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The effect of TGN-020 on penicillin induced epileptiform activity in rats

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Abstract

Objective: Aquaporin-4 (AQP-4) is a water channel protein which is the most abundant aquaporin isoform in the brain. Recent studies indicate the relationship between AQP-4 with epileptogenesis. Therefore, we examined the potential effect of the AQP-4 inhibitor TGN-020 on penicillin-induced epileptiform activity in rats.

Material and Method: Epileptiform activity was induced by intracortical (i.c.) administration of penicillin (200 IU, 1 µl). TGN-020, at doses of 25 µg, 50 µg, 100 µg and 200 µg, was administered by intracerebroventricular (i.c.v.) 30 minutes after penicillin injection. The epileptiform activity was verified by electrocorticographic (ECoG) recordings. Twenty four hours later, animals are decapitated for the collection of blood samples and brain tissue.

Results: The dose of 100 µg TGN-020 decreased the mean spike frequency of epileptiform activity in the 30 min after the injection without changing the amplitude ($p < 0.05$). Serum neuropeptide Y level was up-regulated by 25 µg TGN-020 in comparison with the other groups ($p<0.001$). Plasma levels of calcineurin in the 50 µg dose of TGN-020 were lower than 25 µg and 200 µg doses of TGN-020 ($p<0.01$). Enzymatic ativity of glutathione peroxidase (GP_x-1) in brain tissue was higher in the penicillin and 25 µg TGN-020 group compared with the sham group ($p < 0.05$).

Conclusion: Given all these data, the anticonvulsant effect of TGN-020 which is aquaporin-4 water channel inhibitor in the brain has been studied extensively for the first time in an experimental model of epilepsy. Inhibition of AQP-4 might be useful in the treatment of epilepsy in future.

Key words: TGN-020, Aquaporin 4, Epilepsy, EcoG, DMSO, Neuropeptide Y

Introduction

Aquaporin 4 (AQP-4) is a water channel protein, which is primarily abundant aquaporin isoform in the brain (1). This aquaporin is considered to be an important role in the physiopathology of brain disorders including ischemia, epilepsy, traumatic brain disease, tumor-induced brain swelling, infections and hydrocephalus (2-4). These studies indicate that AQP-4 has a crucial role in the movement of water into and out of the brain tissue (5, 6). Seizure susceptibility of Aqp4-/- mice was found to be less than the all wild-type mice in pentylenetetrazol (PTZ) induced epilepsy and the latency to generalized seizures is also reported to be longer in Aqp4-/- mice (7). This reduction in neuronal excitability is thought to be effective in the extracellular fluid ions and osmolarity changes.

Recently, various chemical structures were defined as AQP-4 water channel inhibitors including 2-nicotinamide-1,3,4-thiadiazole (TGN-020) (8).

In vitro studies indicated that TGN-020 was found the most powerful inhibitor of AQP-4 water channel (9). Pretreatment with TGN-020 significantly reduced brain edema in a mouse model of focal cerebral ischemia using 7.0-T magnetic resonance imaging (MRI) (10). Through increasing the osmolarity of extracellular space, water moves into the cell and ionic edema occurs (11). Therefore, AQP-4 inhibition is a new approach to reduce the cerebral edema and is shown to be promising for the development of effective drugs in the clinical treatment (10). Same as in cerebral edema brain excitability is highly sensitive to acute changes in osmolarity (12). Altering the osmolality of the extracellular fluid changed the amplitude and duration of the epileptiform bursts activity in rat dentate gyrus (13). According to this study decreasing the osmolality increased the amplitude of the spikes within the burst and increasing the osmolality decreased the amplitude in the rat hippocampal slices (14).

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These experimental findings are parallel with various clinical situations such as the dialysis disequilibrium syndrome, compulsive polydipsia and the syndrome of inappropriate ADH secretion (15). Consequently, studies on the role of AQP-4 and its inhibitor, TGN-020, in epileptiform activity is quite limited, therefore it is necessary to investigate the effect of TGN-020 in different experimental models of epilepsy to better understand AQP-4 working mechanism.

In vivo experimental models are frequently used to explain the pathophysiological mechanisms of epilepsy. Application of penicillin G intracortically is one of these models (16). Penicillin is structurally a GABA antagonist that is similar to bicuculline eliminates GABA inhibition and causes the induction of epileptic seizures (17) This model is more similar to the human focal epileptic activity (18). The data concerning the effects of TGN-020 on penicillin-induced epileptic activity under the monitoring of electrocorticography (ECoG) is still not sufficiently reported in the currently available literature. In the present study, we used intracortically injected penicillin method to induce epileptiform activity and investigated the effects of TGN-020 on this epilepsy model in rats.

We measured levels of brain nitric oxide (NO), superoxide dismutase [Cu-Zn] (SOD), general malondialdehyde (MDA), glutation peroxidase (GPx-1) and serum S100B protein, neuron-specific enolase (NSE), Neuropeptide Y (NPY) and calcineurin levels in order to show the effects of TGN0-20 on neuronal excitability.

Recent studies have implicated oxidative stress resulting from excessive free-radical release in the initiation and progression of epilepsy, so it is plausible that excessive free-radical may have a functional role in the hyperexcitability characteristic of epilepsy. Rauca and colleges showed that increased reactive oxygen species in the brain accompanies the development of PTZ kindling and is an important pathogenic factor in the PTZ kindling-induced neuronal death (19). Based on these data, free radicals might be affected from convulsion. Hence in the present study, we investigated the effects of TGN-020 on biochemical parameters including NO, SOD [Cu-Zn], MDA and GPx-1 in the brain tissue of penicillin induced epileptic animals.

Various biochemical markers have been investigated in the context of brain damage and epilepsy. However, S100B protein and NSE are the most widely studied biochemical markers of central nervous tissue damage (20, 21). S100B protein is a large subset of calcium-binding proteins (22). This protein stimulates nerve growth and increase the survival of neurons; thus acting as a protective factor (23). In addition, it is reported to be used as a clinical marker to assess prolactin, NSE and S-100B protein levels in the serum of children and adult patients with temporal lobe epilepsy (24, 25). Thus, we investigated a possible link between AQP-4 activity and serum S-100B, NSE, NPY and calcineurin levels in epileptiform activity of rats using specific inhibitors of AQP-4 (TGN-020).

Materials and Methods

Animals

Experiments were carried out on adult male Wistar albino rats weighing 240-300 g. The animal usage protocol was approved by the local ethics committee of the Bezmialem Vakif University. Along all experiments, local guidelines for the care and use of laboratory animals and the guidelines of the European Community Council for experimental animal care were applied. Animals were housed in temperature of 21 ± 2 oC on a 12 h light/dark period. All animal groups were allowed ad libitum food and water except for the short time when animals were removed from their cages to do experiments. The experiments were materialized between 11:00 and 17:00 periods of day time. Animals were divided into six groups: (1) intracortical (i.c.) delivery of 2.5 µl artificial cerebrospinal fluid [aCSF containing (mM): NaCl, 124; KCl, 5; KH2PO4, 1.2; CaCl2, 2.4; MgSO4, 1.3; NaHCO3, 26; glucose, 10; HEPES, 10; pH 7.4 when saturated with 95% O2 and 5% CO2]; (2) 500 units penicillin (2.5 µl, i.c.); (3) 500 IU penicillin (2.5 µl, i.c.) + TGN-020 (25 µg, i.c.v.); (4) 500 IU penicillin (2.5 µl, i.c.) + TGN-020 (50 µg, i.c.v.); (5) 500 IU penicillin (2.5 µl, i.c.) + TGN-020 (100 µg, i.c.v.); (6) 500 IU penicillin (2.5 µl, i.c.) + TGN-020 (200 µg, i.c.v.); (7) 500 IU penicillin (2.5 µl, i.c.)+dimethylsulfoxide (DMSO, i.c.v); (8) 100 µg TGN-020 (i.c.v.). Each animal group was composed of seven rats.

Induction of epileptiform activity

Induction of epileptiform activity was performed as described previously by Kozan & co-workers (26). Briefly, animals were anesthetized with an intraperitoneal injection of urethane (1.25 g/kg). Rectal temperature was maintained between 36.5 and 37.5 oC using a feedback-controlled heating system (Homeothermic Blanket Control Unit, Harvard Apparatus, MA, USA). The left cerebral cortex was carefully exposed by craniotomy (5 mm posterior to bregma and 2 mm lateral to sagittal sutures). Subsequently, incision of the skull, head of the animal was fixed by utilizing standard stereotaxic methods (Harvard Instruments, South Natick, MA, USA). The epileptic focus was produced by 500 international units of penicillin G potassium injection which is acute in an experimental model of focal epilepsy; 1 mm beneath the brain surface by a Hamilton microsyringe type 701RN; infusion rate 0.5 µl/min).

Drugs and drug administration

TGN-020 and DMSO (Sigma Chemical Co., St. Louis, MO, U.S.A.) were used in this study. All of the solutions were prepared just before experiments. TGN-020 was dissolved in DMSO and the requisite doses were administered intracerebroventricularly. Intracerebroventricular injections were administered into the left lateral ventricle of each rat through a stereotaxic apparatus, with the coordinates of 0.8 mm posterior to the bregma, 2.0 mm lateral to the midline and 4.2 mm ventral to the surface of the skull based on the stereotaxic atlas of the rat brain (27).

First of experiment sets, penicillin was prepared in sterile apyrogen distilled water and 500 IU penicillin was administered intracortically in a volume of 2.5 µl into the left cortex. Secondly, aquaporin-4 water channel antagonist TGN-020, at doses 25, 50, 100 and 200 µg, was administered 30 min after penicillin application (28).

Electrocorticography (ECoG) recordings

All EcoG recordings were obtained in urethane anesthesized animals. Two Ag-AgCl ball electrodes were placed over the left cortex and the coordinates of electrode were first electrode, 1.5 mm lateral to sagittal suture and 1 mm anterior to bregma; second electrode, 2 mm lateral to sagittal suture 5 mm posterior to bregma. Recording electrodes were placed on the cortex surface by means of two different electrode holders. The common reference electrode was stabilized on the pinna. The ECoG activity was continuously monitored by using an eight-channel data acquisition system (PowerLab, 8/SP, AD Instruments, Australia) for at least 120 minutes. During the whole ECoG recording four different corners of the scalp were sutured by surgical threads and stretched as to form a liquid vaseline pool at 37 oC that protects brain tissue from water and electrolyte loss. Recordings were stored on computer. Analysis of epileptiform activity was made off-line. Spike frequencies and amplitudes for each animal were automatically calculated and measured by using the LabChart v 7.3 (PowerLab Software, 8/SP, AD Instruments, Australia). In the study, only the number of spikes with amplitudes greater than threefold baseline activity was taken into consideration.

Biochemical analysis

Following that the ECoG records were completed, the scalp of animal was sutured by surgical threads. Twenty four hours later, animal was harvested under light ether anesthesia for the collection of blood samples and brain tissue. Serum was obtained from blood samples and they were stored at -80ºC degrees until the time of study. Serum levels of S- 100B protein biomarker, NSE, NPY and Calcineurin parameters were studied using a commercial rat specific ELISA kits (ElAab Science Co. Ltd) and each assay was applied (21, 29). Calcineurin Subunit B type level was qualified using ELISA, Rat S100B ELISA kit E1323r, Protein S100-B by Rat S100B ELISA kit E0567r, Pro-neuropeptide Y by ELISA kit E0879r and Neuron-Specific Enolase by Rat NSE ELISA kit E0537r.

The cerebral hemispheres, cerebellum and brain stem of the brains that were extracted were allocated. These structures were separated as right and left hemispheres and stored at -80º C until biochemical studies were completed. Brain tissues dissolved during biochemical studies were broken into pieces, were washed with saline fluid, and were dried with blotting paper. After that, the tissue quantity was weighed and recorded then they were homogenized in TRIS Buffer at a speed of 16.000 rev/min by glass teflon homogenizer in 5 min and the homogenate obtained in this way was used in the studies. From brain tissue, oxidant /antioxidant parameters of the malondialdehyde by MDA ELISA kit, E0597Ge, nitric oxide level by total NO detection kit, (Enzo Life Sciences), ADI-917-020 kit, NO2/NO3 rate was studied and superoxide dismutase by Rat SOD ELISA kit, E0596r, glutathione peroxidase by Rat GPx-1 ELISA kit, E0295r activities were determined using a commercial enzyme-linked immunoassay. The minimal measurable concentrations for these detection systems were 0.15 ng/ml for Calcineurin Subunit B, 15.6 pg/ml for S100-B, 31.2 pg/ml for Pro-neuropeptide Y, 1 pg/ml for NSE, 31.2 U/ml for GPx-1, 0.312 nmol/ml for MDA, 1.56 U/ml for SOD and 20.86 µM/l for NO.

Statistical analysis

All statistical procedures were made by using SPSS statistical software (version 22.0). The differences between the groups were analyzed within the one-way ANOVA. Significant differences were further evaluated through multiple comparisons posthoc Scheffe test for the electrophysiological data and Tukey test for the biochemical parameters. Furthermore, repeated ANOVA test is used to determine difference within each group. Data are expressed as the means ± SEM. Statistical significance was set at $p < 0.05$.

Results

Effects of TGN-020 on epileptiform activity

Baseline activities of each animal were recorded before the administration of intracortical penicillin. Along this period, none of the animals showed spontaneous epileptiform activity (Fig 1A). Intracortical injection of 500 IU penicillin induced an epileptiform ECoG activity which began within 2-4 minutes. The frequency and amplitude of epileptiform activity reached at a constant level in 30 minutes and lasted for 2-3 hours. The means of spike frequency and amplitude were 30.3 ± 3 spikes/min, 940.6 ± 88.3 µV, respectively (Fig 1B).

The intracortical injection of aCSF (2.5 µl) and intracerebroventricular injection of TGN-020 or DMSO (1.5 µl) did not cause any change in the frequency or amplitude of ECoG activity with respect to the control base line in the nonpenicillin injected animals.

Figure 2, indicates the effect of single administration of different doses (25, 50, 100 and 200 µg) of aquaporin-4 water channel antagonist TGN-020 on the penicillin-induced epileptiform activity. Repeated Anova test revealed a significant anticonvulsant effect for TGN-020, at a dose of 100 µg with a maximal effect. Application of TGN-020, at a dose of 100 µg, decreased the mean spike frequency of epileptiform activity in the 30 min after the injection without changing the amplitude. The other three doses of TGN-020 (25, 50 and 200 µg) did not significantly change either the frequency or amplitude of the epileptiform activity. The mean spike frequency of epileptiform activity was 29.68 ± 2.1, 29.64 ± 1.9, 15.78 ± 1.2 and 27.81 ± 2.3 spike/min, and the mean amplitude was 1138 ± 140, 717 ± 72, 1182 ± 128 and 791 ± 85 µV after 70 min from TGN-020 injection in response to dose of 25, 50, 100 and 200 µg TGN-020 respectively (Fig.1C-F).

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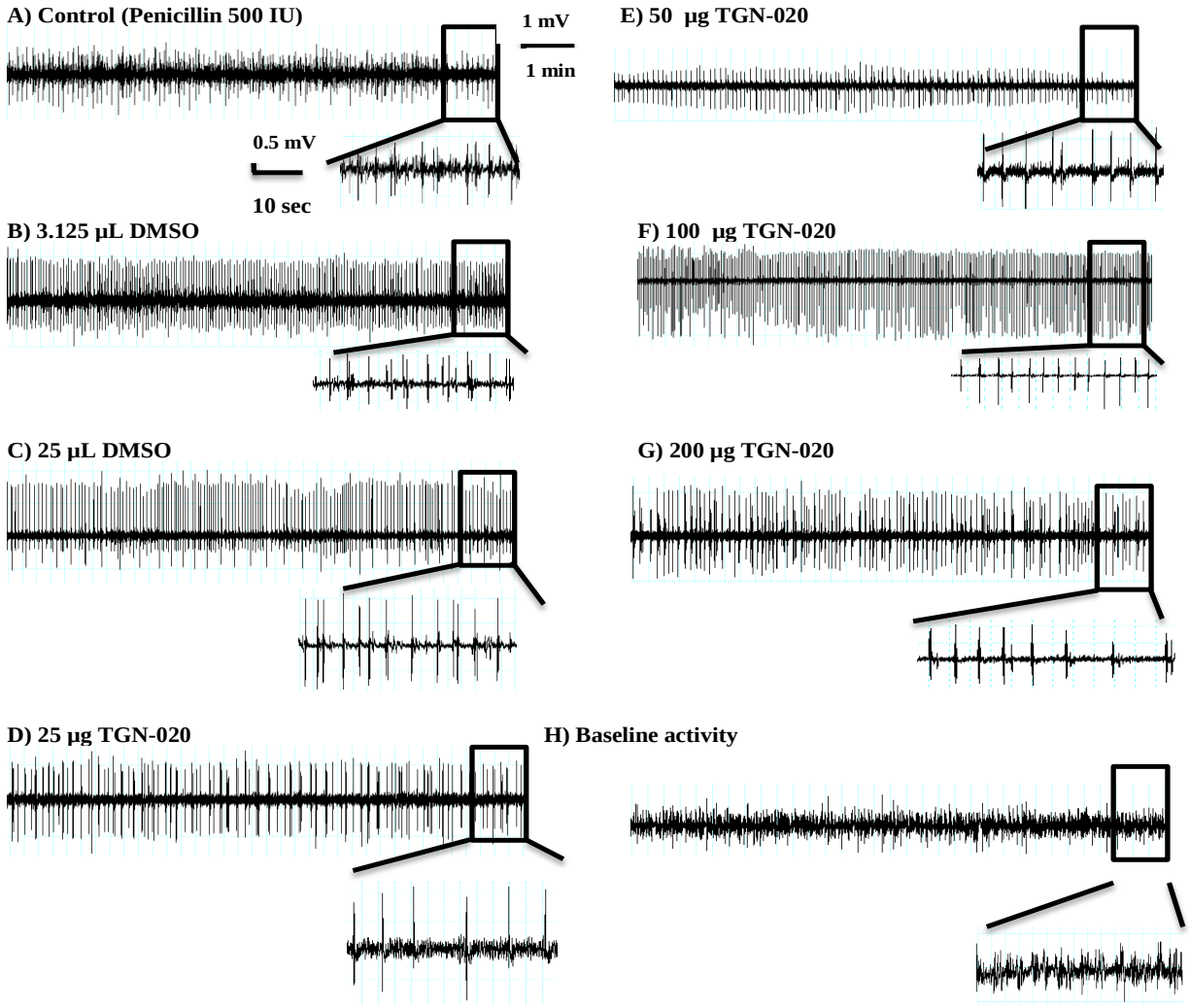
The effects of TGN-020 on biochemical parameters

The intracerebroventricular injection of TGN-020 and DMSO (1.5 µl) did not cause any change in the biochemical parameters of brain tissue and serum levels with respect to the intracortical injection of aCSF (2.5 µl) injected animals.

The serum levels of NSE, S100B protein, NPY and Calcineurin are summarized in Table 1. As shown in Table 1 injection of 25 µg TGN-020 up-regulated significantly serum NPY level in comparison with the penicillin and others doses of TGN-020 groups, respectively ($p<0.001$).

In addition, when compared with the 50 µg dose of TGN-020, plasma levels of calcineurin in 25 and 200 µg doses of TGN-020 were significantly lower ($p<0.01$). There was no significant result in serum Protein S-100B and NSE levels. Table 2 summarizes the activities of brain tissue SOD and GPx-1 enzymes and brain MDA and NO levels in all groups. The activity of SOD and MDA or NO levels did not differ between the groups. However, GPx-1 activity in the brain tissue was higher in the penicillin and penicillin+25 µg TGN-020 groups compared with the sham group ($p < 0.05$).

Figure 1: TGN-020 and DMSO regulates the frequency of epileptiform activity



A) The intracortical injection of penicillin (500 IU) induced epileptiform activity on ECOG. **B)** The intracerebroventricular (i.c.v.) administration of DMSO at a dose of 3.125 µL, significantly decreased the mean frequency of epileptiform activity in the 50 min after DMSO injection without changing the amplitude. **C)** The application of DMSO (i.c.v), at a dose of 25 µL, did not significantly change either the mean frequency or amplitude of penicillin-induced epileptiform activity. **D)** The intracerebroventricular (i.c.v.) administration of aquaporin-4 water channel antagonist TGN-020, at a dose of 25 µg did not significantly change either the mean frequency or amplitude of penicillin-induced epileptiform activity. **E)** The administration of TGN-020, at a dose of 50 µg did not significantly change either the mean frequency or amplitude of penicillin-induced epileptiform activity. **F)** The administration of TGN-020, at a dose of 100 µg with a maximal effect, decreased the mean spike frequency of penicillin-induced epileptiform activity in the 60 min after TGN-020 injection, without changing the amplitude. **G)** The administration of TGN-020, at a dose of 200 µg did not significantly change either the mean frequency or amplitude of penicillin-induced epileptiform activity. **H)** Displays baseline ECoG activity before giving penicillin or the injection of other chemicals.

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Figure 2: The figure 2 is associated with the effects of intracerebroventricular administration of DMSO on the mean spike frequency of penicillin-induced epileptiform activity. DMSO, at a dose of 3.125 µL (i.c.v.), significantly decreased the mean frequency of epileptiform activity in the 10 min after DMSO injection. DMSO, at a dose of 25 µL, did not significantly change the mean frequency of epileptiform activity. *p < 0.05.

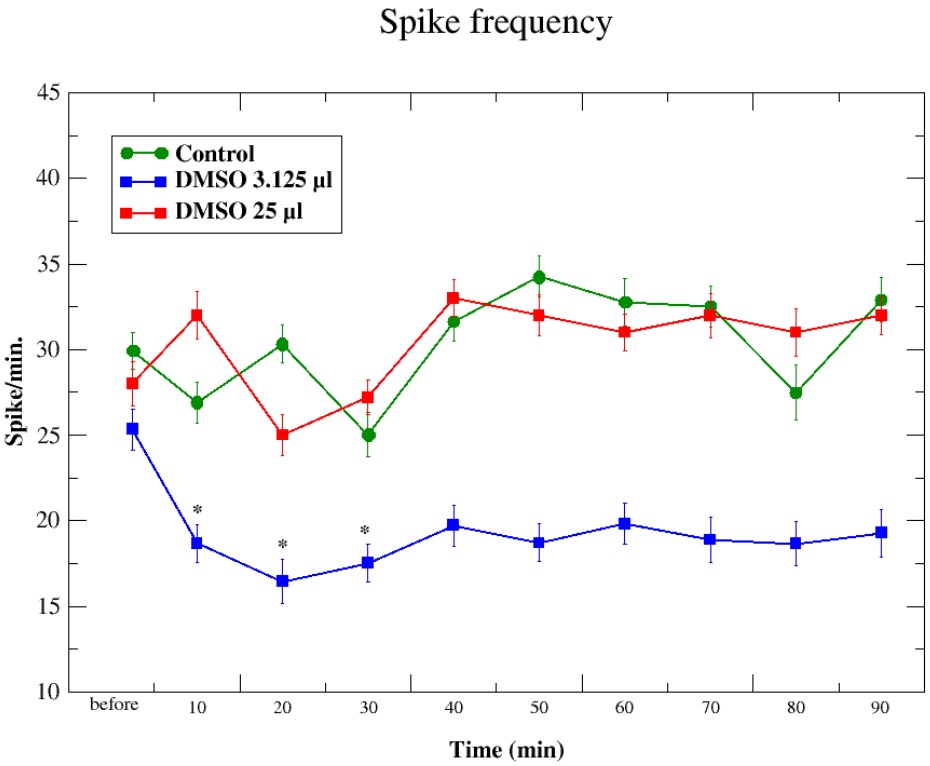

Figure 3: It indicates the effects of intracerebroventricular administration of aquaporin-4 water channel antagonist TGN-020 on the mean spike frequency of penicillin-induced epileptiform activity. TGN-020, at doses of 25, 50 and 200 µg did not change the mean spike frequency. TGN-020, at a dose of 100 µg (i.c.v.), decreased the mean spike frequency of epileptiform activity in the 30 min after TGN-020 injection. The best effect appeared in the 100 µg (i.c.v) administered group. *p < 0.05.

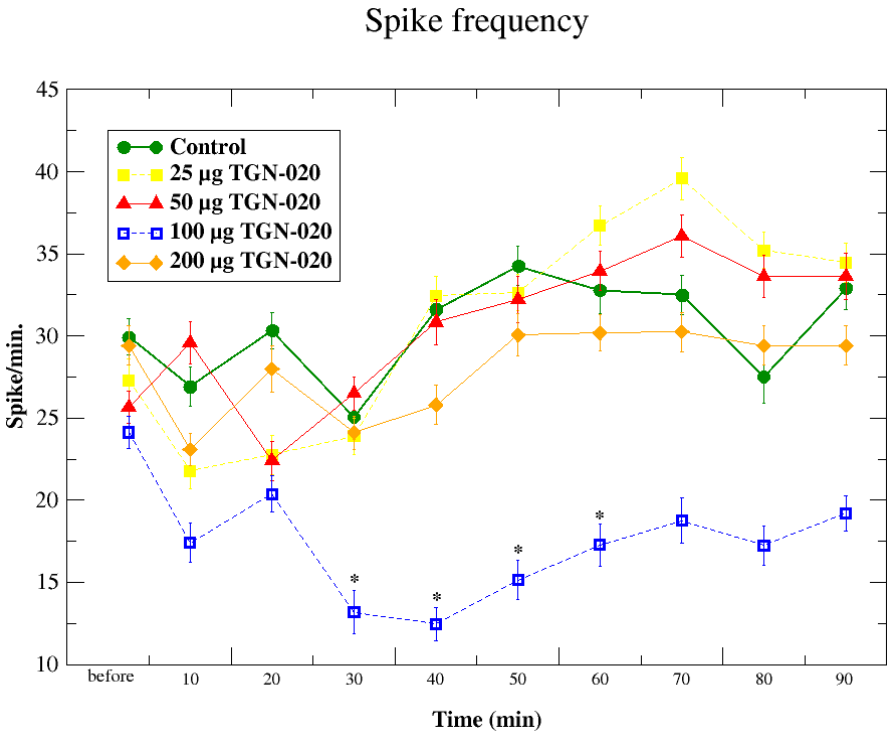

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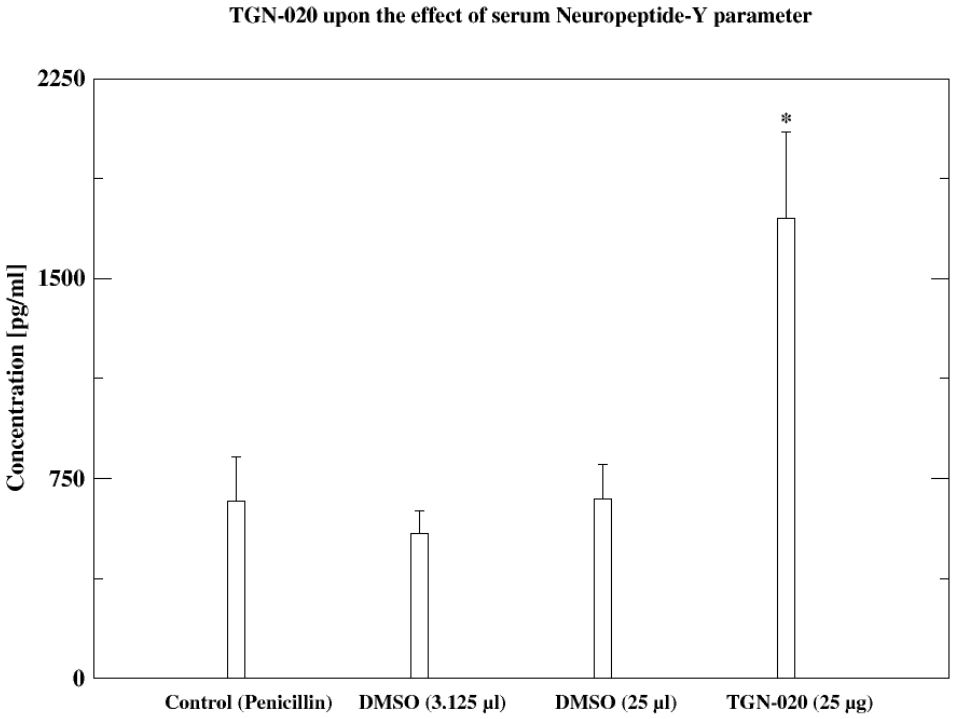
Figure 4: TGN-020 application significantly increased serum neuropeptide Y concentration in comparison with penicillin and both DMSO groups. There were no differences between control and both DMSO groups. *p < 0.05.



Discussion

TGN-020, 2-nicotinamido-1,3,4-thiadiazole, is a potent inhibitor of AQP-4 (9, 28). TGN-020 was identified on the basis of conserved physical and chemical features of several known drugs found to inhibit transport of water channel AQP-4 in vitro. One of the previous studies suggested that, intraperitoneal administration of TGN-020, a dose of 200 mg/kg, significantly reduces ischemic cerebral edema in mice (10). In addition, same workgroup demonstrated that in vivo effect of TGN-020, a dose of 200 mg/kg, on AQP-4 inhibition, namely an increases regional cerebral blood flow in mice (28). On the basis of these studies we used at 25, 50, 100 and 200 µg (i.c.v.) doses of TGN-020 in the current study. TGN-020, a dose of 100 µg, decreased the frequency of penicillin-induced epileptiform activity without changing the amplitude. The remaining doses of TGN-020 (25, 50 and 200 µg) did not affect either the frequency or amplitude of epileptiform activity.

The studies suggest novel roles for water channel AQP-4 in control of seizure susceptibility (30, 31). These suggestions with changes in human epileptic tissues lead to the unifying hypothesis that AQP-4 and its molecular partners may play a functional role in epilepsy (32, 33). K+ accumulates at extracellular space in epilepsy (34). After a certain period with epileptic activity, this accumulation may end up with hyperosmolar state of the extracellular volume.

Hyperosmolar state triggers water imbalance in the brain tissue that is water efflux from the cell. Therefore, we aimed preserve intracellular water by blocking the water channels on the cell membrane. Our data are the first results for the anticonvulsive effects of TNG-020 in experimental model of epilepsy by using electrophysiological method. Intracerebroventricular administration of TGN-020, at a dose of 100 µg, caused significant decrease in mean spike frequency of penicillin-induced epileptiform activity in rat. This finding is in-line with the previous reports on the high expression of AQP-4 in the brain tissue of epileptic rats (35, 36). Song and co-workers (2015) showed that AQP-4 had the high expression in the brain tissue of lithium chloride-pilocarpine epileptic model of rats. Likewise, in another study it was found that AQP-4 expression was higher in 30 minutes of pilocarpine-induced status epilepticus group in comparison with control (35).

It has been reported that Protein S100 B, NSE (21), NPY (37-39) and calcineurin (40) are good markers to be investigated for neuronal damage in epilepsy. The anticonvulsant effect of NPY has been demonstrated in different models of epilepsy (38, 41, 42). NPY prevents seizures by increasing the seizure threshold. NPY occurs this effect by enhancing the GABAergic inhibitory neurotransmission onto pyramidal neurons neocortex and reducing the excitatory neurotransmission (41).

On the other hand, stimulation of metabotropic and ionotropic glutamate receptors induces expression of NPY in hippocampal granule cells (43). Although the main mechanisms affecting of seizure-induced synthesis of NPY are not clearly explained, a gradual increase in NPY was reported up to 7 days after seizure (39). In our study, 24 hours following the epileptiform activity, there was a significant increase in serum levels of NPY in rats treated with TGN-020, a dose of 25 µg. It seemed that low dose of TGN-020 had convulsant effect in terms of the serum NPY level on contrary to anticonvulsant effect of 100 µg TGN-020 on ECoG recordings. There is very limited literature to make further comments on the interaction between NPY and AQP-4 and their roles in neuronal excitability. Only one research was found positive correlations between plasma NPY concentration and gene and protein expression levels of NPY and AQP-4 in the jejunum following traumatic brain injury in rats (44).

Calcineurin is a calcium dependent serine/threonine phosphatase that is widely distributed in the brain with high levels in the hippocampus and caudate putamen (45, 46). It is shown to increase highly expression of calcineurin in epileptic tissue. Consistent with the previous studies (47, 48), we found that the calcineurin level was increased in the plasma of at the 50 µg dose of TGN-020 administration rats compared to 25 µg and 200 µg doses of TGN-020 groups following penicillin induced epileptic rats. Recent data suggest that calcineurin may also involve in the regulation of cAMP mediated PKA-dependent phosphorylation of aquaporin-2 in kidney collecting duct (49). However, there is not yet any data regarding the interaction between calcineurin and AQP-4 and their roles in brain tissue. Thus, our results concerning with the interaction between calcineurin and AQP-4 are the first data from the epilepsy research.

There are numerous evidences linking oxidative stress to the initiation and progression of epilepsy in experimental models and patient with epilepsy (50-52). Oxidative stress is known to be propagating by epileptic seizures. On the contrary, induced severe seizure activity animal models, can lead to neurotoxic effects mediated by oxidative stress. GPx-1 is an endogenous antioxidant enzyme that reacts with the free radicals and prevents the formation of the hydroxyl radical which is the most toxic form of free radicals. We found significantly lower level of GPx-1in the sham group as compared with the penicillin and 25 µg dose of TGN-020 groups. Our findings contradict other reports that PTZ models have oxidant effects (50). However, (53) found no alteration on GPx-1 activity in the hippocampus and cortex of kainic acid induced epileptic animals. The possible explanation could be the diversity in obtaining the results of GPx-1 following epileptic activity, the different strain of animal species, types of the experimental models and difference in duration of chemicals injection.

In conclusion, we showed that TGN-020 which is aquaporin-4 water channel inhibitor in the brain significantly decreased the spike frequency of penicillin-induced epileptiform activity without changing the amplitude. These results firstly indicate the anticonvulsant effect of TGN-020 on an experimental model of epilepsy. In addition, we found a possible link between inhibition of AQP-4 and serum NPY or calcineurin levels and brain GPx-1 activity in penicillin induced epileptic animals.

Conclusion

Given all these data, the anticonvulsive effect of TGN-020 has been studied extensively for the first time in an experimental model of epilepsy. Consequently, present study suggests that inhibition of AQP-4 might be useful in the treatment of epilepsy in the future.

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Conflict of Interest: The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Author's Contributions: EA, MP, RK, HK: Research concept and design; Animal experiments, data collecting, analysis and interpretation of data**. EA:** Preparation of article, and Revisions. All authors approved the final version of the manuscript.

Ethical issues: All Authors declare, Originality and ethical approval of research. Responsibilities of research, responsibilities against local ethics commission are under the Authors responsibilities. The study was conducted under defined rules by the Local Ethics Commission guidelines and audits.

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