B-CA3 region X100 magnification, C-X40, D-CA3 region X100 magnification and, E-CA3 region X100 magnification negative control. **B)** c-Fos immunoreactivity in the CA3 domain (X400). α -Control group, b-PTZ, c-8-OH-DPAT, d-WAY-100135 Yellow arrow; c-Fos positive neurons. **C)** c-Fos positive neuron numbers. * $p < 0.05$, compared to the control group; ** $p < 0.001$, compared with PTZ group.

DISCUSSION

Although GABAergic and glutamatergic systems have been held responsible for the pathogenesis of epilepsy, there is much evidence that cholinergic and dopaminergic systems are effective in this process in recent years^{16;17}. Serotonin and various types of receptors have been found to play essential roles in the development of seizures. Several studies have shown that increasing synaptic 5-HT levels using selective serotonin reuptake inhibitors decreases seizure formation and increases seizure threshold^{18;19}. Although there are several studies on the role of serotonergic receptors in the development of epileptic seizures, this issue is still not fully elucidated.

In our study, we found that 5-HT₁ agonist 8-OH-DPAT increased the duration of FMJ on epilepsy and showed anti-epileptic activity by decreasing the number of epileptic spikes and percentage of epileptic spikes. In contrast, the 5-HT₁ antagonist WAY-100135 decreased FMJ onset time and did not change the number of epileptic spikes and percentage of spikes. Both 8-OH-DPAT and WAY-100135 showed no effect on GABA levels. 8-OH-DPAT and WAY-100135 reduced c-Fos expression in hippocampal DG, CA1, CA2 and CA3 areas.

In general, 5-HT₁ receptors are Gi / Go protein-bound membrane receptors, and their activation decreases cytoplasmic cyclic adenosine monophosphate (cAMP) levels and hyperpolarizes neurons²⁰. In general, with a few exceptions, 5-HT_{1A} receptor stimulation has been shown to reduce the seizure threshold in animal models of absence epilepsy and increase it in other types of seizures. 5-HT_{1A} agonists have been shown to increase the spike-wave discharge number (SWDs) and duration in Wistar Albino Glaxo / Rijswijk (WAG / Rij) rats, a genetically susceptible strain against absence seizures²¹. In contrast, 5-HT_{1A} receptor inhibition reduced SWDs number and duration in WAG / Rij rats²². The results of another study showed that NMDA antagonist MK-801 and AMPA receptor antagonist GYKI 52466 reversed the effects of 8-OH-DPAT on SWDs, indicating that NMDA and, to a lesser extent, AMPA glutamate receptors had a role in this effect^{23;24}. Contrary to these findings, fluoxetine, clomipramine, and 8-OH-DPAT have been reported to significantly reduce the incidence of SWDs in Groggy (GRY) rats, another genetically susceptible species for absence epilepsy. Furthermore, it was found that the effect of these agents on SWDs was eliminated by the 5-HT_{1A} receptor antagonist WAY-100135, indicating the

role of 5-HT_{1A} receptors in this effect²⁵. In this study, we found that 8-OH-DPAT decreased the number of epileptic spikes in accordance with these findings. In the rat model of electroshock-induced kindling epilepsy, 8-OH-DPAT has been shown to delay seizure development²⁶. Similarly, in our study, it was observed that 8-OH-DPAT increased the onset time of FMJ. In the PTZ-induced mouse seizure model, the 5-HT_{1A/1B/1D} agonist RU 24969 has been reported to exhibit anti-epileptic effect, and this effect is inhibited by the 5-HT_{1A} antagonist WAY 100635²⁷. 8-OH-DPAT and indorenate (5-HT_{1A} agonist) have been reported to inhibit seizures in PTZ-induced (60 mg / kg) tonic-clonic seizures, kainic acid-induced status epilepticus seizures, limbic seizures, and the amygdala kindling tonic-clonic seizure model²⁸. In another PTZ-induced (5mg / ml, i.v.) mouse study, 8-OH-DPAT has been shown to lower the seizure threshold²⁹ significantly. It was reported that WAY-100135 increases the firing frequency in bicuculin (GABA_A antagonist) induced seizures in the hippocampal area of rat brain³⁰. In our study, the effect of WAY-100135 on seizure activity was not observed due to the difference in epilepsy model. In addition to the excitatory effect of 5-HT_{1A} agonists on seizure development in models of absence epilepsy, some studies have shown that 5-HT₁ receptors also play a role as pro-convulsants in other models of epilepsy. 8-OH-DPAT has been reported to lower the seizure threshold in PTZ-induced convulsions in mice [182]. In the lithium-pilocarpine-induced status epilepticus model in rats, high-dose (0.5 mg / kg and 1.0 mg / kg) subcutaneous and intrahippocampal administration of 8-OH-DPAT did not cause a significant change in seizure stages but caused an increase in the first myoclonic jerk time and first generalized seizure onset time on EEG. However low-dose 8-OH-DPAT administration (0.01 mg / kg and 0.1 mg / kg) showed no effect on status epilepticus³¹. In the present study, application of 8-OH-DPAT at a dose of 0.3 mg / kg (i.p.) resulted in an increase in FMJ onset time and decreased the number of epileptic spikes and the percentage of epileptic spikes. Genetic studies in mice, however, support that the 5-HT_{1A} receptor has an anti-epileptic role. Genetic deletion of the 5-HT_{1A} receptor has been reported to have lower seizure thresholds and higher mortality rates in kainic acid-induced seizures³². Although 5-HT₁ agonist 8-OH-DPAT and WAY-100135 caused an increase in GABA levels, no significant difference was observed in our study. Despite the anti-convulsive effects of 8-OH-DPAT on seizures, it does not increase GABA levels, suggesting the presence of another mechanism in this effect. Although WAY-100135 does not have

an effect on seizures, it gives similar results in GABA levels, which reinforces this argument. c-Fos is an early-onset gene with protein expression due to neuronal activity. c-Fos expression is an indirect marker of neuronal activity and is usually expressed when neurons generate action potential. It has been widely used to demonstrate stimulus-induced neuronal activation^{33; 34}. Subconvulsive administration of PTZ (10-40 mg / kg) causes c-Fos immunoreactivity in the thalamus and hypothalamus regions of adult rats³⁵. Similarly, in our study, c-Fos expression was significantly increased in PTZ group in DG, CA1, CA2 and CA3 areas. Although 8-OH-DPAT has an anticonvulsant effect on seizures, it has been observed to decrease c-Fos expression in all hippocampal areas in accordance with this effect. Similarly, WAY-100135 has been shown to reduce c-Fos expression in all areas, but not as much as 8-OH-DPAT. These findings suggest that neuronal activation may play a role in the anti-convulsive effect of the 5-HT_{1A} receptor.

CONCLUSION

8-OH-DPAT, a 5-HT_{1A} agonist, showed anti-convulsive effect by increasing FMJ onset time and reducing the number of epileptic spikes and percentage of epileptic spikes. No change in GABA levels was observed in this anticonvulsant effect. 8-OH-DPAT reduced the expression of c-Fos in all hippocampal areas, consistent with the anti-convulsant effect on seizures.

APPRECIATION

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