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Original Article

Changes in the Expression of c-fos and AQP4 in the Hippocampus and Amygdala Regions of Rats with Kainic Acid-Induced Temporal Lobe Epilepsy and Their Role in the Pathogenesis of Disease

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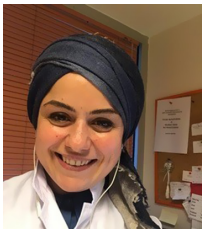

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Abstract

Objective: Aquaporin4 is the main water channel in the brain that is associated with neurological disorders. The role and the expressive changes of aquaporin4 in epilepsy are still limited and controversial. The study aims to evaluate the expression of c-fos and aquaporin4 during epileptogenesis after systemic kainic acid-induced status epilepticus in the temporal lobe epilepsy animal model and to investigate their alterations in both hippocampus and amygdala.
Methods: Intraperitoneal injections of kainic acid (5-15 mg/kg) by repeated low kainic acid protocol were given to young adult 32 Wistar albino rats for status epilepticus. Aquaporin4 and c-fos were investigated in the hippocampus and amygdala on days 1 and 60 after status epilepticus by immunostaining methods in brain slices.
Results: The intensity of c-fos immunostaining rose considerably in the hippocampus CA1 area of rats during the acute period ($P < 0.05$) and in the amygdala during the chronic period. The immunostaining intensity of aquaporin4in the hippocampus of rats with acute kainic acid increased significantly ($P < .05$). It was also raised in the hippocampal region of the rats in the acute sham and chronic kainic acid groups.
Discussion: The results of this study support a link between aquaporin4 and epilepsy. It can be speculated that aquaporin4 change is primarily a defense mechanism immediately after status epilepticus, and then, it can evolve into a causal factor with exhaustion as a result of overuse.
Keywords: c-fos, AQP4, epileptogenesis, temporal lobe epilepsy, animal model, kainic acid

INTRODUCTION

One of the most prevalent brain disorders, which is characterized by spontaneous recurrent seizures, is epilepsy. Temporal lobe epilepsy (TLE) is the most common form of epilepsy. Amygdala and hippocampus, both or alone, are essential sources of epileptic seizures in intractable TLE.[1] Experimental animal models and human brain slices showed functional and structural changes such as neuronal death, axonal sprouting, neurogenesis, and gliosis during epileptogenesis.[2-5]

Aquaporin (AQP) 4 is the main water channel expressed by astrocytes in the brain. Its relationship to neurological disorders has been shown in many cases, including stroke, traumatic injuries, and neurodegenerative disorders.[6-8] Recently, a relationship between AQP4, blood–brain barrier (BBB) dysfunction, and epileptogenesis is suggested.[9-13] However, the data on AQP4 and the mechanisms underlying BBB dysfunction occurring in epileptogenesis are minimal and controversial. In this regard, the role of the alterations in the expression of AQP4 in epilepsy is still deliberated both as a cause and a result of epileptogenic seizures.

Until now, findings of both increase and decrease in the levels of AQP4 in human brain slices have been reported.[11,14-16] Animal models of TLE have also shown some findings on AQP4. In a mice study, in the absence of AQP4, electrographic seizure threshold was higher, seizure duration increased, and potassium (K+) kinetics slowed immediately after electrical stimulation-evoked seizures.[17,18] A marked reduction of AQP4 was observed at the early stage of epileptogenesis in the intrahippocampal kainate model.[12] Aquaporin 4 levels at 1, 4, 7, and 30 days were evaluated after kainic

acid (KA)-induced status epilepticus (SE) in another study.[19] Aquaporin 4 dorsal hippocampal protein expression was significantly downregulated in that study, followed by a gradual return to baseline levels with a significant increase in ipsilateral protein levels.[19] Lastly, the change of AQP4 at week 2 and week 11 was evaluated after systemic KA-induced SE. The study suggested a redistribution of AQP4 from perivascular membranes to other astrocytic plasma membranes.[20]

The immediate early gene c-fos and fos protein has been used as a marker for neuronal activation. They are present under basal levels in the hippocampus of normal adult rat brain, but they increase after neuronal activation.[21] C-fos activation was shown in different animal models following chemically and electrically induced SE.[21-28] According to these studies, the c-fos protein was detected in 15-30 minutes following the activation of neurons, and maximum expression levels were reached in 30 minutes-2 hours and returned to baseline levels in 3-24 hours.[21-24,27,29] However, the status of c-fos protein during epileptogenesis is still not well known.

In the current study, we aimed to evaluate the expression of c-fos protein and AQP4 on days 1 and 60, in the early and late stages, after systemic KA-induced SE in the TLE animal model to investigate their alterations in both hippocampus and amygdala.

METHODS

Animal Protocols

Young adult 32 Wistar albino rats, weighing 220-270 g, were tested. The rats were obtained from Aziz Sancar Institute of Experimental Medicine, Istanbul University. According to the Republic of Turkey Ministry Of Agriculture And Forestry's norms and controls (2011/28141), the research design and all surgical and animal handling methods referred to herein were authorized by the Local Ethics Committee of Animal Experiments of Istanbul University (70/2011).

Two weeks before KA injection, the following procedures were performed: rats were intraperitoneally administered chloral hydrate (350 mg/kg) anesthesia under aseptic conditions. The head of the rats was fixed to the stereotaxy device (Sterotact, World Precision Instruments, Florida, USA). Following the midline, the scalp was incised and opened from back to front. Two stainless steel bipolar electrodes (200 and 260 µm inner and outer diameters; A-M Systems, Inc. #791900, Carlsborg, Wash, USA) were stereotactically implanted into the left and right hippocampus through 0.6 mm-wide burr holes and anchored to the skull using dental acrylic. Based on the Paxinos and Watson rat brain map, stereotaxic coordinates for the hippocampus were obtained (anterioposterior (AP): −3.14 mm, mediolateral (ML): 2.0 mm, and dorsoventral (DV): 2.8).[30] On the skull, 2 stainless steel support screws and 1 stainless steel reference electrode screw (AP: 3.0 mm) were installed and fixed with dental acrylic. Electrodes were connected to an Electro encephalography (EEG) recording system (Micromed S.p.A., Italy) by a mini-universal serial bus connector. Electrical current was continuously controlled during this process in order to determine whether the electrodes conduct electrical current. Finally, rats were individually placed in their cages for awakening. All rats have been subcutaneously injected with 0.2 mL of penicillin (300 000 IU) for 3 days after the surgery, and the recovery period was determined as 1 week before the start of the experiment.

Two weeks after the electrode placement, a repeated low-dose systemic KA administration protocol was adapted from Hellier and used for generating SE.[31] Kainic acid (Sigma Chemical Co., St Louis, Mo, USA) was diluted in 0.9% sterile saline solution to 2.5 mg/mL. The first dose was injected as 2.5-5 mg/kg, and the following doses were given according to the rats' behavior in 3 dosages; the exact dosage, half dosage, or none until developing status. If the rat was excessively active or inactive during those injections, the dose for that hour was omitted to avoid excessive toxicity and morbidity.[31-33] According to Racine's scale, KA-injected rats that exhibited stage 5 convulsions were regarded as having SE.[34] The Racine scale is 0 for no behavioral changes, 1 for facial movements and ear and whisker twitching, 2 for myoclonic convulsions without rearing, 3 for myoclonic convulsions with rearing, 4 for clonic convulsions with loss of posture, and 5 for generalized clonic–tonic seizures. After 4 hours, SE was stopped with diazepam (4 mg/kg, intraperitoneal (i.p.)). In the sham group, rats were given sterile saline solution.

Video-EEG was recorded before all experimental processes for 2 weeks to evaluate regular physiological hippocampal activity concurrent with rat behaviors. After KA or saline treatment, behavioral seizure activities were observed for 24 hours in the acute group and 2 months for the chronic group continuously 24 hours a day, 7 days a week. Two neurophysiologists reviewed recordings after the experimental procedure and evaluated seizures according to the Racine scale. Grooming, rearing, hind limb scratching, wet dog shakes, jaw movements, salivation, urination, defecation, and head-nodding were observed in animals 1 hour after KA injection. Rats were housed in clear polyethylene–carbon cages after receiving KA injections, and motor seizures were assessed and categorized using Racine's scale.

Immunohistochemistry

Aquaporin 4 and c-fos were investigated by immunostaining methods in brain slices, AQP4 in the hippocampus and c-fos in the hippocampus and amygdala. Briefly, high-dosage chloral hydrate (720 mg/kg, i.p.) was administered to the rats at the end of the experiment (at 1 day and 2 months). The animals were perfusion fixed for 15 minutes using a 75 mL saline bolus for 20 seconds, followed by 200 mL fixative (4% paraformaldehyde in 0.1 M phosphate buffer (pH: 7.4)). The animals were decapitated, their brains removed, and preserved in the same fixative for 2 hours after cardiac perfusion. Brains were embedded in paraffin and then 3-4 µm paraffin slices were deparaffinized and hydrated, and later proper antigen recovering process was performed for each antibody. Slices for AQP-4 were boiled in citrate buffer solution at pressure cooker under 1 Atmosphere (ATM) pressure for 1 minute, and slices for c-fos were boiled in the Ethylenediaminetetraacetic acid (EDTA) solution in the microwave oven for 5 minutes, 4-5 times. All slices were treated with 3% H_2O_2 for 10 minutes to inhibit endogen peroxidase activity, after which the slices were washed with buffered phosphate (pH 7.6). First, polyclonal rabbit anti-AQP4 (Millipore, San Francisco, Calif, USA, 1/100, 2 hours), then polyclonal c-fos rabbit antibodies (Santa Cruz, USA, 1/400, 1 hour), and finally anti-polyvalent biotinized secondary antibodies (20 minutes) were incubated. Following streptavidin peroxidase enzyme (ScyTek. Laboratories. Utah, USA.) treatment for 20 minutes on the slices, aminoethyl carbazole chromogen (5-10 minutes) for encoloring and then Mayer hematoxylin (3-5 minutes) for anti encolouring were dropped on the slices. Pictures were taken from those slices under the microscope (Nikon, Coolpix 4500, Singapur) with 40× magnification. The semiquantitative assessment was performed for the quantification of immunohistochemistry. Staining intensities were graded up to 4 (0: no staining, +1: mild intensity, +2: moderate intensity, and +3: high intensity) by 2 researchers who were unknown to the experimental groups.

Processing of Data and Statistical Analysis

For statistical analysis, the Statistical Package for the Social Sciences computer application (Statistical Package for Social Sciences 21.0 version) was utilized. One-way analysis of variance was used to detect group differences, followed by Tukey and Wilcoxon tests. If the *P*-value was less than .05, the differences were judged significant.

RESULTS

Behavioral Analysis

According to the Racine scale in all rats, intraperitoneal injections of KA (5-15 mg/kg) by repeated low KA protocol given to rats resulted in SE, stage 5 seizures. After KA injections, EEG recordings showed an excellent build-up with high-frequency activity at the start of the seizures, which progressed to generalized tonic-clonic seizures (Figure 1). Two rats died during SE at the beginning of the study. Following these deaths, 10 rats were treated with diazepam because of prolonged convulsive seizures. Convulsive SE mean duration was measured as 2 hours 20 minutes. Sometimes SE continued for 20 hours, but its effect was gradually mitigated. In chronic term, 2 rats had non-convulsive SE that consisted of staring and slowing, lasting 24 hours and 15 minutes. No ictal seizure discharges were observed in the sham group.

Immunohistochemical Analysis

Table 1 shows a semiquantitative assessment of the intensity of immunohistochemical staining.

C-fos immunostaining was seen in the hippocampus CA1 region and the amygdala of experimental rats (Figure 2). The intensity of c-fos immunostaining in the hippocampus CA1 area of rats in the acute KA group was substantially higher than in the chronic KA and sham groups (Figure 2; *P* .05). It was also increased in the amygdala in the chronic term but did not reach a statistically significant level.

There were significant increases in immunostaining intensity of AQP4 in the hippocampus region of animals in the KA groups compared to sham groups (Figure 3). However, it did reach the statistically significant level only in the acute KA group ($P < .05$; Figure 3).

DISCUSSION

In the present study, we determined increased AQP4 in the hippocampal CA1 area at the early and late stages in a kainate model of TLE in rats, supporting the activation of astrocytes. Besides, increased c-fos in both stages, early and late, confirmed neuronal activation in this model.

Several studies showed c-fos expression in the brain following the application of some stimuli such as water stress, fear, and injection of convulsing agents to the animals.[21-28] In addition, the onset and the places of c-fos release, as well as the duration of c-fos expression, were observed immediately after acute seizures.[22,23,27,29] Unlike these studies, we evaluated c-fos immunoreactivity 2 months after SE (late-stage) in the kainate model of TLE in addition to early evaluation, which was done 24 hours after SE. Since the hippocampus and amygdala can be alone epileptogenic zones for intractable TLE, both regions were included in the evaluation. The analysis showed that c-fos protein significantly increased in both regions in the acute KA groups and only in the amygdala in the chronic KA group. This finding might suggest that SE in the early stage leads to very intensive neuronal activation in the hippocampus and the amygdala of the rats. However, its first strong effect continues only in the amygdala. In a study focused on mapping the brain structures recruited during the evolution of seizures that follow repeated administration of pentylenetetrazol in rats, it was found that focal seizures did not lead to c-fos expression in the hippocampus. However, intense neuronal stimulation as tonic–clonic generalized seizures caused an increase in c-fos expression.[27] Based on the findings of this study, we can assume why c-fos labeling intensity in our study did not reach significant levels in the hippocampus at the late stage; because we did not see convulsive seizures from the hippocampus for 2 months following early SE. Our data parallel with the previous results which showed that c-fos

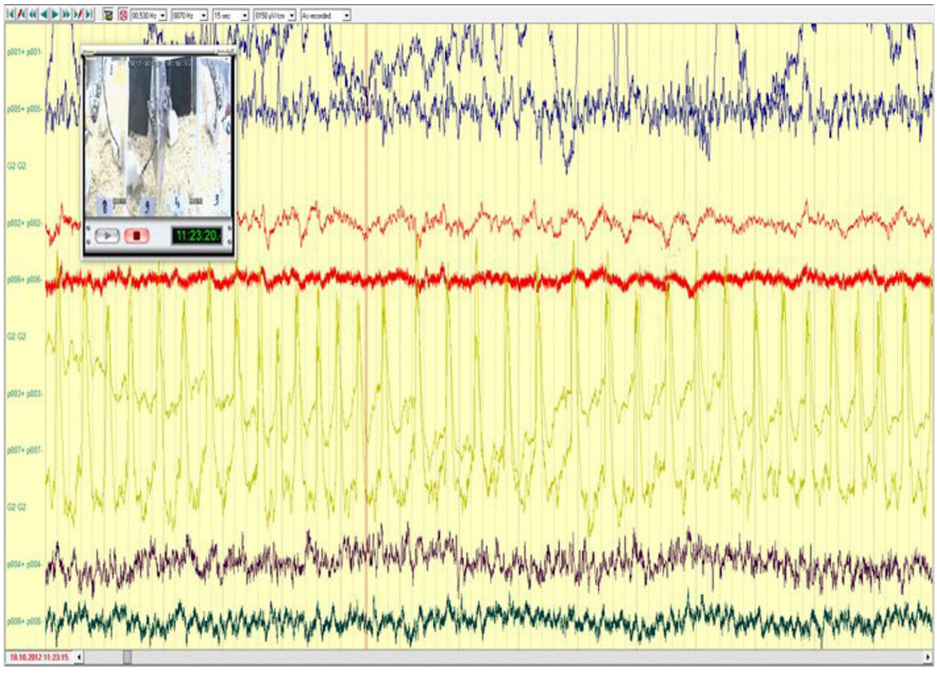

Figure 1 Representative EEG traces from the right and left hippocampus CA1 areas of rats were recorded before and after KA-induced acute generalized seizures. EEG traces in red reveal activity before the seizure in rat #4, while EEG traces in yellow show generalized tonic-clonic seizure activity in rat #9. KA, kainic acid.

Table 1. Semiquantitative evaluation of c-fos and AQP4 immunoreactivity in brain regions

	c-Fos/Hippocampus	c-Fos/Amygdala	AQP4/Hippocampus
Acute KA	+++	+++	+++
Acute Sham	+	++	++
Chronic KA	++	+++	++
Chronic Sham	++	++	+

KA, kainic acid; AQP4, aquaporin 4.

expression after "late" spontaneous seizures in hippocampal neurons was found not to increase in 4 of 11 chronically epileptic rats compared with normal animals.[25] Authors concluded that spontaneous seizures in these animals were originated from extrahippocampal areas because the significant c-fos expression was seen in the piriform cortex and the amygdala but not in the hippocampus.[25] Indeed, in our study, c-fos levels increased only in the amygdala region of the rats in the chronic KA group at the end of 2 months.

Regarding the duration of increased c-fos levels after SE, it was reported that it returned to baseline levels only 16 hours after metrazole-induced seizures.[22] Another study evaluating the duration of increased c-fos expression after KA-induced SE showed different durations for c-fos expression depending on the anatomical sites: 4-5 hours in the dentate gyrus and 24 hours in the hippocampal CA1.[23] We detected increased c-fos levels at the end of 24 hours after SE in concordance with that study. According to these results, this increased duration may also be related to the convulsive agents used for creating seizures along with the intensity of neuronal activity.

In sclerotic hippocampi collected from patients with medically intractable TLE, AQP4 levels were significantly higher.[14,15] In hippocampus tissue taken from mesial TLE patients, however, increased levels of AQP4 were detected, along with a lower density of AQP4 in perivascular membranes.[11] It was concluded that the increase in AQP4 levels was associated with a subcellular redistribution. The astrocyte marker glial fibrillary acidic protein (GFAP) was positively linked with an increase in AQP4 levels, on the other side.[14,15] The overall increase in AQP4 expression levels in mesial TLE was thought to have resulted from the proliferation of astrocytes typical of hippocampal sclerosis. Reactive astrocytosis by increased GFAP immunoreactivity in the hippocampus was also shown in the kainate model of TLE in our previous study.[13]

Aquaporin 4 decreased in the perivascular endfoot membrane of the astrocytes, whereas remained stable or slightly increased in the endfoot membrane facing neuropil (abluminal) in the latent and chronic phase of a previous study.[20] An increase in the M1 isoform of AQP4 in the evaluation was also shown by semiquantitative Western blot analysis. In contrast, we found an increase in AQP4 in the astrocytic cytoplasm and the endfeet of the hippocampal CA1 area in the KA groups compared to shams. However, the increase in AQP4 immunostaining intensity was more significant in the acute group than in the chronic group. That is to say, the increase in immunostaining intensity of AQP4 at the late stage was lower than at the early stage.

On the other hand, it was indicated that AQP4 might be required for the clearance of seizure-induced edema.[35] During rapid neuronal firing, extracellular K+ ions increase from 3 mM to a maximum of 10-12 mM, released by active neurons.[10,36,37] Aquaporin 4 is necessary for the clearance of K+ ions and extracellular solutes. Following the intense neuronal activity accompanying the seizure, K+ ions clearance from the extracellular space in rats without AQP4 was impaired. It was considered that impaired K+ ions clearance due to lack of AQP4 would lead to prolonged depolarization of

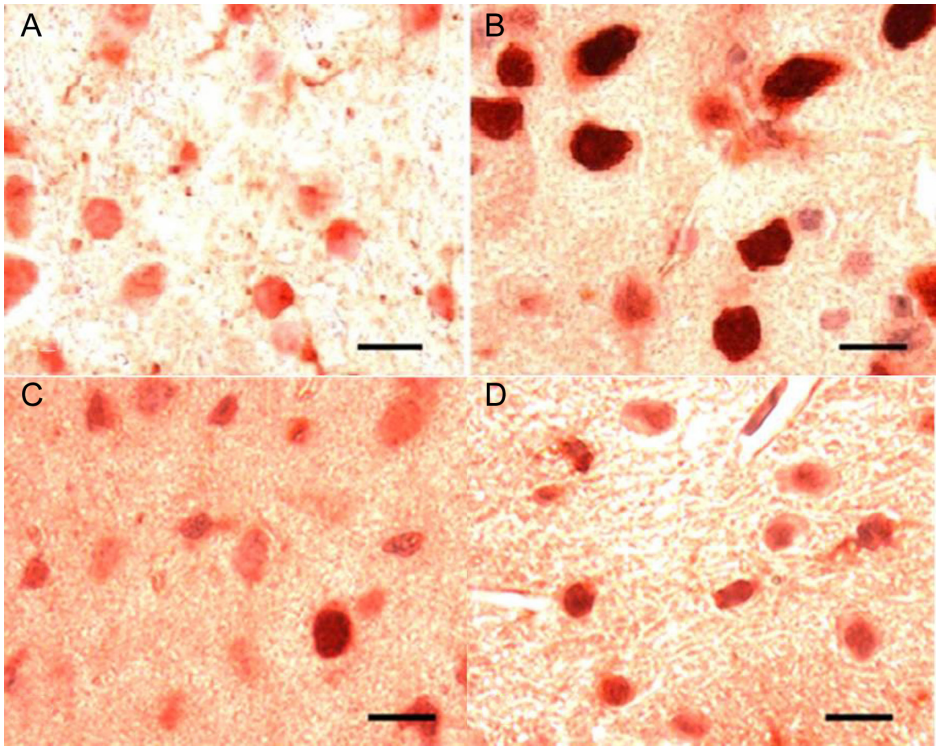

Figure 2 The immunostaining intensity of c-fos protein along the microvessels in the hippocampus region of animals in acute and chronic sham groups and acute and chronic KA groups. (A) Acute sham, (B) acute KA, (C) chronic sham, (D) chronic KA. Note that c-fos immunostaining intensity increased in the hippocampal CA1 region of animals in the acute KA group. Scale bar = 10 µm. KA, kainic acid.

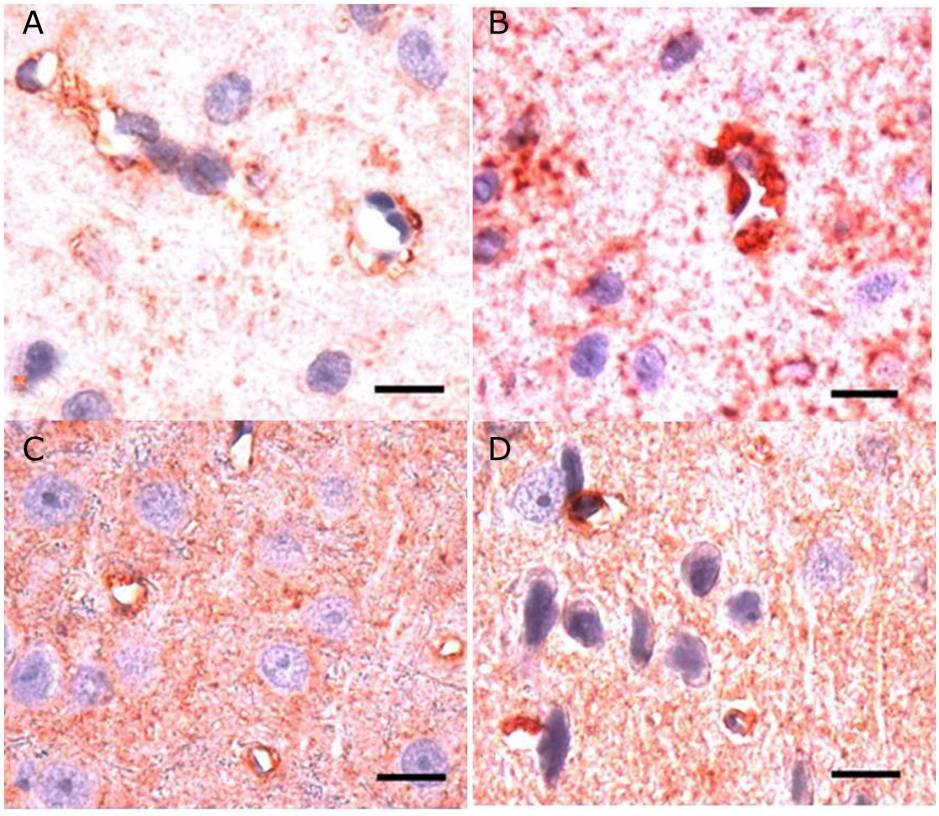

Figure 3 In the hippocampus of animals in the acute sham, chronic sham, acute KA, and chronic KA groups, the immunostaining intensity of AQP4 of astrocytic endfeet and cytoplasm. Note the significant AQP4 staining of astrocytic processes around blood vessels in the hippocampus of rats with acute KA. Only the acute KA group (B) showed the statistically significant increase in AQP4 immunoreactivity as compared to the other groups (acute sham (A), chronic sham (C), and chronic KA (D). Scale bar = 10 µm. KA, kainic acid; AQP4, aquaporin 4.

neurons and inhibit seizure termination.[10] Thereby, the need for AQP4 may increase after intense neuronal activation. A significant increase in the early stage compared to the late stage in our study may be an explanation for this need. As a similar finding, an increase in AQP4 expression was found after seizure activity in preeclamptic rats, and it was assumed that the increase in AQP4 expression altered water and ion distributions in the pericapillary milieu, rendering the BBB more susceptible to disruption by seizure activity.[38]

CONCLUSION

Astrocytes hold a crucial role in neuronal metabolism by AQP4. There is a link between AQP4 and epilepsy. The alterations in AQP4 expression after seizures during epileptogenesis and whether these changes are a causative factor or a defense mechanism remain unclear. According to our findings, we speculate that it can evolve into a causal factor with exhaustion due to overuse even though AQP4 change is primarily a defense mechanism immediately after SE. As for the role of c-fos, increased levels in the study approved the neuronal activation after epileptic seizures.

Ethics Committee Approval: The information on the Ethical Committee approval has been provided as follows: Ethical committee approval was received from the Animal Ethics Committee of Istanbul University (Date: June 30, 2011, Decision no: 70).

Informed Consent: Written informed consent was obtained from all participants who participated in this study.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept - M.K., C.G.; Design - M.K., C.G., E.T.; Supervision - M.K., C.G., B.A.; Funding - M.K., C.G., E.T.; Materials - E.T., C.U.Y., N.O.; Data Collection and/or Processing - E.T., C.U.Y., N.O., M.B.; Analysis and/or Interpretation - M.K., C.G., E.T., N.A.; Literature Review - M.K., C.G., E.T.; Writing - E.T., M.K., C.G.; Critical Review - M.K., C.G., B.A.

Declaration of Interests: The authors stated that they have no conflicts of interest.

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