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ON THE ROLE OF ATP-DEPENDENT POTASSIUM CHANNEL Kir6.2 AND HYPOXIA-INDUCED FACTOR-1α IN THE PENTYLENETETRAZOLE KINDLING PATHOGENESIS

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The purpose of the study was the immunohistochemical examination of the expression of the ATP-sensitive potassium (K^+) channel Kir6.2 and hypoxia-inducible factor 1α in the dorsal hippocampus in kindled rats. Kindling was produced in 19 rats by the three-week pentylenetetrazole administration. The avidin-biotin-peroxidase method was used in 10 control rats for staining. The rest 10 rats were composed of the negative control and stained using only secondary antibodies. The colour intensity of the brain sections of the control and kindling groups was compared with one of the negative control. In rats with pentylenetetrazole kindling, the level of Kir6.2 was 18.12±0.98 relative units and exceeded the corresponding data in control (7.53±0.72 relative units), ($p<0.001$). The expression of hypoxia-inducible factor 1α was 17.62±0.90 against 9.77±1.10 relative units in control ($p<0.001$). Kir6.2 and hypoxia-inducible factor 1α levels in limbic structures can be used as markers of the effectiveness of experimental treatment methods for chronic epilepsy.

Key words: seizures, potassium channels, hypoxia, pentylenetetrazol, hippocamp.

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ДО РОЛІ АТФ-ЗАЛЕЖНОГО КАЛІЄВОГО КАНАЛА Kir6.2 ТА ГІПОКСІЯ-ІНДУКОВАНОГО ФАКТОРА-1αВ ПАТОГЕНЕЗІ ПЕНТИЛЕНЕТЕТРАЗОЛОВОГО КІНДЛІНГА

Метою роботи було імуногістохімічне дослідження експресії АТФ-чутливого калієвого (K^+) каналу Kir6.2 та гіпоксія-індукованого фактора 1α у дорзальному гіпокампі у кіндлінгових щурів. Кіндлінг відтворювали у 19 щурів шляхом тритижневого введення пентиленететразолу. У 10 щурів групи контролю для забарвлення зрізів мозку застосовували авідин-біотин-пероксидазний метод. Ще у 10 щурів, які складали негативний контроль, зрізи забарвлювали, використовуючи тільки вторинні антитіла. Інтенсивність кольору мозкових відділів контрольної і кіндлінгової груп порівнювали з такою у групи негативного контроля. У щурів пентиленететразоловим кіндлінгом рівень Kir6.2 становив 18,12±0,98 умовних одиниць і перевищував відповідні дані в контролі (7,53±0,72 умовних одиниць) ($p<0,001$). Експресія гіпоксія-індукованого фактора 1α становила 17,62±0,90 проти 9,77±1,10 умовних одиниць у контролі ($p<0,001$). Вміст Kir6.2 і гіпоксія-індукованого фактора 1α в лімбічних структурах може бути використано в якості маркерів ефективності експериментальних методів лікування хронічної епілепсії.

Ключові слова: судоми, калієві канали, гіпоксія, пентиленететразол, гіпокамп.

The study is a fragment of the research project "Increasing the effectiveness of epileptic activity control using pharmacological drugs and non-invasive stimulation of brain structures", state registration No. 0121U114510

Epilepsy is one of the most severe diseases of the nervous system, the manifestations of which cannot currently be controlled with the help of pharmacological treatment in a third of patients [12]. Ictal seizures resulted in uncontrollable muscle tension with high oxygen consumption and restricted lung ventilation, which caused brain hypoxia. Periodically precipitated hypoxic damage of neurons underlays progressive epileptogenesis development and chronic epilepsy precipitation [1, 12].

The kindling model of epileptiform manifestations is distinguished by the possibility of reproducing chronic persistent epileptization of the brain and the development of comorbid behavioral disorders and allows the study of seizure termination methods concerning a broad spectrum of chronic epileptization of the brain manifestations [1, 11]. Brain hypoxia is one of the distinct pathogenetic mechanisms of the kindling-induced epileptic syndrome [6]. The high intensity of oxygen absorption, which is necessary to ensure the pathologically increased functional activity of an epileptic neuron, is accompanied by it's relative lack. Accordingly, hypoxia occurs with the production and accumulation of superoxide anion and other peroxide compounds, which further support and develop the pathological process [8].

Also, one of the consequences of hypoxia is a decrease in the content of adenosine triphosphate (ATP) [3]. ATP deficiency is accompanied by the opening of ATP-sensitive potassium (K+) channels. One of these channels is Kir6.2, genetic deletion of which increases ischemia, leading to tissue infarction [13]. Low oxygen tension in cells and tissues leads to the transcriptional activation of hypoxia-inducible factor-1α (HIF-1α), the content of which increases with the occurrence of neurodegenerative and neuroinflammatory processes and is a marker of these processes [14]. Activation of the HIF-1α-VEGF pathway is accompanied by neoangiogenesis and the formation of microvessels with high permeability of the blood – brain barrier, which ensures the further development of brain epilepsy [14].

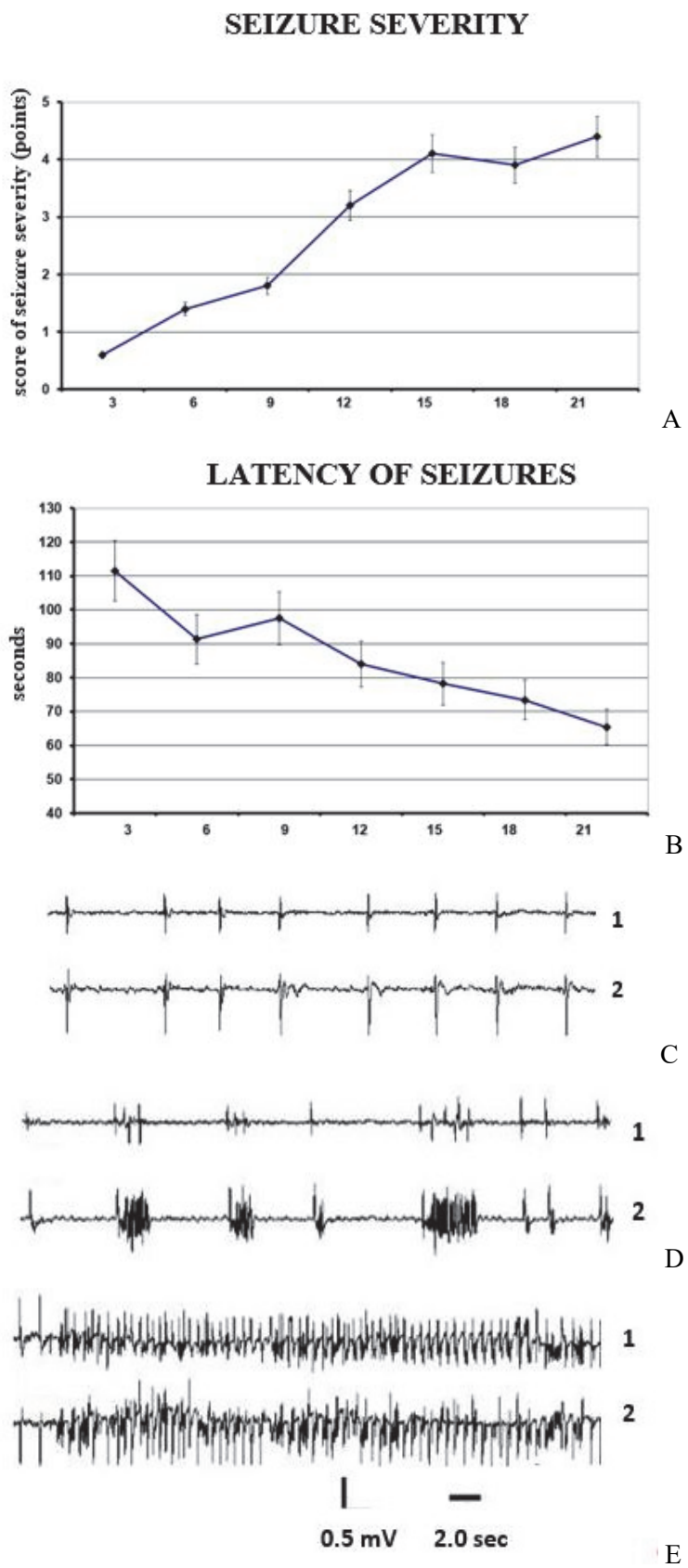

Fig. 1. Dynamics of the behavioral (A, B) and electroencephalographic (C, D, E) characteristics of seizures during the development of pentylenetetrazol-induced kindling. Notes: A, B – the abscissa is the observation period (days); on the ordinate axis are seconds (A) and score of seizure severity (B). C, D, E: C – 17.5 min after 6th dose; D – 15.0 min after 11th dose; E – 25.0 min after 19th dose. First 37 s of ictal potential are presented in both frontal cortex (the 1st line) and dorsal hippocampus (the 2nd line). Calibration – 500 mcV, time marker – 2 sec.

Until recently, immunohistochemical indicators of the content of Kir6.2 and HIF-1α in hippocampal formations under the conditions of experimental modeling of the epileptic syndrome were not investigated.

The purpose of the study was to perform the immunohistochemical examination of the expression of Kir6.2 and HIF-1 α in the hippocampus structures, which plays an important role in the pathogenesis of pharmacological kindling, as a site in which the primary foci of epileptogenic excitation are formed.

Materials and methods. The study was performed on 39 male Wistar rats aged 2–3 months, weighing 180–220 g. The animals were kept under standard conditions of temperature (23±2°C), humidity (60 %) and a 12-hour light cycle with free access to water and food All procedures were carried out in accordance with the Declaration of Helsinki, and in accordance with the permission of the Bioethics Commission of the Odesa National Medical University (protocol No. 3 dated 14.03.2018).

The pentylenetetrazol (PTZ)-induced epilepsy model was induced as previously described [11]. PTZ (P6500, Sigma-Aldrich, USA) was dissolved in 0.9 % NaCl solution ex tempore and administered intraperitoneally at a dose of 35.0 mg/kg for 21 days (n=19). 10 rats included in the immunohistochemical studies and 9 rats were used for EEG registration. Rats of the control groups (n=20, of which ten were in the group of negative immunohistochemical control) were injected with a 0.9 % physiological solution of NaCl. After each injection, the rats were placed alone in an isolated transparent Plexiglas cage, and the severity of seizures was assessed for 30 minutes on a six-point scale [11].

The nichrome electrodes were implanted under ketamine anesthesia (100 mg/kg, i.p., “Farmak”, Ukraine) into the dorsal hippocampus (AP=–4.3; ML=2.5; DV=–3.0), and frontal cortex (AP=1.7; ML=2.0; DV=–1.0) of both

hemispheres (n=9) [11]. Animals were allowed to recover for 10 to 14 days after surgery. The computer electroencephalograph DX-5000 (Kharkiv, Ukraine) with 256 Hz sampling rate was used for monopolar EEG registration. Upon completion of the experiment rats were euthanized with Nembutal (100 mg/kg, i.p.) and the visual quality control of the electrode placement was performed ex tempore.

Brain tissues of 10 kindled rats, which were not implanted with electrodes were fixed in 10 % formaldehyde and subsequently paraffinized. Sections with a thickness of 5 μm were made from the paraffinized blocks, and they were placed on a slide covered with poly-L-lysine [1]. The avidin-biotin-peroxidase method was used according to the previously described method to determine differences in the expression of Kir6.2 and HIF-1α [5]. Images were acquired using an Olympus BX53 light microscope and analyzed using the Image J computer program version 1.46 (National Institutes of Health, Bethesda, USA).

The value of the intensity level of immunohistological staining of the studied samples was calculated as the average signal intensity relative to the background color of the negative control for ten images per experimental animal of the control and kindling groups. The data were evaluated based on staining intensity compared to control quantitative measure of color and were expressed in relative units (RU) [4].

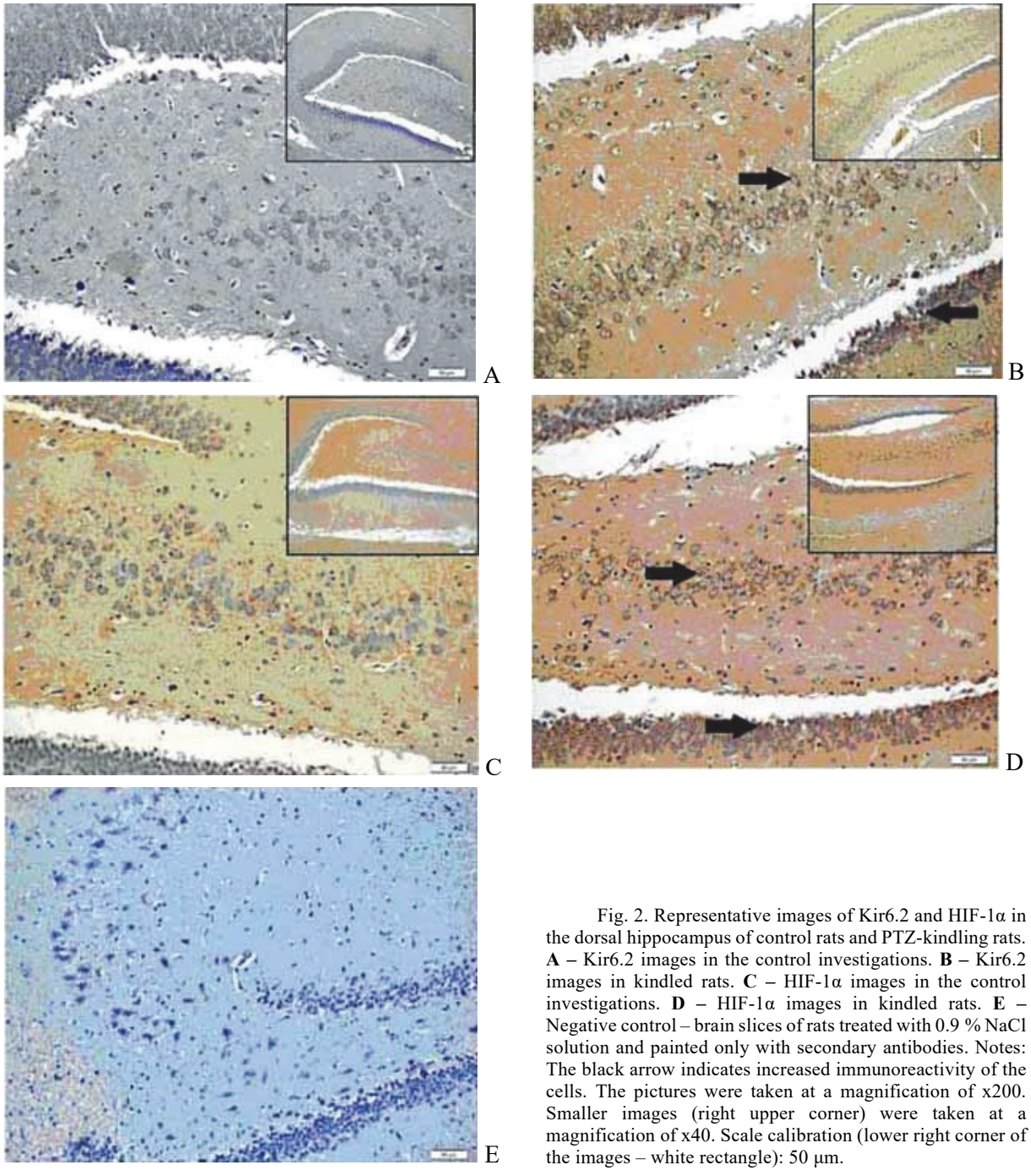

Fig. 2. Representative images of Kir6.2 and HIF-1α in the dorsal hippocampus of control rats and PTZ-kindling rats. **A** – Kir6.2 images in the control investigations. **B** – Kir6.2 images in kindled rats. **C** – HIF-1α images in the control investigations. **D** – HIF-1α images in kindled rats. **E** – Negative control – brain slices of rats treated with 0.9 % NaCl solution and painted only with secondary antibodies. Notes: The black arrow indicates increased immunoreactivity of the cells. The pictures were taken at a magnification of x200. Smaller images (right upper corner) were taken at a magnification of x40. Scale calibration (lower right corner of the images – white rectangle): 50 μm.

The SPSS program for Windows (SPSS Inc., version 24.0, Chicago, USA) was used for the statistical processing of the obtained results. Data were presented as mean values with standard error of the mean (M±SEM). An unpaired two-tailed Student's t-test was used to compare indicators between the control and PTZ-induced kindling groups. Differences between groups were accepted as significant at $p<0.05$.

Results of the study and their discussion. Convulsions began after the second to fourth injection and had a progressive development during the subsequent 2–3 injections of PTZ to the level of the myoclonus of the trunk muscles.

The generation of spikes with amplitude of 0.5–1.5 mV and frequency 15–35 per min was registered during such behavioral manifestations (fig. 1C).

The following 4–9 injections caused the rats to rear up on their hind limbs with clonic convulsions of the rats' forelimbs. The appearance of high frequency after discharges of short duration (2–5 s) was seen in EEG at this stage (it was calculated as score 3) of seizures (fig. 1D). Generalized tonic-clonic convulsive seizures occurred in experimental animals after the 8–17th injections of PTZ with characteristic high-frequency potential generation in brain structures (fig. 1E). During the attacks, the rats lost their balance, fell on their side, and showed post-attack depression. Such manifestation was calculated as score 4. Repeated generalized tonic-clonic fits calculated as score 5 were registered in 3 out of 10 kindled rats after the last 21st PTZ injection.

During the three-week daily administration of PTZ, a characteristic reduction of the latent period of seizure occurrence was observed – to 65.4±7.1 s (fig. 1A), as well as an increase in their severity – to 4.3±0.2 points (fig. 1B). Rats that were included in the subsequent immunohistochemical study showed the development of generalized convulsive seizures in response to each of the last three epileptogen administrations.

Compared to the control group, the PTZ-kindled rats demonstrated upregulation of the Kir6.2 and HIF-1α expression in the dorsal hippocampus: the clear stained immunoractivity was registered in hippocampal neuronal bodies (marked with black arrow) (fig. 2).

The level of Kir6.2 which in the control group was 7.53±0.72 RU, increased to 18.12±0.98 RU ($p<0.001$). The expression of HIF-1α in the tissue of the dorsal hippocampus of rats of the control group (9.77±1.10 RU) increased to 17.62±0.90 RU ($p<0.001$) (fig. 3).

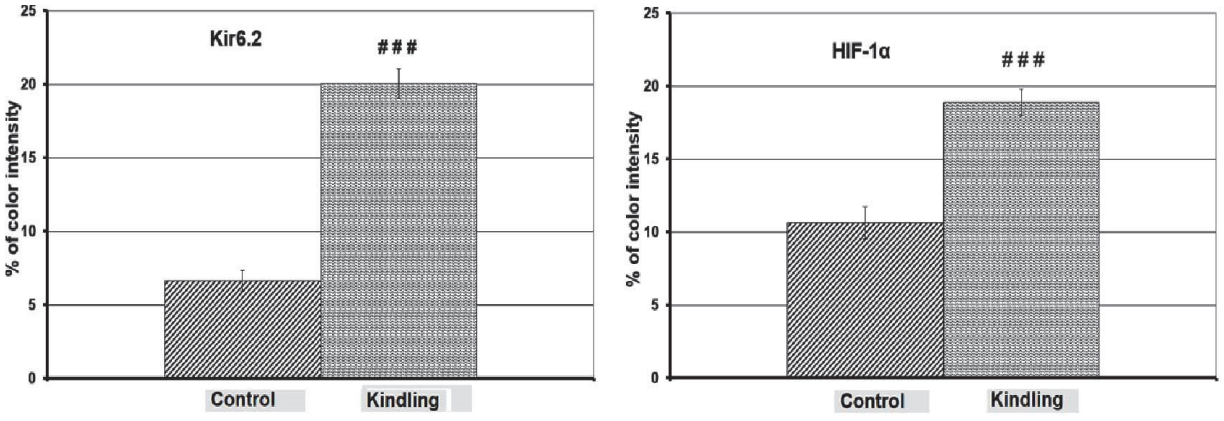

Fig. 3. Intensity of expression of Kir6.2 (A) and HIF-1α (B) in the tissue of the dorsal hippocampus of rats with developed PTZ-induced kindling. Notes: observation groups on the abscissa axis studied indicators on the ordinate axis. ### – $p<0.001$ – the significant differences of the investigated indices compared with the analogous data in the control group (unpaired two-tailed Student's t-test).

Thus, the obtained results proved that the content of hypoxia markers, Kir6.2, HIF-1α, in the structures of the dorsal hippocampus increases in rats with chronic epileptic syndrome caused by PTZ administration. Considering the fact that under the condition of hypoxia, the production of ATP decreases, the growth of Kir6.2 immunoreactivity, as well as HIF-1α, are indicative of the suppression of the energy supply of neurons [3].

It is also possible to consider the growth of Kir6.2 immunoreactivity as a marker of generalized convulsive manifestations, which corresponds to the results of studies [7], which observed a similar pattern under the conditions of studying the culture of hippocampal neurons. Under the conditions of simulating status epilepticus, the protein and mRNA content of Kir6.2 channels in the hippocampus tissue increased [13]. Thus, ictal generalized convulsive manifestations are due to increased expression of Kir6.2. Reciprocal changes in the content and expression of Kir6.2 and Kir6.1 under hypoxia are observed both in nervous tissue and in cardiomyocytes, which allows us to consider a similar mechanism as universal for

excitable tissues [2]. It should be noted that pharmacological regulation of Kir6.2 may effectively prevent cytotoxicity during the development of generalized epileptic activity [1].

The results presented in the actual study showed that the level of HIF-1α protein was significantly increased in the dorsal hippocampus. HIF-1α plays a significant role in ensuring the resistance of cells to the damaging effects of hypoxia. In epilepsy, the transcription factor HIF-1α can be activated in association with seizures. Thus, the study of HIF-1α content in the status epilepticus model determined its increase in the hippocampal tissue, which correlated with the apoptosis of hippocampal neurons [10].

Also, increased expression of HIF-1α in the structures of the hippocampus was determined in [16] on a model of status epilepticus. Moreover, the authors note a decrease in the expression of TNFα against the background of suppression of the expression of HIF-1α [16]. Similar data testify to the importance of the activation of the HIF-1α – TNFα pathway in the mechanisms of neuronal damage under conditions of chronic epileptization of the brain [9].

Further studies of the interaction of neuroimmune mechanisms and markers of hypoxia in the mechanisms of formation of chronic epileptic activity, as well as targets of the influence of pharmacological drugs, can be considered promising.

Conclusion

The content of Kir6.2 and HIF-1α in the dorsal hippocampus of rats with developed PTZ-induced kindling seizures increased, indicating these factors' pathogenetic role in the formation and development of chronic epileptization of the brain.

Prospects for furthers researches include a subsequent comprehensive experimental and clinical studies on the dynamics of Kir6.2 and HIF-1α as markers of the effectiveness of pharmacological epilepsy treatment.

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