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INTERRELATIONS BETWEEN EPILEPTIC SEIZURES AND BRAIN NEURONS' BACK REMODELING: CLINICAL AND EXPERIMENTAL STUDY

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The article concerns the problem of interrelationship between epileptic activity and brain neurons' back remodeling process. The studies were carried out on chronic epileptic patients of pre-puberty period, of both sexes. Healthy persons of the same age served as a control group. The blood samples were taken from the patients in free-of-seizure timeframe and platelets and serum were saved. The levels of collapsin-response mediator protein 2 (CRMP2) and βIII tubulin were evaluated in the patients' platelets and the levels of natural anti-CRMP2 autoantibodies and nerve growth factor (NGF) were evaluated in the patients' serum with indirect ELISA test. Downregulation of CRMP2 and βIII tubulin in the platelets, sharp downregulation of natural anti-CRMP2 autoantibodies and upregulation of NGF in the serum was revealed. In the next series we studied the effect of intra-cerebral administration of CRMP2 to stress-tolerant Wistar male rats on their tolerance to the audiogenic stress effect (90-120 dB, 3 min). CRMP2 administration to the rats did not induce epileptic seizures under their exposure to audiogenic stress. The results give grounds to coming to a conclusion about engagement of brain neurons' back remodeling in epileptic activity and that epileptic activity itself brings to induction of neurons' back remodeling in the epileptogenesis-engaged neurons.

Keywords: *epileptic patients, platelets, serum, collapsin-response mediator protein 2 (CRMP2), natural anti-CRMP2 autoantibodies, βIII tubulin, nerve growth factor, ELISA test*

To-date although plenty of studies has already been undertaken to clarify the underlying mechanisms of epileptic seizures, they are still left unclear. One of the most arguable questions is the cause-effect relations between newly formed neurons (neurogenesis) and initiation of epileptic seizures. In particular, it is unclear, whether epilepsy itself brings to induction of adult brain neurogenesis or, conversely, adult neurogenesis comprises relevant conditions for the development of epileptic seizures. The scientists standing on opposite standpoints regarding this question refer to quite different experimental arguments supporting their positions [1]. Surely, these debates can be judged and final conclusion can be made only basing on convincing and unequivocal experimental data on the subject.

The goal of the present study was analysis of the levels of collapsin-response mediator protein 2 (CRMP2), involved in regulation of axonal sprouting and long-term memory formation [5, 7], and marker of mature neurons βIII tubulin [3] in the platelets of epileptic patients having this pathology for a long time, in free-of-seizure timeframe, the level of anti-CRMP2 autoantibodies and nerve growth factor (NGF) in their serum. Besides, we studied the effect of intracerebral administration of anti-CRMP2 antibodies into the Wistar rats on their tolerance to exposure to audiogenic stress that induces epileptic seizures in the stress-intolerant rats.

Materials and Methods

1.Biochemical methods

The studies of the first series were carried out on the patients having epilepsy of traumatic origin for a long time (n=20). Healthy persons of the same age were used as controls (n=15). All the patients were of pre-puberty period, of both sexes and their EEGs were permanently recorded and analyzed. The blood sampling of the patients were conducted in free-of-seizure timeframe at the moment of their regular blood

sampling in polyclinic conditions. The blood samples were collected into sample tubes containing 5% solution of EDTA as anticoagulant in v/v ratio of blood to EDTA solution as 9:1. Thereafter blood samples were centrifuged at 600 RCF for 7 min, plasma was saved and centrifuged at 9000 RCF for 15 min for precipitating platelets. Platelets and serum samples were saved in separate Eppendorf sample tubes and analyzed in indirect ELISA test on 96-well polystyrene plates with moderate level of adsorption (Sigma, Germany). The water-soluble proteins were extracted from the platelets by extraction buffer, leveled to a concentration 20 μg/mL with 0.1 M Tris-HCl buffer (pH 8.6) and used as antigens in indirect ELISA test. Rabbit anti-CRMP2 and rabbit anti-βIII tubulin polyclonal immunoglobulins were used as the primary antibodies in the buffer for antibodies (pH 7.3). Anti-rabbit goat immunoglobulins conjugated to horseradish peroxidase (Sigma Immunochemicals, Germany) were used as the secondary antibodies in the buffer for antibodies (pH 7.3). Orthophenylendiamine was used as a substrate for peroxidase at a concentration of 0.5 mg/mL in 0.05 M citrate-phosphate buffer (pH 4.5). The reaction was stopped by adding 3 M NaOH in the wells 30 min after pouring substrate buffer. The results of the reactions were recorded in photometer for ELISA test "Molecular Devices Spectra Max 250" (MTX Lab Systems, Inc., USA) on wavelength 492 nm (relative wavelength 630 nm).

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 and 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 X 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 ending 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 [4]. 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 [4].

Anti-CRMP2 polyclonal immunoglobulins were produced through 5-6-month immunization of the male rabbits of Chincilla species 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 ear vein, serum was separated and polyclonal immunoglobulins G were precipitated by adding 100% ammonium sulfate to equal volume of serum (final concentration 50% of ammonium sulfate).

Patients' serum was used for evaluation of the level of natural anti-CRMP2 autoantibodies in indirect ELISA test. In this series of studies, purified CRMP2 was added into the polystyrene plates with moderate level of adsorption (Sigma, Germany) at concentration 20 μg/mL. The serum samples were diluted with buffer for antibodies (pH 7.3) in v/v ratio 1:75 and used as the first antibodies. Mice anti-human immunoglobulins conjugated to horseradish peroxidase were used as the second antibodies.

Besides, we defined the level of nerve growth factor (NGF) in the epileptic patients' serum in indirect ELISA test. For this purpose the serum samples were used as antigens at a concentration 20 μg/mL in 0.1 M Tris-HCl buffer (pH 7.3) and rabbit polyclonal anti-NGF antibodies were used as the primary antibodies (2.5 S; Sigma Immunochemicals, Germany). Further steps of indirect ELISA test were realized as described above.

Anti-CRMP2 polyclonal antibody was purified from the solution of anti-CRMP2 immunoglobulins through a technique of immune-affinity chromatography carried out on the column (0.5 x 3 cm) of CNBr-Sepharose (Sigma, Germany) with covalently immobilized CRMP2 [2]. After application of anti- CRMP2 immunoglobulins onto the column at a low speed of 8 mL/h, the column was thoroughly washed with 20 column volumes of 0.01 M phosphate buffer (pH 7.2) and under the control of spectrophotometric extinction at the wavelength 595 nm (Bradford method for measurement of protein concentration) specifically bound anti-CRMP2 antibody was eluted from the column through application of a chaotropic agent – 3 M KCNS. The eluted antibody was dialyzed against 0.15 M NaCl, buffered to the value of pH 7.2, and frozen. In a single cycle, up to 6 mg of antibody was eluted from the affinity column.

2. Physiological Methods

The experiments were carried out on epileptic patients (n=20) and on the Wistar male rats of 180-230 g body mass (n=20).

The epileptic patients were subjected to EEG recording on electroencephalograph "Neuron-Spector-2" (Russia) with traditional "10-20" system of application of registration electrodes on the human skull.

Audiogenic stress was formed by exposure the rats to a strong sound of 90-120 dB for 3 min in a box (30 x 40 x 30 cm) made from organic glass. In this series of studies, the animals tolerant to audiogenic stress were selected and divided into 2 groups: 1) control group (n=10) – administration of rabbit non-immune γ-globulins; 2) experimental group (n=10) – administration of polyclonal rabbit anti-CRMP2 antibodies. Preparations were administered under animal's anesthesia with sodium etaminali (40 mg per 1 kg of body mass) at a dose of 1 mg/mL, in a volume of 10 μL in buffered saline (pH 7.3) into left lateral brain ventricle.

The results were averaged within each group under study and analyzed with application of Student's t-criterion [8].

Results

EEG analysis of the selected epileptic patients demonstrated existence of epileptiform activity in different recordings from their skull (Fig. 1). Hence, we have got reliable electrographic conformation of epileptic activity in their brains.

Analysis of the level of CRMP2 in the platelets and the level of natural anti-CRMP2 autoantibodies in the serum of the epileptic patients relative to healthy persons of the same age revealed significant downregulation of these indices. In particular, the level of CRMP2 in the platelets of epileptic patients was equal to 0.242±0.002 optical units of extinction (OUE), while its level in the healthy persona was equal to 0.256±0.002 OUE ($p < 0.001$; Fig. 2). The sharpest decrease was noted in the level of natural anti-CRMP2 autoantibodies in the serum of the epileptic patients relative to the healthy persons. In particular, while in the healthy persons their level was equal to 0.020±0.001 OUE, in epileptic patients their level downregulated 2.5 times and reached 0.008±0.001 OUE ($p < 0.001$; Fig. 3).

Evaluation of the neural differentiation marker βIII tubulin in the platelets of the epileptic patients revealed its noticeable downregulation. In particular, its level in the platelets of the healthy persons was 0.014±0.001 OUE, whereas in the epileptic patients this index was equal to 0.008±0.001 OUE ($p < 0.001$; Fig. 4).

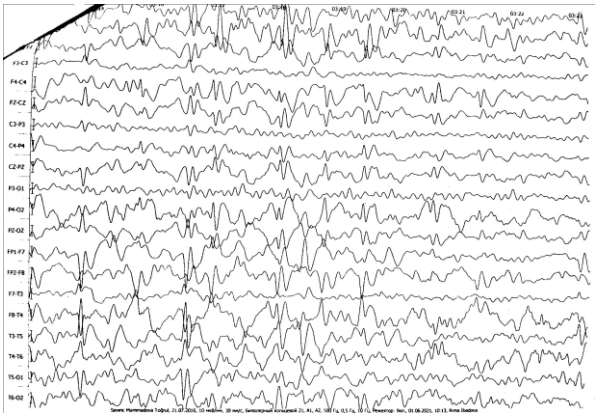

Fig. 1. EEG registration of the epileptic patient in free-of-seizure timeframe

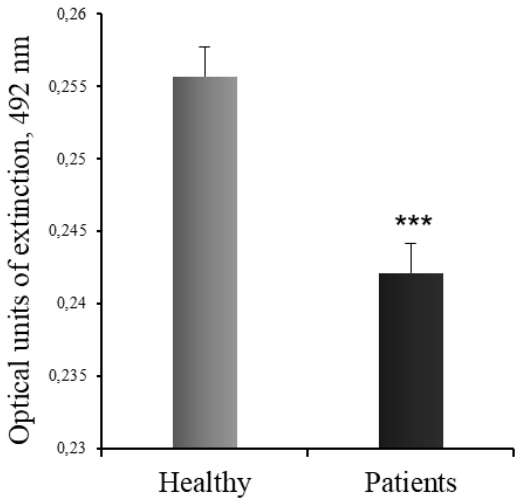

Fig. 2. Changes of the level of CRMP2 in the platelets of the epileptic patients. ** - $p < 0.001$

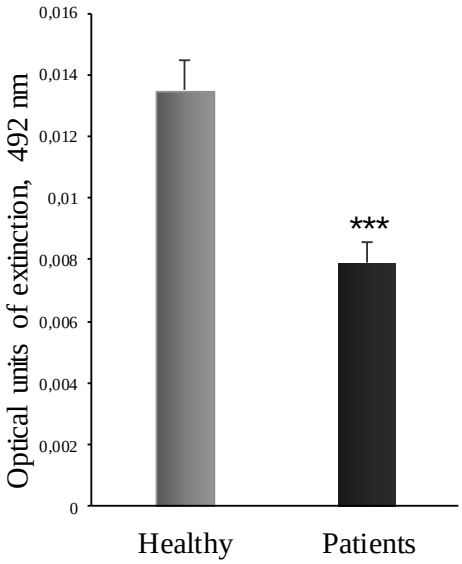

Fig. 4. Changes of the level of βIII tubulin in the platelets of the epileptic patients. ** - $p < 0.001$

Analysis of the level of NGF in the serum of the epileptic patients in opposite to other indices measured in the blood samples of these patients showed its upregulation relative to its level in the healthy persons. In particular, if its level in the serum of the healthy persons was equal to 0.037±0.003 OUE, in the serum of the epileptic patients its level made 0.051±0.003 OUE ($p < 0.01$; Fig. 5).

In the next series of studies we selected the audiogen stress-tolerant rats and administered intra-cerebrally anti-CRMP2 antibody to them. 24 h later from anti-CRMP2 antibody administration they were again subjected to the effect of 3-minute audiogenic stress. The results of the experiments showed to remaining tolerance in these animals towards audiogenic stressful effects.

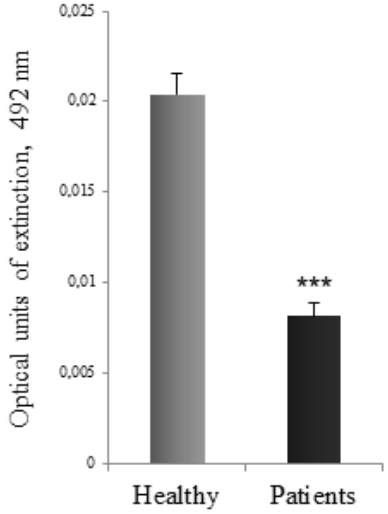

Fig.3. Changes of the level of natural anti-CRMP2 autoantibodies in the serum of the epileptic patients. *** - $p < 0.001$

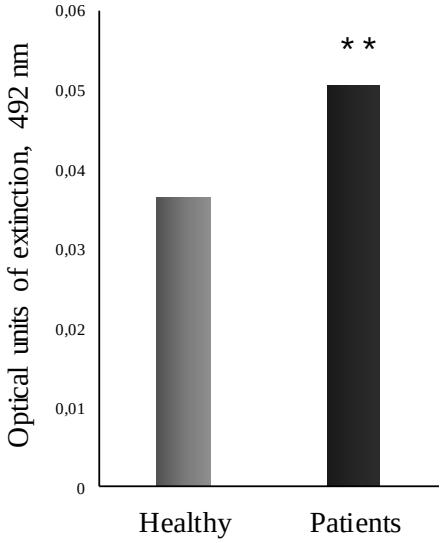

Fig. 5. Changes of the level of nerve growth factor in the serum of the epileptic patients.
*** - $p < 0.01$

Discussion

While analyzing the experimental results, downregulation of CRMP2 in the platelets and sharp downregulation of the natural anti-CRMP2 autoantibodies in the serum of the epileptic patients was noted. As demonstrated the results of different researchers, changes of serotonergic system activity in the platelets reflects the same character changes occurring in the brain cortex of the organism. Hence, the changes of the level of CRMP2 in the patients' platelets reflect similar changes in their brain cortex. Furthermore, our earlier experimental results showed that the level of natural anti-CRMP2 autoantibodies in the serum of the depressed rats perfectly corresponds to the level of CRMP2 in their subcortical structures of the brain [6]. So, it means that downregulation of the natural anti-CRMP2 autoantibodies in the serum of the epileptic patients indicates to correspondent downregulation of CRMP2 in their subcortical structures such as hippocampus and amygdala, which are closely related to initiation of epileptic activity in the patients' brains.

Along with it, significant decrease of the neuronal differentiation marker – βIII tubulin – in the platelets and upregulation of NGF in the serum of the epileptic patients was revealed. Some researchers attribute to upregulation of NGF in neurons as an indicator of neurons' back remodeling [9]. The observed directions of changes of the levels of βIII tubulin and NGF give grounds to making a conjecture concerning engagement of the process of neuronal back remodeling in prolonged epileptogenesis. During the process of back remodeling neurons undergo dedifferentiation process, losing their differentiation markers and getting back to neuron's precursor state. From such secondarily formed neuron's precursor state, the newly dedifferentiated neurons can again pass through differentiation process tuning it up to the challenges of epilepsy-disturbed neuronal environmental signals.

Significant downregulation of neuronal differentiation marker βIII tubulin in the platelets of the epileptic patients reflects its engagement and, speaking more broadly, engagement of the process of neuron's back remodeling in epileptogenesis. Again, cause-effect question is sharply raised: what is the first, triggering event in these relationships. Does neuron's back remodeling create adequate conditions to induction of epileptic seizures or, conversely, do epileptic seizures themselves bring to neuron's back remodeling? Our last experimental series was undertaken for finding an answer to this question.

As showed our earlier experimental data (Mekhtiev, *unpublished data*), 3 days later from rabbit anti-CRMP2 antibodies intra-cerebral administration to the Wistar male rats, prominent downregulation of the neuronal differentiation marker – βIII tubulin, apparently, indicating to neuron's back remodeling, was observed in their hippocampus. Bearing in mind such effect of anti-CRMP2 antibodies and the exact timeframe between antibodies administration and sampling hippocampus, we tried to reveal the role for downregulation of βIII tubulin in development of epileptic seizures. However, intracerebral administration of anti-CRMP2 antibodies to the audiogen stress-tolerant Wistar male rats did not bring to induction of epileptic seizures during their exposure to 3-min audiogen stress 3 days after antibodies administration. Thus, this result demonstrates that neuron's back remodeling does underlie the molecular mechanism of epileptic seizure initiation. It appears, that opposite cause-effect relationship is probable and epileptic activity and following correspondent changes of neurochemical background of the neurons involved into epileptic seizures are capable to induce their back remodeling.

The results of the study indicate that chronic epilepsy brings to decline of the level of CRMP2 in the patients' platelets, to promi-

nent decline of the level of natural anti-CRMP2 autoantibodies in their serum, to prominent decline of the level of βIII tubulin in their platelets and to upregulation of NGF in their serum. Downregulation of βIII tubulin in the platelets and upregulation of NGF in the serum, probably, indicates to epileptic seizures-engaged neurons' back remodeling. Experiments on the rats administered intra-cerebrally with CRMP2 and subjected later to audiogenic stress give grounds to state primary role for epileptic activity in induction of neurons' back remodeling.

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EPİLEPTİK QICOLMALARIN VƏ BEYİN NEYRONLARININ GERİ REMODELLƏŞMƏSİNİN QARŞILIQLI ƏLAQƏSİ: KLİNİK VƏ EKSPERİMENTAL TƏDQİQAT

R.H. İbadova, A.A. Mehdiyev

Bu məqalə epileptik aktivlik və beyin neyronlarının geri remodelləşmə prosesi arasındakı qarşılıqlı əlaqə problemini öyrənir. Tədqiqata pre-pubertə yaşında olan, hər iki cinsə mənsub xroniki epilepsiyalı pasientlər daxil edilmişdir. Eyni yaşda olan sağlam şəxslər kontrol qrup yerinə istifadə olunub. Pasientlərdən qan nümunələri qıcolmaarası dönəmdə götürülərək trombositlər və serum ayrılmışdır. Dolayı immuno-enzim analizi üsulu ilə pasientlərin trombositlərində collapsin-response mediator protein 2-nə (CRMP2) və βIII tubulinə, zərdablarında isə təbii CRMP2 qarşı autoanticisimlər və sinir böyümə faktoru (SBF) səviyyəsinə baxılmışdır. Trombositlərdə CRMP2 və βIII tubulin səviyyəsinin azalması, zərdabda isə təbii CRMP2 qarşı autoanticisimlər səviyyəsinin ciddi azalması və SBF-nin səviyyəsinin artması müəyyən olunmuşdur. Növbəti mərhələdə CRMP2-nin beyindaxili yeridilməsinin təsirinin öyrənilməsi üçün stressə dözümlü Vistar erkək siçovulları seçilmiş və audiogenik stress effektinə dözümlülüyü öyrənilmişdir (90-120 db, 3 dəq). Siçovullarda CRMP2-nin tətbiqi zamanı audiogenik stressə məruziyyət sırasında epileptik qıcolma yaranmamışdır. Nəticələrə əsasən, epileptik aktivlik zamanı beyin neyronlarının geri remodelləşməsinin baş verməsi və eyni zamanda epileptik aktivliyin özünün epileptogenezə qoşulmuş neyronların remodelləşməsinin səbəb ola biləcəyi söylənilə bilər.

Açar sözləri: *epileptik pasientlər, trombositlər, qanın zərdabı, collapsin-response mediator protein 2 (CRMP2), CRMP2-nə qarşı təbii autoanticisimlər, βIII tubulin, sinir böyümə faktoru, immuno-enzim analizi*

ВЗАИМОСВЯЗЬ МЕЖДУ ЭПИЛЕПТИЧЕСКИМИ СУДОРОГАМИ И ОБРАТНЫМ РЕМОДЕЛИРОВАНИЕМ НЕЙРОНОВ ГОЛОВНОГО МОЗГА: КЛИНИЧЕСКОЕ И ЭКСПЕРИМЕНТАЛЬНОЕ ИССЛЕДОВАНИЕ

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Статья посвящена вопросу взаимосвязи между эпилептической активностью и процессом обратного ремоделирования нейронов головного мозга. Исследования были выполнены на больных хронической эпилепсией препубертатного возраста и обоего пола. В качестве контрольной группы использовали здоровых лиц того же возраста. Пробы крови, из которой выделяли тромбоциты и сыворотку, забирали у больных в интервале времени между судорогами. Методом непрямого иммуноферментного анализа определяли уровни collapsin-response mediator protein 2 (CRMP2) и βIII тубулина в тромбоцитах и уровни естественных аутоантител к CRMP2 и фактора роста нервов (ФРН) в пробах сыворотки больных. Было выявлено снижение уровня CRMP2 и βIII тубулина в тромбоцитах, а также резкое снижение уровня естественных аутоантител к CRMP2 и увеличение уровня ФРН в пробах сыворотки. В следующей серии исследовали влияние внутримозгового введения CRMP2 стресс-устойчивым крысам-самцам линии Вистар на устойчивость к воздействию аудиогенного стресса (90-120 дБ, 3 мин). Введение CRMP2 крысам не вызывало возникновения эпилептических судорог под влиянием аудиогенного стресса. Полученные результаты позволяют прийти к заключению о задействованности процесса обратного ремоделирования нейронов головного мозга в эпилептической активности и о том, что сама эпилептическая активность приводит к индукции обратного ремоделирования в вовлечённых в эпилептогенез нейронах.

Ключевые слова: *эпилептические больные, тромбоциты, сыворотка крови, collapsin-response mediator protein 2 (CRMP2), естественные аутоантитела к CRMP2, βIII тубулин, фактор роста нервов, иммуноферментный анализ*