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The protein profiles in human brain structures underlying epileptic seizures

Abstract

The article is a pilot study that addresses the problem of molecular basis of epilepsy. The studies were carried out on the platelets, serum of the severe course epileptic patients and on the brain regions (hippocampus, amygdala and temporal cortex) removed from the epileptic patient's during the appointed neurosurgery. The selected patients were subjected to EEG analysis and their epileptic pathology was confirmed by recording of the fusiform patterns of the brain electric activity. Through indirect ELISA test, the levels of Collapsin Response-Mediated Protein 2 (CRMP2), being in linear relations with serotonin, in the platelets and the natural anti-CRMP2 autoantibodies in the serum were evaluated. Both of these indices were significantly downregulated in the epilepsy patients relative to the healthy peers. We extracted the proteins from hippocampus, amygdala and temporal cortex, removed from the patient during neurosurgery, and fractionated them through Isoelectric Focusing (IEF) with application of wide pH range ampholines. The interaction of the IEF protein fractions with serum samples of two other severe course epileptic patients, used as a primary antibody, was analyzed by indirect ELISA test. The upregulation of protein fractions with pI beyond 8.0 was revealed. The author propose that the upregulated protein fractions might belong to epileptogenic proteins whose synthesis is over-expressed on the background of decreased levels of CRMP2 in the epileptic patients' brain structures.

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Keywords: Epilepsy patients; Platelets; Serum; Collapsin response-mediated protein 2 (CRMP2); Natural anti-CRMP2 autoantibodies; Brain regions.

Introduction

Presently, there are multitude of studies concerning molecular basis of epileptic seizures. One of the mostly often-published results obtained in such kind of studies are related to disturbances of serotonin turnover in the brain structures of the epileptic patients or experimental animals with induced epilepsy. In particular, the studies on epileptic patients revealed that decline of serotonin level in the brain structures brings to induction of epileptic seizures [1,2]. Though up-to-date the underlying molecular mechanisms leading to induction of epileptic seizures remain unclear, nevertheless, the downstream of the known molecular events may create a valid background bringing to de-repression of certain genes and consequently to initiation of synthesis of the epileptogenic proteins, that are specifically responsible for triggering epileptic seizures in the engaged brain structures.

According to the results of several postmortem studies undertaken on humans, the level of serotonin, its receptor subtypes, and its synthesizing and degrading enzymes' activities in the platelets are very much similar to these indices in the human brain cortex [3,4]. Hence, analysis of serotonergic system indices in the platelets might give to researchers an opportunity to evaluate their levels in the brain cortex.

As showed the results of experimental studies, the levels of natural autoantibodies, that are present in the serum of healthy individuals without intentional immunization with any antigen, serve for the purpose of homeostasis regulation of all proteins existing in the organism including membranous and nuclear ones, keeping their levels unchangeable. Furthermore, the levels of natural autoantibodies to different proteins in the serum exactly reflect these proteins' levels in the organism tissues [5]. Besides, as showed our earlier studies carried out

on the rat depression model, the levels of natural autoantibody to the Collapsin Response-Mediated Protein 2 (CRMP2) are in direct relationship with these proteins' levels in the brain subcortical structures of the depressive rats [6].

The main goals of the present pilot study were, first, evaluation of the levels of CRMP2, being in linear relations with serotonin [7], in the platelets and the levels of natural anti-CRMP2 autoantibody in the serum of chronic epileptic patients and, second, identification of the protein fractions, whose levels are significantly increased during epileptic seizures in the human brain structures.

Material and methods

CRMP2 was purified from the cow brains. The brains were homogenized in the extracting buffer containing 0.05 M phosphate buffer (pH 7.2), 0.3 M NaCl, 5 mM EDTA and 0.1% Triton X-100 in a volume ratio of tissue to buffer as 1:4. The main stages of fractionations were as follows: 1) protein partial precipitation by ammonium sulfate under the final concentration 40%, 2) gel-chromatography on the column (3×60 cm) of Sephadex G-150, 3) exposure to the effect of 40 mM of deionized EDTA throughout the night on the end-to-end shaker under 4ºC, 4) isoelectric focusing on the gel with application of ampholines of narrow pH range (pH 4-6). After finalization of isoelectric focusing 1 cm width gel strips were collected, pH values were measured in each gel strip and the fraction with pH value that was equal to *pI* value of CRMP2, was eluted from the gel and analyzed in SDS electrophoresis with protein standards [8]. The process of fractionation and selection of the immune-positive protein fractions was realized under the screening control by the indirect ELISA test with application of anti-CRMP2 polyclonal immunoglobulins [8].

Anti-CRMP2 polyclonal immunoglobulins were produced through 5-6-month immunization of the male Chinchilla rabbits of 2.2-2.5 kg body mass by sub-cutaneous administration of 300 µg of the purified protein per animal, always in a mixture with complete Freund adjuvant (Sigma, Germany). The first three injections were done within a timeframe of 14 days; the following injections were done one per month. Ten days after the third and following injections blood samples were taken from the ear vein, the serum was separated and polyclonal immunoglobulins G were precipitated by adding 100% ammonium sulfate to the equal volume of serum (final concentration 50% of ammonium sulfate).

The first series of experiments were carried out on the patients with severe course of epilepsy with frequent manifestations of epileptic seizures. All the selected patients (n=21) were subjected to EEG analysis and their epileptic pathology was confirmed by recording of fusiform patterns of their brain activity. The healthy volunteers-peers (n=15) were used as controls. The blood samples were taken from their veins in a seizure-free timeframe into the 5-mL sample tubes, containing 5% EDTA solution as anticoagulant, at a ratio of 1:4 of EDTA solution to blood sample (v/v). The blood samples were centrifuged under 800 rcf for 10 min, supernatant (plasma) was saved and centrifuged under 9000 rcf for 15 min. The supernatant fractions were saved as serum, whereas pellets were saved and used as platelets. The serum samples after dilution by the antibody's buffer (0.04 M phosphate buffer, 0.15 M NaCl, 0.5 mg/mL Tween-20 and 10 mg/mL, pH 7.2), at a ratio 1:75 (v/v) were used as primary antibody in the solid-phase indirect ELISA test on 96-well polysterene plates with moderate adsorption (Sigma, Germany), to evaluate the levels of natural anti-CRMP2 autoantibody. In this reaction the purified CRMP2 [8] was used as an antigen at a concentration of 20 µg/mL (Bradford method) in 0.1 M Tris-HCl buffer (pH 8.6). Each sample was repeated three times (three wells for each sample). Mouse anti-human IgG with conjugated horseradish peroxidase were used as the secondary antibody at a dilution rate of 1:1000 in the antibody buffer. Orthophenylendiamine at a concentration of 0.5 mg/mL in 0.05 M citrate-phosphate buffer (pH 4.5) was used as a substrate for peroxidase for reaction results visualization. The reaction was stopped by addition of 50 µL of 3 M NaOH into each well 30 min after substrate addition and digital recordings of the reaction the plates were obtained into the photometer for ELISA-test "Spectra Max 250" (Molecular Devices Co., USA) at the wavelength 492 nm (wavelength of comparison 630 nm).

In the second series of experiments, the water-soluble proteins were extracted from the platelets and used as the antigens in indirect ELISA test at a concentration of 20 µg/mL in 0.1 M Tris-HCl buffer (pH 8.6). The polyclonal rabbit anti-CRMP2 immunoglobulins were used as a primary antibody in the antibody buffer (pH 7.2) diluted at a ratio of 1:40. The goat anti-rabbit immunoglobulins with conjugated horseradish peroxidase were used as a secondary antibody at a dilution ratio of 1:20000 in the antibody buffer (pH 7.2). The following steps of the reaction were fulfilled as described above.

In the third series of experiments, the brain regions including hippocampus, amygdala and temporal cortex were removed from one epileptic patient's brain because of the appointed neurosurgery as a therapeutic measure and thereafter frozen under -70°C. Then, the water-soluble proteins were extracted from each of the removed brain structure in the extraction buffer, dialyzed twice against 300 volumes of distilled water and fractioned by isoelectric focusing technique with application of ampholines within the pH range 3-10 (Pharmacia, Sweeden) in the Ultragel (Pharmacia, Sweeden), for 6-7 h, under stable power regime of 8 W, until electric current stabilization at low values. After isoelectric focusing finalization, we collected the parallel gel strips of 1 cm width and put them into the sample tubes, containing distilled water. Basing on pH measures of the collected gel strips, the pH gradient was plotted and the protein fractions were eluted from each gel strip and used as antigens levelled to 20 µg/mL with 0.1 M Tris-HCl buffer (pH 8.6) in the indirect ELISA test. The serums of the two epileptic patients that demonstrated the lowest values of natural anti-CRMP2 antibodies among all studied patients of the first series (the most severe epilepsy manifestation), were used as the primary antibody diluted at a ratio 1:50 with the antibody buffer. The following stages of the reaction were similar to the reaction of the first series of experiments.

The results of the experiments were analyzed by calculation of the variants mean values and the mean value errors of each group (M±S.D.) and through comparison of intergroup differences basing on Student's *t*-criterion and Wilcoxon-Mann-Whitney *U*-criterion. Prior to application of Student's *t*-criterion for statistical analysis of the results, we checked samples of all the studied groups for their suitability to normal distribution and the samples' values responded to the main requirements of normality criterion – they did not exceed 3 σ value [9].

Results

EEG analysis of the selected epileptic patients demonstrated registered epileptiform activity (fusiform pattern) in different

recordings from their skull (Figure 1). Hence, we have got to our disposal reliable electrographic conformation of epileptic activity in their brains.

In the first series of experiments, the analysis of the level of natural anti-CRMP2 autoantibodies in the serum of the epileptic patients in the seizure-free timeframe revealed its drastic decrease relative to the healthy persons of the same age. In particular, in the healthy persons their level was equal to 0.020±0.001 Optical Units of Extinction (OUE), while in the epileptic patients their level declined 2.5 times and reached 0.008±0.001 OUE (*p*<0.001; Figure 2).

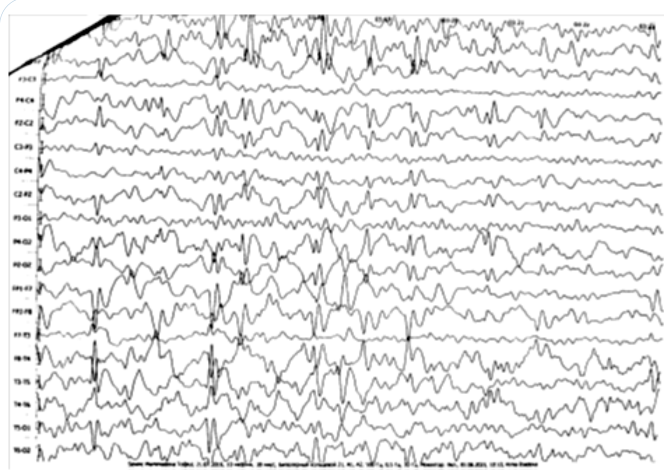

Figure 1: EEG registration of the analyzed epileptic patient in a seizure-free timeframe.

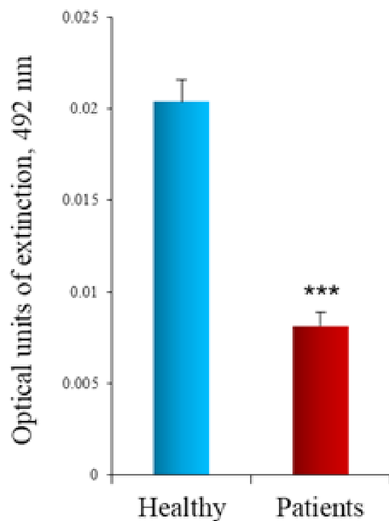

Figure 2: Changes of the level of natural anti-CRMP2 autoantibody in the serum of the epileptic patients (n=21), used as primary antibody in indirect ELISA test, in a seizure-free timeframe.
*** - *p*<0.001.

In the second series of experiments, the downregulation of CRMP2 in the platelets of epilepsy patients relative to healthy persons was observed. In particular, the level of CRMP2 in the platelets of epileptic patients was equal to 0.242±0.002 OUE, while its level in the healthy persons was equal to 0.256±0.002 OUE (*p*<0.001; Figure 3).

In the third series of experiments, we analyzed immunochemical reaction of the isoelectric focusing-fractionated samples of the hippocampus, amygdala and temporal cortex removed from the epileptic patient's brain with the serum samples (separately) of the two other epileptic patients. The serum-sampled patients demonstrated their therapeutic tolerance to administration of two and more kinds of anti-epileptic drugs and had the lowest values of natural anti-CRMP2 antibodies in their serum revealed by indirect ELISA test in the first series of experiments (the severest manifestations of epilepsy). The pH gradients at the end of isoelectric focusing of three brain areas fractionations bore plain configuration, reflecting their good deployment within all used pH intervals (Figure 4).

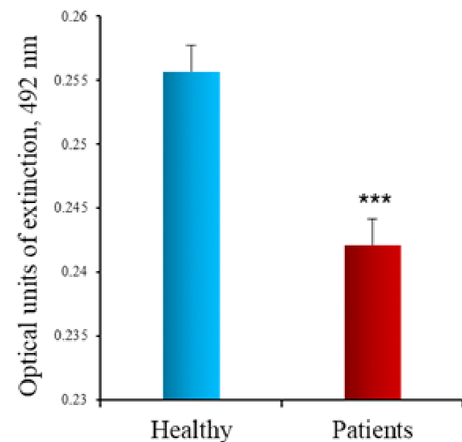

Figure 3: Changes of the level of CRMP2 in the platelets of the epileptic patients (n=21), whose extracts were used as antigens in indirect ELISA test, in a seizure-free timeframe.
*** - *p*<0.001 on Student's *t*-criterion and ** - *p*<0.01 on Wilcoxon-Mann-Whitney *U*-criterion.

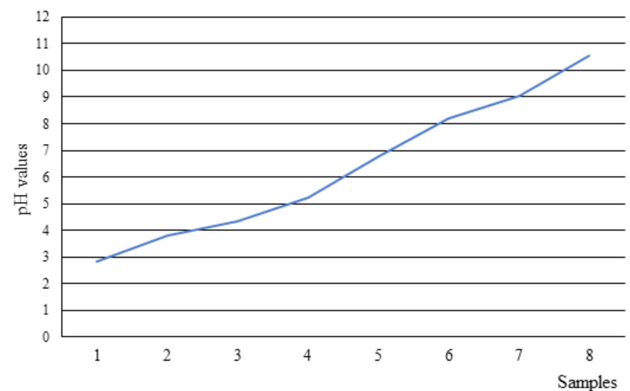

Figure 4: pH gradient deployed in the gel strips after isoelectric focusing of the human hippocampus protein extract.

The results of indirect ELISA test revealed high similarity of the protein profiles' reaction of the each studied brain structure throughout the whole pH gradient range with the two serum samples taken from the two different patients. At the same time, we revealed drastic differences in the character of the protein profiles' immunochemical reactions throughout the whole pH gradient range between three studied brain structures (Figures 5-7).

Such similar character of differences between three studied brain structures we observed repeatedly with application of two patients' serum samples used as a primary antibody in the indirect ELISA test. However, comparison of the results of protein profiles' reactions of all the studied brain structures revealed common patterns for all of them. In particular, noticeable upregulation of the alkaline protein fractions with *pI* close to and beyond 8.0 was observed. Furthermore, prominent downregulation of the protein fractions having *pI* near 5.3 region was revealed in the isoelectric focusing-protein profiling of the hippocampus.

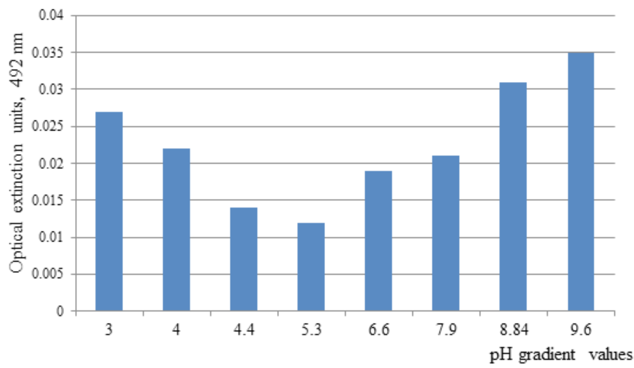

Figure 5: Optical extinction values of autoantibody from the serum of severe epileptic patients (n=2) after their binding to isoelectric focusing-protein fractions of the epileptic patient's hippocampus.

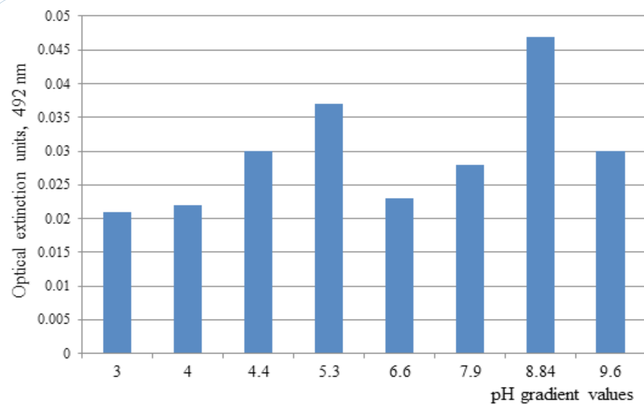

Figure 6: Optical extinction values of autoantibody from the serum of severe epileptic patients (n=2) after their binding to isoelectric focusing-protein fractions of the epileptic patient's amygdala.

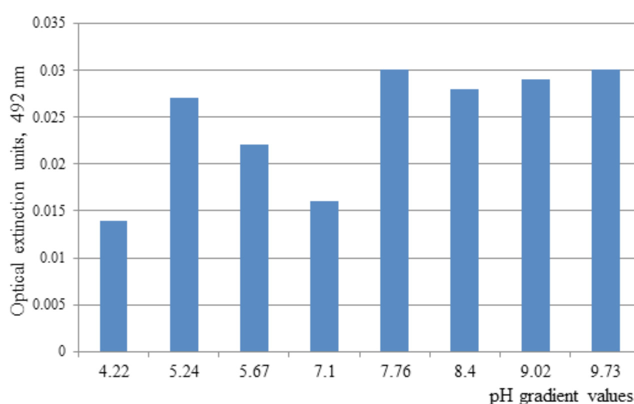

Figure 7: Optical extinction values of autoantibody from the serum of severe epileptic patients (n=2) after their binding to isoelectric focusing-protein fractions of the epileptic patient's temporal cortex.

Discussion

As the studied patients had frequent epileptic seizures incidence and valid electrographic confirmation (fusiform activity) of epilepsy, they could be qualified as chronic epileptic patients with persistent epileptiform activity in their brain structures. Basing on that, the indirect ELISA test evaluation of the levels of natural anti-CRMP2 autoantibody in the patients' serum, as revealed our earlier studies on the rat depression model [5], apparently, might have direct correlation with the CRMP2 level in their subcortical structures on the background of continuous course of epilepsy. Hence, as the mean level of natural anti-CRMP2 autoantibody in the chronic epilepsy patients' serum was 2.5 times lower than in the healthy persons' serum, then such drastic decline of CRMP2 level is as well supposed to be present in the patients' subcortical structures. The revealed high correlation between the level of natural anti-CRMP2 autoantibody in the serum and its level in the brain subcortical structures might be the result of increased permeability of Blood-Brain Barrier (BBB) in the region of these brain structures allowing passing the brain cells proteins to the blood circulation and following induction of immune response to them [10]. It should be noted that such subcortical structures as hypothalamus, area postrema, etc. are deprived completely of BBB and the most dyes administered into the animal's vein easily pass through BBB [10]. Indicated noticeable differences in BBB permeability, apparently, is related to significant heterogeneity of endothelial cells alignment in different brain structures, especially in the mentioned brain regions [11]. As CRMP2, apart from its well-known role in stimulation of axon elongation and nerve cells precursors' migration, participates actively in vesicular trafficking and autophagy aimed to removal of worked out cellular and molecular debris from the nerve cells [12], its intracellular level should be rather high in the functioning neurons allowing its easy release into intercellular space.

Though organism's immune system is involved in realization of defensive response against pathogenic microorganisms and alien proteins through humoral (synthesis of specific antibody by B-lymphocytes) and cellular immunity activation leading to development of inflammation processes in tissues, this immunological response bears highly specific character towards target antigens and in most cases does not show cross-reactivity with organism's host antigens. For this reason, the declined level of natural anti-CRMP2 autoantibody in the serum of epilepsy patients cannot be the result of inflammation or other accompanying pathological process. If, by any reason, inflammation process with involvement of mighty anti-CRMP2 production occurs in the patient's organism, it would surely bring, in opposite, to sharp elevation, but not decline of the anti-CRMP2 antibody level. Different strength of the studied patients' individual immunological system reactivity as well did not have significant impact on the anti-CRMP2 autoantibody production, because in all the studied epilepsy patients unidirectional and low scattering decline of its level was observed.

As the level of serotonergic sytem activity in the human platelets directly reflects the level of serotonergic system activity in the brain cortex [3,4], the decreased level of serotonergic CRMP2 in the platelets of chronic epleptic patients relative to healthy peers means similar decrease of CRMP2 level in their brain cortex cells.

The similarity within each studied brain structure of the results of indirect ELISA test patterns of the isoelectric focusing-fractionated samples of the epileptic patient's brain with the serum samples of the other two severe-course epileptic patients used as a primary antibody allows to propose an engagement character of the same antigens, revealed through their level changes, within each of the brain structure into epileptogenic process. Hence, these data obtained through application of two patients' serum, make grounds to putting forward a conjecture about commonness of the brain protein profiles within each brain structure in the epileptic patients. At the same time,

the differences of the protein profiles reactions between the studied brain structures of the epileptic patients might reflect the different character of involvement of their composing antigens into the pathological process and, correspondently, the different degree of their expression within these brain structures in the epileptic patients.

The noticeable increase of the levels of protein fractions with *pI* values beyond 8.0 in each of the studied brain structures of the epileptic patient, apparently, indicates their engagement in triggering the epileptic activity. Indeed, there are evidences in the literature confirming these results by demonstrating upregulation of the protein fractions with these *pI* values in postmortem studies of the epileptic patients' brains [13]. In addition, noticeable decrease of the levels of proteins having *pI* values close to *pI* value of CRMP2 – 5.95 – in the hippocampus and amygdala protein profiles once more confirms the CRMP2 prominent downregulation in these brain subcortical structures of the epileptic patients, which we observed in the first experimental series while evaluating the levels of natural anti-CRMP2 autoantibody in the patients' serum.

Conclusion

We propose the existence of the following downstream of consecutive molecular events in the brain structures of epileptic patients: noticeable downregulation of CRMP2 in the nerve cells of the hippocampus and amygdala brings to de-repression of epileptogenic genes triggering upregulated expression of the putative epilepsy-associated protein fractions with *pI* values beyond 8.0 in these subcortical structures. Indeed, in our earlier studies, we found the existence of such way of negative regulation of protein expression realized by CRMP2, when intracerebral administration to the rats of the polyclonal anti-CRMP2 antibody, leading to the prolonged blockade of CRMP2 activity, brought to the noticeable upregulation of nerve growth factor in their hippocampus and left parietal cortex [14]. Hence, the putative epilepsy-associated protein fractions might be upregulated in the same way on the background of decreased level of CRMP2 in the brain structures of the epileptic patients. At the same time, the epileptogenic nature of the said proteins should be proven in further studies with analysis of their capability on initiation of epileptic seizures in the model experiments on the animals.

Human and animal rights policy: The authors declare that all experiments on human subjects were conducted in accordance with the Declaration of Helsinki https://www.wma.net/policies-post/wma-declaration-of-helsinki-ethical-principles-for-medical-research-involving-human-subjects/ and that all procedures were carried out with the adequate understanding and written consent of the subjects.

The study protocol was approved by the Institutional Review Board (IRB) of the Academician Abdulla Garayev Institute of Physiology (Protocol No. 1. Dated 10 February, 2023).

Author declaration: The authors declare that they do not have any competing interests regarding intellectual property of the data used in the article.

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