Immunomorphologic Assessment of Changes in Functional Astroglial Proteins in a Kainate-Induced Hippocampal Sclerosis Model

Dmitry N. Voronkov[1], Anna V. Egorova[1,2], Evgenia N. Fedorova[1,2], Alla V. Stavrovskaya[1], Ivan A. Potapov[1], Anastasiya K. Pavlova[1], Vladimir S. Sukhorukov[1,2]

[1]Research Center of Neurology, Moscow, Russia;
[2]Pirogov Russian National Research Medical University, Moscow, Russia

Abstract

Introduction. *Astrocytes are involved in mediator metabolism, neuroplasticity, energy support of neurons and neuroinflammation, and this determines their pathogenetic role in epilepsy.*

Aim. *This study aimed at evaluating region-specific changes in the distribution of functional astroglial proteins in reactive astrocytes in a kainate-induced model of mesial temporal lobe epilepsy.*

Materials and methods. *The localization and expression of functional astroglial proteins (i.e. aquaporin-4, connexin-43, EAAT1/2, and glutamine synthetase) in the hippocampus CA3 region, dentate gyrus, and stratum lucidum layer were evaluated by immunofluorescence 28 days after intra-hippocampal administration of kainic acid to animals.*

Results. *Changes were heterogeneous in different hyppocampus subregions. Astrocytes of the stratum lucidum associated with mossy fibers showed the highest vulnerability and decreased content and/or disturbed localization of the channels and transporters that form membrane complexes in the processes. Disturbances in homeostatic functions of astrocytes aggravated the adverse processes both on the side where the toxin was injected and in the contralateral hippocampus.*

Key words*: hippocampal sclerosis; kainic acid; astrocytes*

Ethics approval. The experiment was carried out in compliance with bioethics standards for experiments with laboratory animals. The study was approved by the Ethics Committee of Research Center of Neurology (Protocol 5-3/22 dated 06/01/2022).

Source of funding. This study was not supported by any external sources of funding.

Conflict of interest. The authors declare no apparent or potential conflicts of interest related to the publication of this article.

For correspondence: 80 Volokolamskoye shosse, Moscow, 125367, Russia. Research Center of Neurology.
E-mail: voronkov@neurology.ru. Voronkov D.N.

For citation: Voronkov D.N., Egorova A.V., Fedorova E.N., Stavrovskaya A.V., Potapov I.A., Pavlova A.K., Sukhorukov V.S. Immunomorphologic assessment of changes in functional astroglial proteins in a kainate-induced hippocampal sclerosis model. *Annals of Clinical and Experimental Neurology*. 2024;18(4):34–44. (In Russ.)

DOI: https://doi.org/10.17816/ACEN.1102

Received 07.03.2023 / Accepted 02.05.2023/ Published 25.06.2024

Иммуноморфологическая оценка изменений функциональных белков астроглии на индуцированной каинатом модели склероза гиппокампа

Д.Н. Воронков[1], А.В. Егорова[1, 2], Е.Н. Федорова[1, 2], А.В. Ставровская[1], И.А. Потапов[1], А.К. Павлова[1], В.С. Сухоруков[1, 2]

[1]Научный центр неврологии, Москва, Россия;
[2]Российский национальный исследовательский медицинский университет им. Н.И. Пирогова, Москва, Россия

Аннотация

Введение. *Участие астроцитов в медиаторном обмене, нейропластичности, энергетической поддержке нейронов и нейровоспалении определяет их патогенетическую роль при эпилепсии.*

Цель *исследования – оценка регионально-специфических изменений распределения функциональных белков астроглии в реактивных астроцитах при каинат-индуцированной модели мезиальной эпилепсии височной доли.*

Материалы и методы. *Иммунофлуоресцентным методом оценивали локализацию и экспрессию функциональных белков астроглии: аквапорина-4, коннексина-43, EAAT1/2 и глутаминсинтетазы в поле CA3 гиппокампа, зубчатой извилине, слое stratum lucidum у животных через 28 сут после интрагиппокампального введения каиновой кислоты.*

Результаты. *Выявленные изменения носили неоднородный характер в исследованных субрегионах гиппокампа. Астроциты stratum lucidum, ассоциированные с мшистыми волокнами, демонстрировали наибольшую уязвимость и снижение содержания и/или нарушение локализации каналов и транспортёров, формирующих мембранные комплексы в отростках. Нарушение гомеостатических функций астроцитов отягощает патологический процесс как на стороне введения токсина, так и в противоположном гиппокампе.*

Ключевые слова*: склероз гиппокампа; каиновая кислота; астроциты*

Этическое утверждение. Эксперимент проводили с соблюдением биоэтических норм по работе с лабораторными животными. Исследование одобрено этическим комитетом ФГБНУ НЦН (протокол № 5-3/22 от 01.06.2022).

Источник финансирования. Авторы заявляют об отсутствии внешних источников финансирования при проведении исследования.

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Адрес для корреспонденции: 125367, Москва, Волоколамское шоссе, д. 80. Научный центр неврологии.
E-mail: voronkov@neurology.ru. Воронков Д.Н.

Для цитирования: Воронков Д.Н., Егорова А.В., Федорова Е.Н., Ставровская А.В., Потапов И.А., Павлова А.К., Сухоруков В.С. Иммуноморфологическая оценка изменений функциональных белков астроглии на индуцированной каинатом модели склероза гиппокампа. *Анналы клинической и экспериментальной неврологии*. 2024;18(4):34–44.

DOI: https://doi.org/10.17816/ACEN.1102

Поступила 07.03.2024 / Принята в печать 02.05.2024 / Опубликована 25.06.2024

Introduction

About a third of epilepsy cases are drug-resistant [1]; therefore, mechanisms of epileptogenesis and new therapeutic targets are the most important objectives of translational neuroscience. At the molecular and morphological levels, epilepsy is characterized by neurodegeneration, abnormal neuroplasticity, impaired neurogenesis, changes in the topology of neuronal connections, neurotransmitter imbalance, abnormal functional ability of several receptor complexes, and modified molecular structure of ion channels [2].

Hippocampal sclerosis (HS) with neuronal death in several hippocampal regions is the most common histological diagnosis in adult patients who undergo surgery for focal structural drug-resistant epilepsy [3, 4]. Glutamatergic excitotoxicity caused by excessive release of glutamate during epileptic activity is considered to be the leading cause of neuronal death in HS [5]. Participating in homeostasis and regulating levels of extracellular glutamate, astroglia is directly involved in excitotoxic reaction cascades. By modulating synaptic transmission, astrocytes provide energy support to neurons and participate in neuroinflam-

mation and synaptic plasticity [6]. Impairment of astrocytic functions is a key cause of epileptogenesis [7].

In HS, there are several patterns of damage that are associated with astrogliosis (astrocyte proliferation and hypertrophy) of various severity. International League Against Epilepsy (ILAE) type 1 HS is the most common (i.e. 60–80% of cases); it is associated with severe damage in CA1 and CA3 regions and affects CA2 and *dentate gyrus* (DG) [4]. Type 2 HS is mostly associated with damage in CA1, while in type 3 HS hilus (also known as CA4) neurons are affected most significantly. Pre-operative seizure burden and outcome after hippocampal resection positively correlated with the degree of gliosis, in particular in the CA3 region [8].

No pronounced neuronal death is found in up to 20% of cases of temporal lobe epilepsy. This is explained by reactive changes in astroglia, which, according to A. Grote et al., does not precede types 1–3 HS but is a separate condition [9]. Relationship between reactive changes in astroglia and damage to hippocampal neurons has not been elucidated yet.

Epileptogenesis in temporal lobe epilepsy is associated with abnormal invasion of mossy fibers (i.e. DG granular cell axons) into the molecular layer of the DG and formation of new excitatory synapses. Reorganization of the hippocampal neural network results in oversynchronization and generation of epileptic discharges [10], where dysfunction of glial cells plays a key role.

Administration of kainic acid (KA), an agonist of kainate receptors (i.e. a subtype of ionotropic glutamate receptors), is a common chronic model of temporal lobe epilepsy that reproduces pathomorphological signs of HS. Besides increased excitability of CA3 glutamatergic pyramidal neurons and suppression of GABA release, effects of KA, depending on its interaction with presynaptic, postsynaptic or glial kainate receptors, include "reactive" plasticity of DG granular neurons, pro-inflammatory glial responses, and changes in the release of neurotrophic factors and gliotransmitters (signaling molecules that ensure communication between glial cells and control the excitability of neurons) [11].

Astrocytes are involved in regulation of glutamatergic neurotransmission; they control levels of extracellular glutamate using EAAT1 (GLAST) and EAAT2 (GLT-1) transporters and participate in the metabolism and detoxification of glutamate using a gliospecific enzyme called glutamine synthetase (GS) [12]. Astrocytes form so-called "tripartite synapses" by encompassing with their processes the area of the synaptic contact between neurons, which allows astrocytes to modulate neurotransmission. Astrocytes also release gliotransmitters, such as purines, D-serine, and various glutamate receptor ligands, which affect neuronal excitability [6]. Neuronal group activity is regulated by interastrocytic networks formed with gap junction proteins connexins (Cx30, Cx43), which are involved in the transport of small molecules and organization of the glial network, regulation of glutamate transport, and diffusion of energy metabolites and gliotransmitters [6, 13]. Factors that cause neuron hyperexcitability include disturbed water balance in the nervous tissue, which is regulated by a water channel protein called aquaporin-4 (AQP4) [12, 14]. Localized in the distal parts of astrocyte processes, AQP4, as well as Cx43, is associated with redistribution of ions and water in the intercellular area; it affects levels of neurotransmitters and regulates the volume of astrocytic perisynaptic sheaths. AQP4 and Cx43 are also involved in migration of astrocytes and regulation of the motility of their processes [13], which suggests their importance in gliosis and tissue remodeling.

Structural and functional characteristics of neuro-glio-vascular interactions in brain structures are determined by regional characteristics of astroglia [12]. The glioarchitecture of the hippocampus, as well as other brain structures, is closely related to its synaptic organization. Astrocytes of different hippocampus regions and layers have morphological, neurochemical, and functional heterogeneity [15, 16]. Region-specific characteristics are likely to be associated with normal functioning of the structures and determine astrocyte response to pathological processes; reactive astroglia maintains a regional profile of homeostatic gene expression [17]. Genes that determine regional specificity of hippocampal astroglia include *slc1a2* (EAAT2), *slc1a3* (EAAT1), *Gja1* (Cx43), *Glul* (GS), and *Aqp4* [16, 18]. Dysregulation of several groups of genes can affect specific astroglial subpopulations [19]. Relationship between AQP4, Cx43, and EAAT1/EAAT2, taken together with macromolecular complexes they form on astrocyte membranes [20], require a joint assessment of changes in these functional proteins in HS.

Therefore, data from modern experimental studies showed a significant contribution of astroglia to epileptogenesis; however, the role of astroglia in the pathogenesis of HS has not been sufficiently studied yet.

Aim. This study aimed at evaluating region-specific changes in the distribution of functional astroglial proteins in reactive astrocytes in kainate-induced HS.

Materials and methods

The study was performed in 10 male Wistar rats aged 3.5 to 5.0 months weighing 300 to 350 g, which were obtained from Stolbovaya Laboratory Animals Breeding Facility of Biomedical Technology Research Center. The animals were kept in a vivarium with constant access to

water and food. The experiment was carried out in compliance with bioethics standards for experiments with laboratory animals according to the European Convention for the Protection of Vertebral Animals Used for Experimental and Other Scientific Purposes (CETS No. 170). The study was approved by the Ethics Committee of Research Center of Neurology (Protocol No. 5-3/22, dated 06/01/2022).

Stereotactic injections

Zoletyl-xylazine anesthesia was used for anesthesia after premedication with atropine (Dalkhimfarm JSC) 0.04 mg/kg s.c. for 10–15 minutes. Then, a mixture of zolazepam hydrochloride and tiletamine hydrochloride (0.3 mg/kg, Zoletil-100, Virbac) was administered i.m. followed by xylazine hydrochloride (3 mg/kg, Xyla, De Adelaar) i.m.

KA solution (Sigma) 0.5 μg in 3 μL of normal saline was injected using an RWD stereotaxic manipulator into the CA1 region of the rostral hippocampus on the right (n = 5) according to the coordinates (AP = –3.0; ML = 2.0; DV = 2.8) in the rat brain atlas[1]. The same volume of normal saline was injected on the left. Sham-operated (control) rats (n = 5) were administered bilaterally with 3 μL of normal saline.

Immunofluorescence staining

Twenty-eight days after KA injection, the animals were decapitated using a guillotine; their brain was removed, dissected in the frontal plane, and fixed for 24 hours in 4% neutral formalin. After soaking in a 30% sucrose solution, samples were placed in O.C.T. (Tissue-Tek), and a series of frozen frontal sections (12 μm thick) was prepared at the level of the frontal third of the hippocampus. The sections were heated in a steamer in citrate buffer pH 6.0 (Leica) for 15 min. Staining was performed according to the recommendations of the primary antibody manufacturers. The following antibodies were used: antibodies to NeuN (Abcam, ab104224) and synaptophysin (SF, Sigma, S5768) neuronal proteins; antibodies to astrocyte proteins, i.e. EAAT1 (GLAST, Abcam, ab181036) and EAAT2 (GLT1, Abcam, ab203130) glutamate transporters, GS (Sigma, G2781), Cx43 (Abcam, ab11370), AQP4 (Sigma, HPA014734), vimentin (Vim, Abcam, ab92547), and gliofibrillar protein (GFAP; Abcam, ab207165). Microglia was detected using anti-IBA1 antibody (GeneTex, GTX635399). Primary antibodies were incubated with the sections in a humidified chamber for 18 hours at room temperature. Corresponding anti-murine or rabbit Ig antibodies (Invitrogen) labeled with Alexa Fluor 488 or Alexa Fluor 555 fluorochromes were used to detect binding. Sections were mounted in Fluoroshield medium (Abcam) containing 4',6-diamidino-2-phenylindole (DAPI) to stain cell nuclei.

[1] Paxinos G., Watson Ch. The Rat Brain in Stereotaxic Coordinates. Amsterdam; Boston, 2005.

Morphometry

From each animal, 6–12 sections were examined at the level of the rostral third of the hippocampus, which were taken at equal intervals along the rostrocaudal axis. Sections were documented using a Nikon Eclipse Ni-U microscope (×20), and average tissue fluorescence intensity (adjusted for background staining) in gradations of brightness of the 8-bit image was evaluated using ImageJ software.

Besides regions (sectors) of the hippocampus (CA1, CA2, CA3, DG), staining for neuronal and glial proteins allowed differentiating the layers of the CA3 region: *stratum lacunosum-moleculare*, *stratum radiatum*, *stratum lucidum*, *stratum pyramidale*, *stratum oriens*. The granular cell layer (*str. granulare*) and the polymorphic layer (*hilus*, *stratum polymorphe*) were detected in the DG [21].

Measurements were performed in the CA3 region as a whole and in the *stratum lucidum* separately, as well as in the polymorphic layer of the DG of the right (ipsilateral to the damage) and left (contralateral) hippocampus for the animals administered with KA and on the right (on the side of administration of 0.9% NaCl) for control animals. Areas of interest were manually segmented in the images. To estimate the area of $AQP4^+$ vessels using local threshold segmentation in ImageJ, the area of the vessels was isolated in relation to the area of the visual field. Gray level co-occurence matrix (GLCM) image texture analysis was used to assess Cx43 distribution in the tissue. GLCM contrast is inverse to changes in the homogeneity of marker distribution. The use of GLCM for histological images was reported earlier [22].

Statistical analysis

Mean values were calculated for each animal. Statistical analysis was carried out using GraphPad Prism 7.0. Two-way analysis of variance (ANOVA) with Tukey's post-hoc test was used to identify between-group differences. Data were presented as $M \pm SD$, where M is mean and SD is mean-square deviation. If results of Shapiro–Wilk test deviated from normal distribution (data for SF), Wilcoxon test was used to compare the hemispheres. Data for SF were presented as median (Me) and interquartile range [Q_1; Q_3]. Differences were considered significant at $p < 0.05$.

Results

Neuronal damage in CA3 was seen in all animals that received KA (Fig. 1, *A*). Assessment of staining intensity for NeuN neuronal marker showed a significant ($p < 0.001$) decrease (to 60.63% ± 22.11% vs. control) in the pyramidal layer of the CA3 region on the side where the toxin was injected but not on the opposite side (to 89.8% ± 16.8% vs. control). Neuronal damage was also detected in the CA1

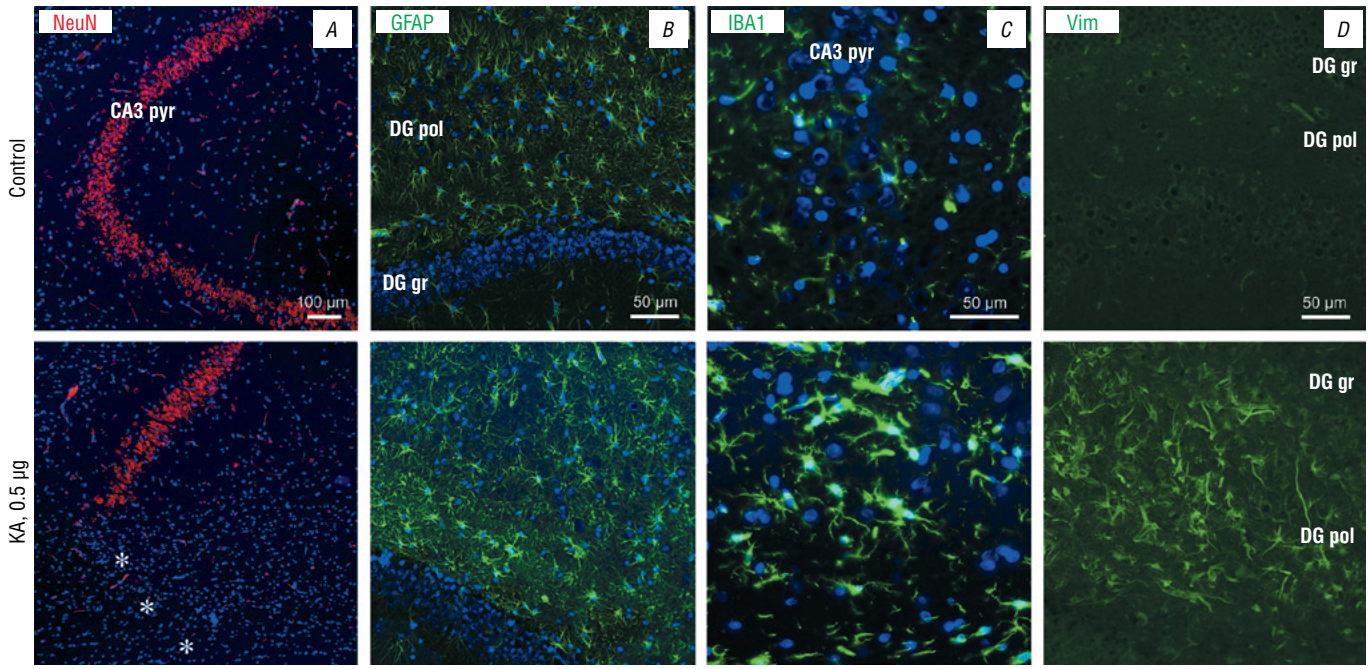

Fig. 1. Administration of KA into the hyppocampus resulted in neuronal damage in the CA3 region and glia activation in the DG.
A, detection of NeuN neuronal marker (stained with red), CA3, × 10;
B, astrocyte activation, GFAP (stained with green), DG, × 20;
C, microglia hypertrophy, IBA1 (stained with green), CA3, × 40;
D, expression of vimentin (stained with green) by reactive astrocytes of the polymorphic layer of the hippocampus, × 20.
CA3 pyr, pyramidal layer of CA3; DG pol, polymorphic layer, DG gr, granular layer, * damage area. Nuclei stained with DAPI (blue).

region; however, due to the proximity of the needle track and glial activation caused by mechanical damage, this region of the hippocampus was excluded from the analysis in our study. No significant staining for NeuN was seen in the DG. Staining intensity for SF in the *stratum lucidum* on the side where the toxin was injected (Me = 73.44 [67.76, 87.15]) was significantly ($p < 0.05$, Wilcoxon test) higher compared with the contralateral hippocampus (Me = 68.12 [56.67; 77.81]).

In both CA3 and DG of the hippocampus, pronounced gliosis, increased staining for GFAP, hypertrophy and deformation of astrocyte processes were seen (Fig. 1, *B*), as well as activation of microglia (Fig. 1, *C*). Some astrocytes in both CA3 and DG expressed Vim, which is typical for immature and reactive astrocytes, with the highest staining intensity for Vim seen in the DG. It is of note that Vim^+-astrocyte bodies were changed to the highest extent (Fig. 1, *D*).

Therefore, KA caused activation of astrocytes both directly in the area of maximum neuronal damage (in the CA3 region) and in the polymorphic layer of the DG, without neuronal loss in the latter.

The boundaries of the *stratum lucidum*, which is formed predominantly by the axons of DG granular neurons, were detected by both SF staining and glial marker identification (Fig. 2), with the exception of relatively uniform staining for AQP4. The greatest differences in staining between CA3 layers were visually noted for EAAT1 and Cx43, with the stratum lucidum having their lowest content.

Besides a dramatic increase in immunofluorescence intensity for GFAP ($p < 0.001$) in the CA3 region (without considering layers, compared with the sham-operated control), the side of KA administration demonstrated increased levels of Cx43 ($p < 0.001$) and GS ($p < 0.001$) and significantly decreased staining for AQP4 ($p < 0.001$) (Fig. 2, 3).

Together with a decrease in immunofluorescence intensity for AQP4 in the CA3 region, the bodies of glial cells were more clearly identified and a decrease in staining intensity of blood vessels were seen, which indicates AQP4 redistribution in the cells and a decrease in its content in the astrocyte endfeet. This was confirmed by percentage area of stained vessels in the CA3 in the visual field: it was significantly ($p = 0.022$) reduced from 0.99% ± 0.48% in the control group to 0.55% ± 0.15% on the side of KA administration. Similar changes were found in the DG.

The increase in total fluorescence intensity (Fig. 3) for Cx43 in CA3 was predominantly due to glial cells in the *stratum oriens* and *stratum lacunosum molecularis*. Changes in the distribution of Cx43 in the tissue was confirmed by assessing the "contrast" of the image, i.e. a parameter inverse to homogeneity of marker distribution

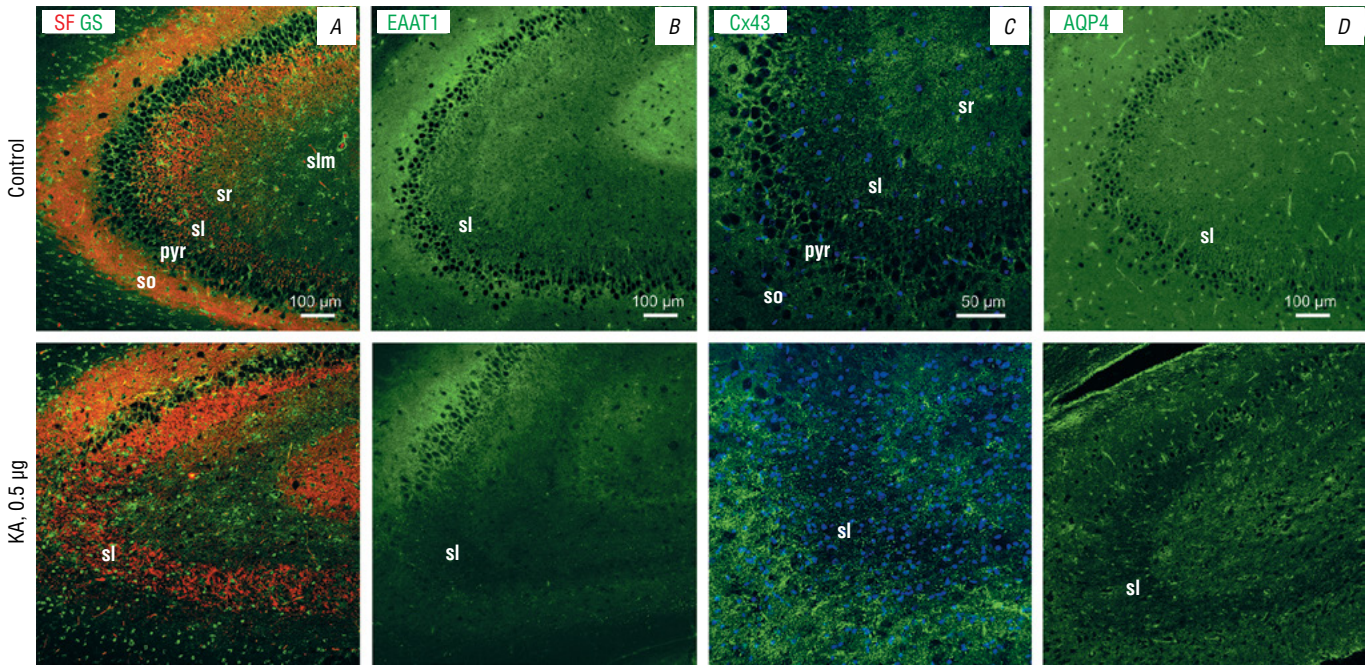

Fig. 2. Changes in expression and localization of astrocyte functional proteins in hippocampal CA3 layers after administration of KA.
A, identification of GS localization (stained with green) and SF (stained with red) in hippocampus layers, × 10;
B, EAAT1 detected, × 10;
C, Cx43 detected (stained with green), nuclei further stained with DAPI (stained with blue), × 20;
D, AQP4 detected, × 10.
so, *stratum oriens;* **pyr**, *stratum pyramidalis;* **sl**, *stratum lucidum;* **sr**, *stratum radiatum;* **slm**, *stratum lacunosum moleculare.*

in the sample. This parameter significantly (p = 0.021) increased threefold (214.49 ± 116.51) in CA3 compared with control (70.97 ± 2.41) on the side where KA was injected. Demonstrated decrease in the uniformity of Cx43 distribution appeared to reflect Cx43 redistribution in astrocyte processes. It is of note that large $Cx43^+$ staining clusters around vessels were seen in animals treated with KA, which may be due to increased expression of Cx43 by endothelial cells or impaired distribution of Cx43 in the astrocyte endfeet.

GS distribution also changed differently across hippocampal layers. On the side where KA was injected in the CA3 region, a large number of ovoid GS^+-glial cells with intense staining of the cytoplasm were detected, which were likely to be oligodendroglia. These cells were rare in control animals. Decreased GS staining was observed in astrocyte processes in the *stratum lucidum* (Fig. 3, 4). At the same time, GS+-reactive astrocytes with hypertrophied processes and intensely stained cytoplasm were detected in the stratum oriens, *startum radiatum*, and *stratum lacunosum moleculare*.

For EAAT1 glutamate transporter, the average intensity in the CA3 region (without considering the layers) increased significantly (p = 0.0149) under the influence of KA on the damaged side, and areas with both increased and decreased EAAT1 expression were visually noted, which may be related to varying degrees of damage to neurons in the CA3 region.

Immunofluorescence in the *stratum lucidum* on the side of KA administration (Fig. 3) demonstrated a significant decrease in levels of functional astrocyte proteins vs. sham-operated control (i.e. EAAT1, EAAT2, GS and AQP4 but not Cx43, for which a trend to increased staining was detected; p = 0.053). In the *stratum lucidum* of the hippocampus of the contralateral hemisphere, immunofluorescence was also decreased with this effect being the greatest for GS and AQP4.

Similar to the changes in the *stratum lucidum*, a less pronounced decrease in levels of evaluated proteins (i.e. EAAT1, GS, and AQP4) was also seen in the polymorphic layer of the DG in the hippocampus (Fig. 3), which is probably related to the general direction of changes in astroglia in the areas of synaptic contacts of mossy fibers with neurons. It is noteworthy that opposite changes (increased immunostaining) were detected only for EAAT2 in the DG compared with the *stratum lucidum.* Increased staining intensity for EAAT2 was likely to be related to intense staining of reactive astrocyte soma (Fig. 4).

Therefore, KA administration led to unilateral damage to pyramidal neurons of hippocampus CA3 and gliosis; these reactive changes in astroglia were accompanied with

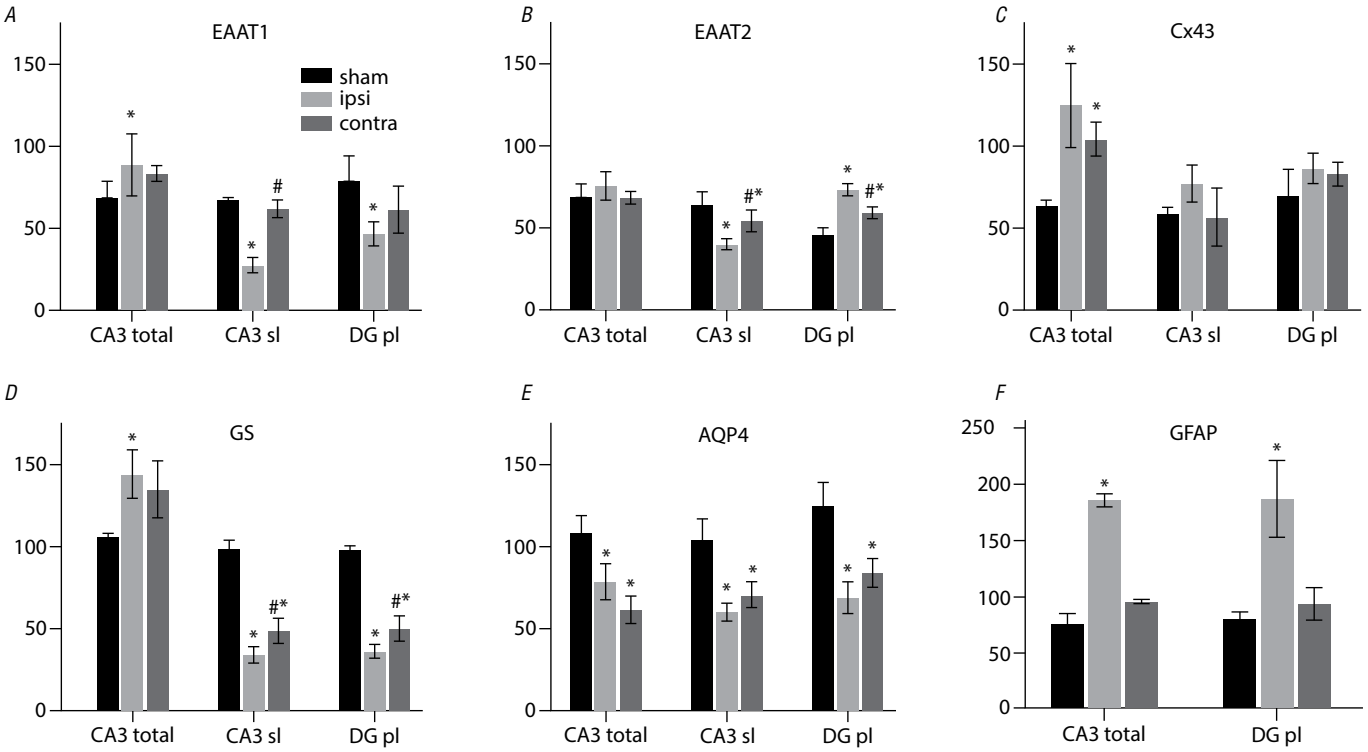

Fig. 3. Changes in immunofluorescent staining intensity for functional astrocyte proteins in CA3 of the hippocampus (CA3 total), *stratum lucidum* (CA3 sl), and the polymorphic layer of DG (DG pl) after administration of KA.
A, EAAT1 glutamate transporter (GLAST); *B*, EAAT2 glutamate transporter (GLT-1); *C*, Cx43; *D*, GS; *E*, AQP4; *F*, GFAP.
sham, sham-operated animals; ipsi, on damage side; contra, contralateral to damage side; *$p < 0.05$ compared with sham-operated animals; #$p < 0.05$ compared with damage side (ANOVA, Tukey's post-hoc test).

decreased expression of its functional homeostatic proteins in the *stratum lucidum*, namely in a layer of mossy fibers both on the side where the toxin was injected and in the contralateral hemisphere. In the CA3 region, KA-induced damage resulted in increased expression of GFAP, Vim, GS and Cx43, together with AQP4 redistribution with a moderate decrease in immunostaining intensity. At the same time, reactive changes in astroglia of the *stratum lucidum* differed from the total CA3 region and were associated with a significant decrease in glutamate metabolism proteins (GS, EAAT1/2) and AQP4, while levels of Cx43 did not change significantly although tended to increase. Besides changes in Cx43, AQP4, GS and EAAT2 levels, changes in their cellular localization and distribution in the tissue were also found, including those associated with disruption of glio-vascular contacts.

Discussion

Intrahippocampal administration of KA caused gliosis in the CA3 and DG regions and pronounced changes in the regional expression of functional astrocyte proteins to varying degrees in the CA3 region as a whole and in the *stratum lucidum layer* in particular. Neuronal damage was detected in CA1 and CA3 on the side where the toxin was injected, corresponding to type 1 HS [4]. The changes detected in astroglia were also seen in the contralateral hemisphere, where no statistically significant neuronal damage was recorded.

The *stratum lucidum* of the hippocampus CA3 region is represented by mossy fibers, i.e. axons of granular cells of the DG, which form many glutamatergic "detonator" synapses on the pyramidal neurons of the CA3 region. Their functional significance in epileptogenesis and mossy fiber rearrangements observed in epilepsy determine interest in changes in glial cells in this area [10]. Our data on increased immunostaining for SF in the *stratum lucidum* are consistent with literature data [23] that showed increases SF levels 30 days after KA administration and may indicate "reactive" plasticity.

Astrocytes of different regions of the hippocampus differ in their characteristics, which depend, among others, on the synapses they "serve" [15]. The hilus and stratum lucidum of the hippocampus have relatively high astrocyte density [24]. Astrocytes in the stratum lucidum change their intracellular $Ca2^{+}$ levels only in response to burst activity of neurons and significant increases in glutamate levels, in contrast to DG astrocytes, which provide fine regulation of synaptic transmission [25]. Compared with synapses in other areas of the hippocampus, contacts of

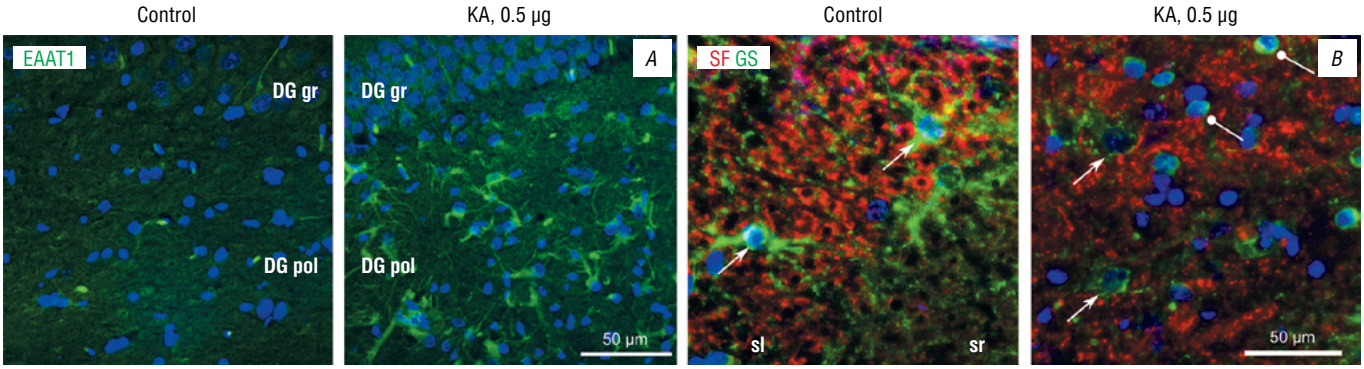

Fig. 4. Changes in intracellular EAAT2 and GS location in astrocytes after KA administration.
A, intense staining for EAAT2 glutamate transporter (green) in reactive astrocyte bodies of the polymorphic layer, × 40;
B, GS detected (stained with green) and SF (stained with red) in the processes and bodies of reactive astrocytes (arrows) in *stratum lucidum and* cells without processes identified (line segments with a dot at the end), × 40.
DG gr, granular layer; DG pol, polymorphic layer; sl, *stratum lucidum*; sr, *stratum radiatum*.

astrocytes with synaptic buttons on mossy fibers are less close [25]. It is of note that the *stratum lucidum* was shown to have extremely high levels of brain-derived neurotrophic factor [26], which is involved in neuroinflammation and regulation of astroglial morphogenesis [27, 28].

Changes in the levels and localization of AQP4, glial glutamate transporters (EAAT1/EAAT2), GS, and Cx43 in our study were associated with increased neuronal excitability, as well as initiation and maintenance of epileptic activity [7]. Inconsistent changes in the levels of these proteins in publications of various authors were associated with differences in measurement methods, evaluated areas, and timing of KA administration. For example, AQP4 levels in rat hippocampus were decreased one day but increased one month post KA-induced status epilepticus [29]. On the other hand, total content of AQP4 was increased but its immunostaining in the perivascular endfeet was decreased in patients with lobe epilepsy and HS [30]. Our study showed redistribution of AQP4 in astrocytes and decreased area of $AQP4^+$ perivascular staining, consistent with other authors, and an overall decrease in APQ4 levels. Previously, the kainate model in CA1 and CA3 showed decreased AQP4 levels in the perivascular compartment both in the latent and late periods of epileptogenesis [31]. Subcellular redistribution of AQP4 in the neuropil but not in the astrocyte endfeet was also shown in a model of posttraumatic epilepsy [32].

A macromolecular complex of AQP4 with EAAT2 and Kir4 determines possible AQP4 participation in the exchange of K^+ and glutamate [29, 33]. Disrupted association of AQP4 and EAAT2 glutamate transporter in astrocytes was suggested to lead to neuronal dysfunction [34, 35]. Evaluation AQP4 and EAAT2 levels in a KA model of epilepsy showed a decrease in their expression in the early period of epileptogenesis [29], which is consistent with our results for the *stratum lucidum*. Changes in the distribution of these proteins in astroglial processes, which were also shown in our study, indicated disturbances in the organization of gliovascular and glioneuronal contacts in the astrocyte endfeet. According to publications, EAAT2 (but not EAAT1) glial glutamate transporter was directly inhibited by KA [36], and an increase in their levels in the DG that we found may reflect a compensatory increase in their expression. Heterogeneous changes in EAAT2 in the *stratum lucidum* and DG may be related to astrocyte heterogeneity in these areas of the hippocampus or different intensity of their reaction.

Several studies showed an increase in EAAT1 expression after KA administration, which was considered by the authors as a compensatory reaction [36]. We detected an increase in EAAT1 levels in CA3, which, however, was not due to the astrocytes of the *stratum lucidum*, where, on the contrary, staining was reduced for both transporters. Overall, dysfunction of EAAT1/2 transporters in epileptic foci led to impaired utilization of extracellular glutamate [37]; however, the changes in the expression of these proteins in patients with temporal lobe epilepsy were often inconsistent and demonstrated different directions of changes [38, 39].

GS, an astroglia-specific enzyme of glutamine-glutamate metabolism, also plays a key role in preventing the accumulation of toxic glutamate. In patients with mesial temporal lobe epilepsy, the intensity of GS staining was reduced in the CA1, CA3, and DG regions of the hippocampus, demonstrating an association with seizure patterns [40]. We found decreased GS staining intensity in astrocyte processes in the *stratum lucidum* but not in other layers where GS^+-reactive astrocytes had hypertrophied processes. Previously, GS redistribution in astrocyte processes in CA1 and CA3 of the hippocampus was shown in models of temporal lobe epilepsy [40, 41].

Neuronal excitability and synchronization are also largely regulated by coordinated activity of the astrocytic network

together with connexins. Cx43 gene knockout led to seizures and motor disorders in animals [42]. Disruption of connections between astrocytes promotes epileptogenesis by reducing buffering of K^+ and Na^+ followed by inhibition of glutamate clearance from the synaptic space. The experiment showed disruption of communication between astrocytes via Cx43 already at the early stages of epileptogenesis [43]. On the other hand, functioning of the astrocytic network is required for delivery of energy substrates [44], and, therefore, reduced astrocyte communication may inhibit seizure activity and be protective in the later stages of epileptogenesis [45].

Evaluation of Cx43 levels in hyppocampus specimens from HS patients showed increased expression of Cx43, which, however, did not form functional channels [46]. This was related to subcellular Cx43 redistribution in the perivascular endfeet together with post-translational protein modifications that affected channel permeability. We observed similar changes in our study: total content of Cx43 in CA3 increased, and although changes in staining intensity in the *stratum lucidum* were not statistically significant, Cx43 redistribution and formation of Cx43 clusters around the vessels were seen in this layer. Changes in AQP4 and Cx43 expression and localization was previously shown to disrupt BBB permeability [20] and result in negative effects. It is of note that AQP4 and Cx43 changed their localization in different directions, while Cx43 was accumulated around the vessels with AQP4 immunoreactivity decreased. These results demonstrated disturbed organization of water channels and connexins in reactive astrocytes, which, in its turn, may cause disturbances in ionic and water homeostasis [32] and contribute to epileptogenesis.

Conclusion

1. Astrocyte reaction in kainate-induced HS was heterogeneous and had regional differences, with *stratum lucidum* astrocytes associated with mossy fibers demonstrating the greatest vulnerability.
2. Changes in the expression of homeostatic proteins in hippocampal astrocytes were observed in both hemispheres (i.e. ipsilateral and contralateral to the damage), which aggravated epileptogenesis in the intact hippocampus.
3. Changes in astrocytic glutamate metabolism proteins in KA-induced HS enhanced neurotransmission disturbances and may induce secondary excitotoxic damage to neurons during epileptogenesis.
4. Disturbances in the localization and expression of AQP4 and glial glutamate transporters were unidirectional, indicating their common regulatory mechanisms and local dysregulation of water and ion homeostasis by astrocytes in HS.
5. Disturbances in the perivascular localization of Cx43 and AQP4 showed that the two classes of membrane proteins were interdependent with glio-vascular interactions reconfigured in HS.

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Information about the authors

Dmitry N. Voronkov – Cand. Sci. (Med.), senior researcher, Laboratory of neuromorphology, Brain Institute, Research Center of Neurology, Moscow, Russia, https://orcid.org/0000-0001-5222-5322
Anna V. Egorova – Cand. Sci. (Med.), researcher, Neuromorphology laboratory, Brain Institute, Research Center of Neurology, Moscow, Russia; Associate Professor, Department of histology, embryology and cytology, Pirogov Russian National Research Medical University, Moscow, Russia,
https://orcid.org/0000-0001-7112-2556
Evgenia N. Fedorova – research laboratory assistant, Neuromorphology laboratory, Brain Institute, Research Center of Neurology, Moscow, Russia; assistant, Department of histology, embryology and cytology, Pirogov Russian National Research Medical University, Moscow, Russia,
https://orcid.org/0000-0002-2128-9056
Alla V. Stavrovskaya – Cand. Sci. (Biol.), Head, Laboratory of experimental pathology of the nervous system and neuropharmacology, Brain Institute, Research Center of Neurology, Moscow, Russia,
https://orcid.org/0000-0002-8689-0934
Ivan A. Potapov – junior researcher, Laboratory of experimental pathology of the nervous system and neuropharmacology, Brain Institute, Research Center of Neurology, Moscow, Russia, https://orcid.org/0000-0002-7471-3738
Anastasiya K. Pavlova – research laboratory assistant, Laboratory of experimental pathology of the nervous system and neuropharmacology, Brain Institute, Research Center of Neurology, Moscow, Russia,
https://orcid.org/0009-0006-5653-5524
Vladimir S. Sukhorukov – D. Sci. (Med.), Head, Laboratory of neuromorphology, Brain Institute, Research Center of Neurology, Moscow, Russia; Professor, Department of histology, embryology and cytology, Pirogov Russian National Research Medical University, Moscow, Russia,
https://orcid.org/0000-0002-0552-6939

Author contribution. *Sukhorukov V.S., Stavrovskaya A.V., Voronkov D.N.* – study design, methodology development; *Voronkov D.N., Fedorova E.N., Egorova A.V., Potapov I.A., Pavlova A.K.* – conducting the study, curation and analysis of data; *Sukhorukov V.S.* – research management and coordination.

Информация об авторах

Воронков Дмитрий Николаевич – к.м.н., с.н.с. лаб. нейроморфологии Института мозга Научного центра неврологии, Москва, Россия,
https://orcid.org/0000-0001-5222-5322
Егорова Анна Валериевна– к.м.н., н.с. лаб. нейроморфологии Института мозга Научного центра неврологии, Москва, Россия; доцент каф. гистологии, эмбриологии и цитологии РНИМУ им. Н.И. Пирогова, Москва, Россия, https://orcid.org/0000-0001-7112-2556
Федорова Евгения Николаевна – лаборант-исследователь лаб. нейроморфологии Института мозга Научного центра неврологии, Москва, Россия; ассистент каф. гистологии, эмбриологии и цитологии РНИМУ им. Н.И. Пирогова, Москва, Россия,
https://orcid.org/0000-0002-2128-9056
Ставровская Алла Вадимовна – к.б.н., зав. лаб. экспериментальной патологии нервной системы и нейрофармакологии Института мозга Научного центра неврологии, Москва, Россия,
https://orcid.org/0000-0002-8689-0934
Потапов Иван Александрович – м.н.с. лаб. экспериментальной патологии нервной системы и нейрофармакологии Института мозга Научного центра неврологии, Москва, Россия,
https://orcid.org/0000-0002-7471-3738
Павлова Анастасия Кирилловна – лаборант-исследователь лаб. экспериментальной патологии нервной системы и нейрофармакологии Института мозга Научного центра неврологии, Москва, Россия,
https://orcid.org/0009-0006-5653-5524
Сухоруков Владимир Сергеевич – д.м.н., зав. лаб. нейроморфологии Института мозга Научного центра неврологии, Москва, Россия; профессор каф. гистологии, эмбриологии и цитологии РНИМУ им. Н.И. Пирогова, Москва, Россия,
https://orcid.org/0000-0002-0552-6939

Вклад авторов: *Сухоруков В.С., Воронков Д.Н., Ставровская А.В.* – концепция исследования, разработка методологии; *Воронков Д.Н., Федорова Е.Н., Егорова А.В., Потапов И.А., Павлова А.К.* – проведение исследования, курирование и анализ данных; *Сухоруков В.С.* – руководство и координация работ.