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Study of Neuroinflammation in the Rat Hippocampus during Ethanol Exposure and Pharmacological Correction with Azithromycin: New Data and Future Perspectives

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Abstract—With prolonged ethanol ingestion, disturbances in the emotional spectrum develop, and memory problems are noted. These symptoms could be mediated by the development of neurochemical changes in the hippocampus of the brain. Although there is evidence that hippocampus is vulnerable to chronic alcohol intoxication and that neuroinflammation and neurodegeneration develop in this brain region, the key molecular mechanisms have not been identified. The aim of the study was to investigate changes in the immune system in the periphery as well as in the hippocampus of rat brain during ethanol exposure and during pharmacological correction with azithromycin (AZM). Long-term ethanol exposure was modeled by injecting rats with a 20% ethanol solution (4 g/kg) for 4 weeks. General biochemical and clinical blood analysis was performed in animals. Expression levels of the cytokine genes (*Il1β*, *Ccl2*, *Il6*, *Il11*, *Il13*, *Tnfα*, *Tgfβ*), Toll-like receptor system genes (*Tlr3*, *Tl4*, *Tlr7*, *Nfkb1*, *Hmgb1*), and TLR system-related microRNA molecules (miR-182, miR-155-5p, miR-96-5p, miR-let-7b) were evaluated in the hippocampus. IL-1β protein content was also assessed in the hippocampus. Prolonged exposure to alcohol caused increase in the mRNA and protein levels of IL-1β, and decrease in the mRNA levels of *Tnfα*, *Il11*, *Tlr3*, and *Tlr7*. The contents of miRlet7b, miR96, and miR155 were downregulated in the hippocampus after long-term alcohol exposure. Elevated levels of THE *Il1β* mRNA and protein and *Hmgb1* mRNA were maintained under conditions of ethanol abstinence. The *Tlr3* mRNA levels were decreased after abstinence. Administration of AZM reduced the IL1β, TLR3, and HMGB1 mRNA levels under conditions of ethanol abstinence; and at higher doses of the drug decrease in the IL-1β protein levels in the hippocampus of rat brain was observed. Thus, the study provided new insights into the mechanisms of neuroinflammation in the hippocampus during prolonged exposure to ethanol and upon abstinence. The obtained results allowed us to suggest a number of tasks for further studies in this direction.

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Keywords: ethanol, hippocampus, Toll-like receptors, cytokines, IL-1β, microRNAs, azithromycin

INTRODUCTION

New publications appear periodically, which expand our current knowledge on the neuroinflammation theory of alcoholism suggested more than 20 years ago that the long-term exposure of an organism to ethanol activated neuroinflammation mechanisms thus causing development of neurodegeneration in the brain and, as a result, disruption of the coordinated executive control at the higher level of brain

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functioning, which, in turn, exacerbate course of the disease [1-4]. The fact that chronic alcohol consumption causes death of nerve cells is considered nowadays as fully established [1-8], however, first observations of this have been reported in the study by S. S. Korsakoff in 1887 [9]. Later this disorder was termed Korsakoff's syndrome (also known as Korsakoff's amnesic syndrome) that develops due to chronic alcohol intoxication. Almost irreversible memory disorder develops in this state, pharmacological interventions are ineffective, and rehabilitation of the patients with Korsakoff's syndrome mostly involves adaptation of the individual to life with limited memory capabilities [6, 8-10]. From the emotional point of view the patients could exhibit either euphoria and placidity, or, vice versa, apathy and lethargy [11]. This syndrome is acute manifestation of neurodegeneration, which could develop on the background of chronic consumption of ethanol. However, it was shown in the recent studies that development of the signs of neurodegeneration could be observed in the course of experiments with less severe exposure of an organism to ethanol, i.e., more often, not only in the case of Korsakoff's syndrome [8, 9]. It has been suggested that such pathogenetic processes could occur during transition from the state of excessive drinking (binge drinking) to early stages alcoholism, which is followed by the development of more severe forms of alcoholism [8, 9]. It is likely associated with the fact that death of the cells in different regions of the brain chronically exposed to ethanol disrupts coordinated functioning of brain thus affecting processes of psycho-physiological control of an organism behavior, which is manifested as symptoms characteristic of alcoholism – inability to stop drinking, cognitive and emotional disorders [1, 2, 12, 13].

The data obtained in the course of examination of the *post mortem* samples from the patients with chronic alcohol consumption reveal presence of neuroinflammation developed in the cerebral cortex [8, 9]. These data allowed suggesting that formation of alcohol dependence could be associated not only with dysfunction of neurotransmitter and neuropeptide systems, but also is mediated by various factors of neuroinflammation [8-11]. The system of Toll-like receptors (TLR), being a key component of innate immunity, participates in regulation of synthesis of numerous inflammatory response factors [1, 2, 14, 15]. This system includes endogenous TLR agonists (such as Hmgb1 and a number of microRNAs), Toll-like receptors, and intracellular adapter proteins (including MyD88, TRIF, IRF, NF-κB1, and others), which initiate activation of the genes associated with neuroinflammation [1, 2, 14, 15].

Abbreviations: AZM, azithromycin; TLR, Toll-like receptors.

Modeling of long-term exposure to ethanol both *in vitro* and *in vivo* allowed establishing that the system of Toll-like receptors in nervous tissue cells indeed responds to ethanol exposure – overexpression of the TLR genes proteins, of their endogenous agonists has been reported, as well as overexpression of the genes of proinflammatory cytokines [1-4]. However, despite the existence of such knowledge, there is no full understanding of the mechanisms of the effects of long-term ethanol consumption on this system in different regions of the brain. In this regard, the structures comprising elements of the so-called brain reinforcement system associated with the development of alcohol addiction attract the most attention [16-18]. One of such key structures is hippocampus [16-18]. It seems interesting to evaluate levels of expression of the elements of TLR system and associated molecules in the hippocampus during modeling of long-term ethanol exposure and ethanol abstinence, as well as during pharmacological correction with the help of azithromycin (AZM), which, according to the literature data, is an effective agent for treatment of neuroinflammation [19-22]. This was the goal of our study.

MATERIALS AND METHODS

Animals. Three-months old male Wistar rats (n = 56) with average body mass 250-300 g were used in the study, which were purchased in the Rappolovo laboratory animal facility (Russia). Prior to the experiment animals were divided into groups (8 animals in each group): control group, group with long-term exposure to ethanol, five groups with ethanol abstinence (ethanol abstinence on the day 7; ethanol abstinence + water; ethanol abstinence + AZM, 40 mg/kg; ethanol abstinence + AZM, 80 mg/kg; ethanol abstinence + AZM, 160 mg/kg). Animals have free access to standard animal feed.

Modeling of long-term ethanol exposure and state of abstinence was performed according to the models described in the literature with some modifications [23-25]. Long-term exposure to ethanol was modeled by intragastric administration of 20% ethanol solution through a gastric tube at a ratio 4 g of pure ethanol per 1 kg of body weight on Monday-Friday of each week for 4 weeks (20 administrations in total). Control animals received equivalent volumes of water according to the same scheme. Animals have free access to water all the time. Animals in 'ethanol group' were decapitated on the last day of ethanol exposure 2 h after the last administration of ethanol. To model ethanol abstinence, the animals exposed to ethanol were kept for 7 days without administration of ethanol and next were sacrificed by decapitation (group 'ethanol abstinence, 7 days').

Table 1. Sequences of primers

Gene	Primers	
	Forward (5′→3′)	Reverse (5′→3′)
Tlr3	AACTGGAGAACCTCCAAGA	CACCCTGGAGAAAACTCTTT
Tlr4	ACTCTGATCATGGCATTGTT	GTCTCAATTTCACACCTGGA
Tlr7	TGAAAATGGTATTTCCAATGTG	TAAGGGTAAGGTTGGTGGTA
Nfκb1	ATACTGCTTTGACTCACTCC	AGGTATGGGCCATCTGTT
Hmgb1	CTCTGATGCAGCTTATACGA	AAAAGACTAGCTTCCCCTTG
Il1β	TGTCTGACCCATGTGAGCTG	TTTGGGATCCACACTCTCCAG
Ccl2	AAGATGATCCCAATGAGTCG	TGGTGACAAATACTACAGCTT
Tnfα	CACGTCGTAGCAAACCAC	TATGAAATGGCAAATCGGCT
Il13	TGTAACCAAAAGGCCTCGGA	TGGCCATAGCGGAAAAGTTG
Tgfβ	GGACTACTACGCCAAAGAAG	GGACTACTACGCCAAAGAAG
Il11	GGGACATGAACTGTGTTTGT	GGTAGGTAGGGAGTCCAGAT
Il6	ACTTCACAAGTCGGAGGCTT	AATTGCCATTGCACAACTCTTTTC
miR-182	TTTGGCAATGGTAGAACTCACACCG	GCGAGCACAGAATTAATACGAC
miR-155-5p	TTAATGCTAATTGTGATAGGGGT	GCGAGCACAGAATTAATACGAC
miR-96-5p	TTTGGCACTAGCACATTTTTGCT	GCGAGCACAGAATTAATACGAC
miR-let-7b	GCGGCGGCTATACAACCTACTGC	GCGAGCACAGAATTAATACGAC
U6	TGCTTCGGCAGCACATATAC	AGGGGCCATGCTAATCTTCT
Gapdh	GCCAGCCTCGTCTCATA	GTGGGTAGAGTCATACTGGA

Injection of pharmacological agents. After the long-term exposure to ethanol the animals were subjected to daily intragastric administration of azithromycin through a gastric tube for three days [40 mg/kg, 80 mg/kg, 160 mg/kg; Hemomycin, powder for preparation of suspension for oral administration (Hemofarm, Serbia)]. Corresponding volume of water was administered to the rats in control groups (group "ethanol abstinence + water"). Animals in all 'ethanol abstinence groups' were decapitated on the day 7 after the last ethanol administration.

Sample preparation. Boundaries of brain structures were determined in accordance with the rat brain atlas [26]. Brain samples were immediately frozen at –80°C. Blood was sampled at the moment of animal decapitation into special tubes for biochemical and clinical analysis.

Blood analysis. Blood parameters was analyzed out within first 2 h after sampling with a veterinary hematological analyzer (Mindray, China). Determination of biochemical parameters in blood serum was carried out with an Erba Mannheim XL-100 automatic analyzer (Germany).

RNA isolation from brain samples. Isolation of total RNA was carried out using an ExtractRNA reagent (Evrogen, Russia) in accordance with the manufacturer's instruction. Purity and concentration of obtained RNA were evaluated with an Implen NanoPhotometer P330 (Implen, Germany) from the A260/A280 ratio (normal level ≥ 1.8).

RT-PCR. Synthesis of cDNA was carried out using reverse transcription (RT) in 20 μl of reaction mixture of a MMLV RT kit (Evrogen) in accordance with the manufacturer's instruction. Prior to conducting RT microRNA were polyadenylated with the help of poly(A)-polymerase *Escherichia coli* (New England Biolabs Inc., USA) according to the previously described technique [27]. RT of microRNA was carried

in 10 μl volume reaction mixture with a MMLV RT kit and specific PolyT-adapter (5′-GCGAGCACAGAAT TAATACGACTCACTATAGGTTTTTTTTTTTTVN-3′).

Concentration of cDNA obtained in the course of RT was measured with an Implen NanoPhotometer P330.

Polymerase chain reaction (PCR) with real-time detection was carried out with a Mx3005P amplifier (Stratagene, USA) in a 10-μl reaction mixture containing SYBR Green (Evrogen) and a mixture of specific of forward and reverse primers (Table 1) (Beagle, Russia). Relative levels of mRNA and miR were calculated using $2^{-\Delta\Delta Ct}$ method, mRNA content was normalized to the level of expression of the *Gapdh* gene, level of miR was normalized to the level of expression of the *U6* gene. All reactions were conducted in triplicate.

Enzyme-linked immunoassay. Frozen hippocampus samples were homogenized with a CRYOMILL (China) prior to conducting enzyme-linked immunoassay (ELIA); next, the obtained frozen powder was dissolved in 1 ml of PBS (phosphate-buffered saline, pH 7.4) and centrifuged for 10 min at 3000*g*. Aliquots (100 μl) of the collected supernatants were stored at –80°C before conducting ELIA. Content of IL1β was determined with the help of an ELIA kit (Cloud-Clone Corp., USA) according to the provided protocol. Absorption in the reaction mixture was recorded with a Synergy 2 plate reader (Bio Tek, USA). Total protein content was determined using the Bradford method with a Bio-Rad Protein Assay Kit (Bio-Rad, USA). Measurements were performed in triplicate.

Statistical data processing was carried out using the Graph Pad Prizm v.6 software. All data are presented as a mean ± standard deviation of the mean. Results in different groups were compared using the Mann–Whitney *U*-test for small samples. Differences were considered statistically significant at $p \le 0.05$.

RESULTS AND DISCUSSION

Biochemical and clinical parameter in blood after long-term exposure to ethanol. To evaluate general state of an animal organism during long-term exposure to ethanol we considered reasonable to assess biochemical and clinical blood parameters in animals.

Results of analysis demonstrated increased levels of alanine aminotransferase (ALT) and albumin in blood serum of animals in the experimental group (Table 2). Increased level of albumin could reflect changes in the degree of dehydration of the organism, which is typical for the patient with chronic alcoholism, and the increased level of ALT serves as an indicator of possible damage of hepatocytes in the experimental animals, which is also characteristic for an organism with long-term alcoholic intoxication [8, 28, 29].

Table 2. Biochemical parameters of rat blood serum upon long-term exposure to ethanol

Indicator	Control (n = 8)	Ethanol (n = 8)
ALT, IU/l	18.63 ± 2.91	28.14 ± 5.64*
Glucose, mmol/l	4.39 ± 0.43	4.01 ± 1.08
Creatinine, μmol/l	25.43 ± 7.65	28.16 ± 3.58
Urea, mmol/l	3.48 ± 0.54	4.07 ± 0.36
Albumin, g/l	44.87 ± 3.99	55.43 ± 4.86*
AST, IU/l	69.53 ± 14.67	64.44 ± 6.35
Total protein, g/l	59.30 ± 8.59	67.38 ± 4.48

Note. ALT, alanine aminotransferase; AST, aspartate aminotransferase; * $p \le 0.05$ – in comparison with the control group.

Table 3. Results of clinical analysis of blood samples of the rats subjected to long-term exposure to ethanol

Indicator	Control (n = 8)	Ethanol (n = 8)
Leukocytes, 10^9/l	8.52 ± 1.82	6.12 ± 2,22
Neutrophils, 10^9/l	0.61 ± 0.51	0.98 ± 0,53
Lymphocytes, 10^9/l	7.37 ± 2.10	4.01 ± 1.76*
Monocytes, 10^9/l	0.5 ± 0.46	1.10 ± 0.69*
Eosinophils, 10^9/l	0.03 ± 0.01	0.04 ± 0.02
Basophils, 10^9/l	0 ± 0.00	0 ± 0.00
Erythrocytes, 10^{12}/l	8.04 ± 0.41	7.73 ± 0.51
Hemoglobin, g/l	141.5 ± 8.10	145.0 ± 8.74
Hematocrit, %	41.4 ± 1.84	41.9 ± 2.44
Thrombocytes, 10^9/l	977.33 ± 156.42	754.01 ± 265.26

Note. * $p \le 0.05$ in comparison with the control group.

The results of clinical blood analysis demonstrated decrease of the level of lymphocytes and increase of the level of monocytes in the animals subjected to long-term exposure to ethanol (Table 3).

The presented results show existence of immune system response to the long-term exposure of the animal to ethanol. Increase of the level of monocytes could reflect to a certain degree presence of chronic peripheral inflammatory response, and decrease of the level of lymphocytes could indicate decrease of activity of adaptive immune system, which is observed often under conditions of long-term exposure of the organism to ethanol [29-31].

Hence, modeling of the long-term exposure of an organism to ethanol conducted in this study resulted in certain changes of blood parameters in the animals, which manifest development of a pathological state. It should be mentioned that body mass of the animals in both experimental and control group did not differ significantly in the course of the experiment, and the animals with signs of one or other disease (such as acquired trauma) were removed from the experiment.

Expression of the genes of cytokines and genes of the TLR system in the hippocampus of rats after long-term exposure to ethanol. Long-term exposure to ethanol resulted in the increase of the level of expression of the *Il1β* gene and decrease of expression of the *Tnfα* and *Il11* genes in the rat brain hippocampus (Fig. 1). No significant differences at the mRNA levels were revealed in the expression of *Il6*, *Tgfβ*, and *Il13* (Fig. 1). Product of the *Il1β* gene is a key proinflammatory cytokine, and increase of its content is often associated with the development of neuroinflammation. Increase of its expression in the brain exposed for a long time to ethanol has been reported in the previous studies [12, 32, 33]. The results obtained in this study on the enhanced expression of this gene in the hippocampus of rats subjected to long-term exposure to ethanol are similar to the previously reported data for different regions of the brain including hippocampus, but using different conditions for modeling long-term ethanol exposure [12, 32, 33]. The level of expression of the *Il6* gene in hippocampus in our experiment did not change, while expression of the *Tnfα* gene was downregulated (Fig. 1). Both cytokines, according to the latest data, exhibit dual action with regard to the development of inflammation – in some situations they play a role of proinflammatory factors, while under other conditions they could exert anti-inflammatory effects [15, 34]. Hence, no unambiguous conclusions could be made with regards to the decrease of the level of expression of the *Tnfα* gene, further research is needed to resolve this issue.

Downregulation of expression of the *Il11* gene seems very interesting (Fig. 1). There are data in the literature indicating that this cytokine in the nervous tissue exhibits anti-inflammatory effects, which was demonstrated in the studies modeling various pathologies [35]. It is likely that in this case we observe manifestation of decreased activities of the mechanisms of inhibition of proinflammatory processes developing on the background of chronic consumption of ethanol. The decreased level of expression of the *Il11* gene could be one of the reasons for dysfunction of these mechanisms, and further investigation of this cytokine could help to identify a new molecular target to correct neuroinflammation. This hypothesis requires future verification.

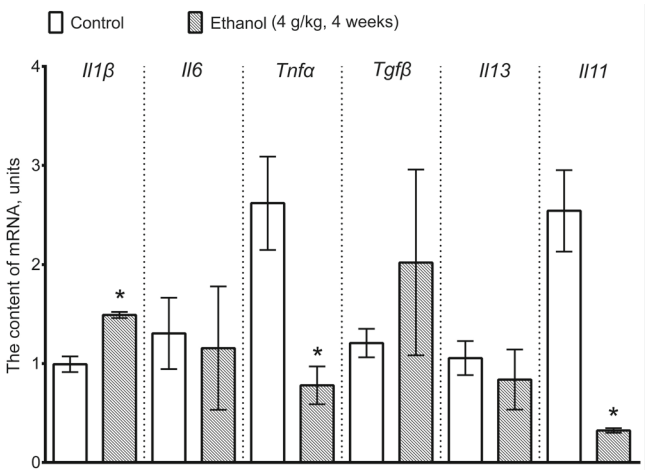

Fig. 1. Content of cytokine mRNAs in the hippocampus of rats subjected to long-term ethanol exposure; * $p \le 0.05$ in comparison with the control group.

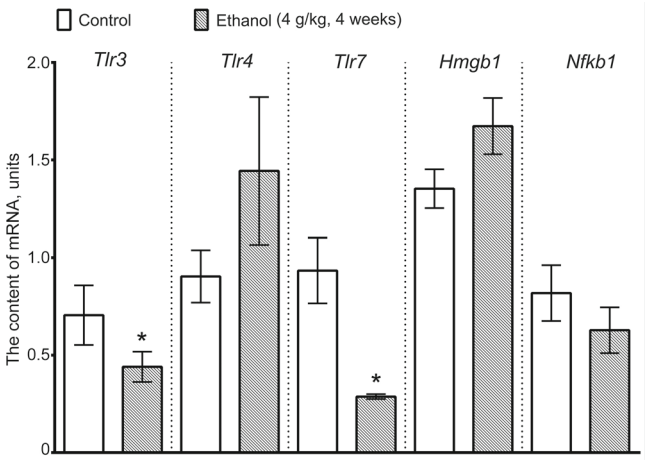

Fig. 2. Relative level of mRNAs of the TLR system genes in the hippocampus of rats subjected to long-term exposure to ethanol; * $p \le 0.05$ in comparison with the control group.

In addition to cytokines, expression of some genes of Toll-like receptors was assessed in this study. We did not observe any significant changes in the expression of the *Tlr4*, *Hmgb1*, and *Nfκb1* genes, while expression of the *Tlr3* and *Tlr7* genes was downregulated (Fig. 2). In the previous studies the researchers reported increase in the level of expression of the *Tlr7* gene, which was associated with manifestations of neurodegenerative process [1, 5]. It is likely that the observed differences between the results of ours and other studies is due to the regional peculiarities of expression of this gene, or due to the use of different model of long-term exposure to moderate doses of ethanol.

It was observed in our previous studies that there were only minimal changes in the TLR system under condition of long-term exposure to ethanol, however,

Table 4. Relative level of expression of microRNAs in the hippocampus of the rats, arb. units

miR	Control (n = 8)	Ethanol (n = 8)
miRlet7b	0.78 ± 0.13	0.43 ± 0.19*
miR96	1.08 ± 0.34	0.38 ± 0.15*
miR155	0.96 ± 0.37	0.51 ± 0.25*
miR182	1.01 ± 0.25	0.79 ± 0.17

Note. * $p < 0.05$ in comparison with the control group.

abstinence from the long-term ethanol exposure caused pronounced disbalance in their expression [36-38]. In particular, the similar results were obtained for in the nucleus accumbens of the rat brain in the similar model of long-term exposure to ethanol [37]. Hence, it seems interesting to investigate the mRNA and protein levels of cytokines, as well as other components of TLR-signaling pathway not only under conditions of long-term exposure to alcohol, but also under conditions of alcohol abstinence.

Investigation of the content of microRNAs in hippocampus of the rats subjected to long-term exposure to ethanol. Analysis of relative content of microRNA molecules in the hippocampus of the rats revealed that the levels of miRlet7b, miR96, and miR155 changes significantly in the group of animals subjected to long-term exposure ethanol: the level of the miRlet7b decreased 1.8-fold, of the miR96 – 2.8-fold, and of the miR155 – 1.9-fold (Table 4).

The miR molecules tested in our study were selected on the basis of their functional association with the TLR-signaling pathways [39-43]. It is known that miR-let7b is an endogenous agonist of TLR7 [1, 12]. It has been reported that the change in expression of miR-let7b could have functional association with the TLR7-signaling cascade [5, 12].

Increase of the content of miR-155 in the microglia cells of the mice was observed with increase of activity of TLR-signaling, while in the mice with knockout of the *Tlr4* gene (TLR4-KO) no such increase was observed [41]. Content of miR-96 in the cerebral cortex does not change in the TLR4-KO mice, while long-term exposure of the wild type mice to alcohol decreased the level of miR-96 [42, 43]. The data are available exemplified with miR-182-5p that indicate existence of functional interactions of the miR-183C cluster (that include miR-96 and miR-182) with the TLR4 protein [42, 43]. It is also worth mentioning that not only the content of miR-let7b (endogenous agonist for TLR7) was decreased in our experiments, but also the level of *Tlr7* mRNA, which emphasizes importance of further research aiming at establishing possible functional association between the observed changes.

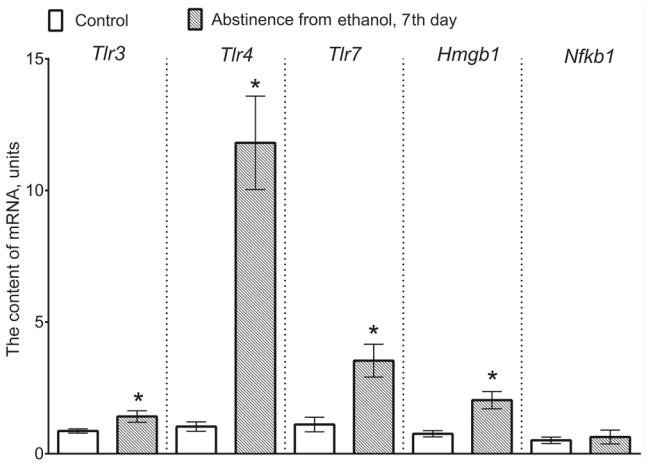

Fig. 3. Content of mRNAs of the cytokines genes and of the genes of TLR system in the hippocampus of rat brains during ethanol abstinence; * $p \leq 0.05$ in comparison with the control group.

Expression of the genes of cytokines and of the TLR system in rat brain hippocampus under conditions of ethanol abstinence. There is no information on the state of the investigated system of genes under conditions of ethanol abstinence. Few facts available in this regard were mainly obtained either during modeling of chronic and sub-chronic forms alcohol addiction or on the next day after abstinence [2, 13]. In our present experiment we analyzed expression of the investigated genes on the day 7 after abstinence from the long-term ethanol exposure (Fig. 3).

The obtained results revealed that 7 days after abstinence expression levels of the genes *Tlr3*, *Tlr4*, *Tlr7*, and *Hmgb1* were increased, while expression of the *Nfκb1* gene did not change (Fig. 3). The highest increase of expression was observed for the *Tlr4* gene. The obtained data allows suggesting involvement of the system of Toll-like receptors into pathogenic mechanisms in the brain hippocampus developing on the background of long-term exposure of an organism to ethanol. It was observed in our experiments that the levels of expression of the *Tlr3* and *Tlr7* genes in hippocampus decreased in the group of animals subjected to long-term exposure to ethanol (Fig. 2), while increase of the levels of their expression was observed under conditions of ethanol abstinence (Fig. 3). The obtained results are in agreement with the data obtained by other researchers for other brain structures, as well as for the biopsies from the patients with chronic alcohol intoxication [2, 13, 24]. It was shown in a series of studies devoted to elucidation of functional significance of such changes that the increase of expression of the *Tlr4* and *Hmgb1* genes resulted in activation of the genes of proinflammatory cytokines and, as a result, to the development of neuroinflammation [12, 13, 24, 33, 41]. Increase of activity

of the *Hmgb1* and *Tlr7* genes leads to activation of the mechanisms of programmed cell death in the neuronal cell cultures (*in vitro* studies), i.e., to manifestation of the signs of neurodegeneration [5]. With regard to the *Tlr3* gene, information is available that activation of the product of expression of this gene, endosomal protein receptor TLR3, with specific agonists results in increase of the level of voluntary consumption of ethanol by animals [2]. Hence, the observed changes in the expression of these genes indicates presence of persistent pathological processes in the hippocampus of rat brain 7 days after abstinence of ethanol, and the system of investigated genes participate in their realization. The obtained data emphasize once more significance of studies in this research area.

Use of azithromycin for correction of expression of the genes of cytokines and of the system of Toll-like receptors under conditions of abstinence of ethanol. Here we present results of our first observations aimed at evaluation of expression of the cytokine genes and of the genes of TLR system in the rat brain hippocampus under conditions of abstinence after long-term exposure to ethanol and during pharmacological correction by azithromycin. AZM has been recognized as an effective neuroprotector in different models of pathological states, which decreases the levels of inflammatory factors and slows down the process of neuronal cell death [19-22]. Exact mechanism is not known. In particular, it has been suggested that the effect could be realized through the protein complex NF-κB [19-22], which is a key component in the pathway of transduction of intracellular signals from TLR [14, 15]. We have set a goal to assess possible neuroprotective effect of AZM on the investigated system of the TLR genes in the hippocampus under conditions of abstinence of long-term exposure to ethanol.

In this study we also obtained the data on expression of the *Tlr3*, *Hmgb1*, and *Nfκb1* at the day 7 after abstinence of long-term exposure to ethanol – expression of the *Tlr3* and *Hmgb1* genes was increased, and the level of expression of the *Nfκb1* gene did not change (Fig. 4). Treatment with AZM provided correction of the levels of expression of the *Tlr3* and *Hmgb1* genes (Fig. 4). With respect to the *Hmgb1* gene, all three used doses of the preparation exhibited positive effect, while use of the lowest dose of AZM (40 mg/kg) did not affect expression of the *Tlr3* gene (Fig. 4). The AZM preparation did not affect expression of the *Nfκb1* gene (Fig. 4). In addition, we evaluated effects of AZM on the levels of expression of the genes of proinflammatory cytokines *Il1β* and *Ccl2* under conditions of ethanol abstinence (Fig. 5). Previously, the effects of AZM on these cytokines were investigated in other pathological states; it was shown that AZM is capable of correcting levels of these cytokines [19-22]. In our earlier study we also reported our observations on expression of the genes of these cytokines on the day 7 after ethanol abstinence, but the obtained data on their expression were different – increase of expression of the *Ccl2* gene was revealed, and there were no changes in the expression of the *Il1β* gene [36]. These differences could be explained by the fact that different protocols of modeling long-term ethanol exposure were used. In the present experiment increase of the level of the *Il1β* gene expression was demonstrated, while there were no significant changes in the expression of the *Ccl2* gene (Fig. 5).

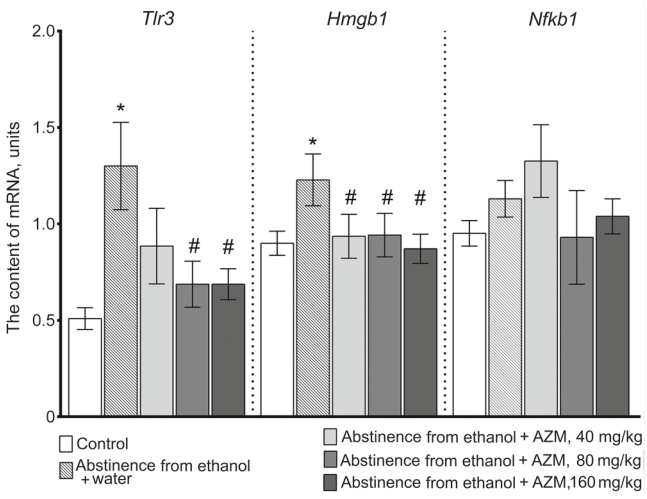

Fig. 4. Contents of mRNAs of the genes of TLR system in the hippocampus of rats under conditions of ethanol abstinence followed by pharmacological correction with azithromycin; * $p < 0.05$ in comparison with the control group; # $p < 0.05$ in comparison with the group "Abstinence from ethanol + water."

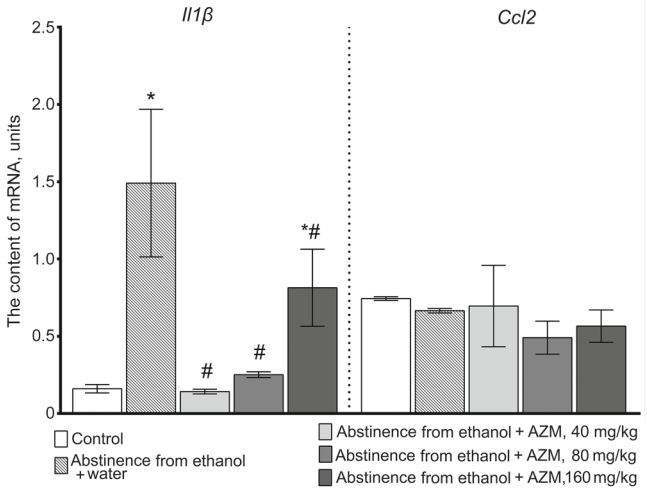

Fig. 5. Contents of mRNAs of the cytokine genes in the hippocampus of rats under conditions of ethanol abstinence followed by pharmacological correction with azithromycin; * $p < 0.05$ in comparison with the control group; # $p < 0.05$ in comparison with the group "Abstinence from ethanol + water."

Treatment with AZM allowed to decrease the elevated level of the *Il1β* gene expression at all doses of the preparation used (Fig. 5).

Level of the IL-1β protein under condition of long-term exposure to ethanol and pharmacological correction with azithromycin in the rat hippocampus. We concentrated our attention on measuring of the level of IL-1β protein in the rat hippocampus. This protein is a key proinflammatory cytokine; level of this protein increases in the brain tissues during neuroinflammation development [1, 2, 13].

Role of the IL-1β protein, which mediates development of cognitive and emotional disfunctions, has been investigated in different pathologies. In particular, in the experiment with mice subjected to psycho-social stress, reduced social interactions and disruptions of working memory was observed, which was prevented with knockout of the *Il1r1* gene (gene of the IL-1 protein receptor); and the virus-mediated selective deletion of the *Il1r1* gene in the hippocampus neurons also demonstrated its crucial role in the stress-induced behavioral dysfunctions. In addition, it was shown that the stress-induced transport of monocytes into brain was also blocked by the knockout of the *Il1r1* gene [44]. In another study a lentiviral construct was used to induce hyperexpression of IL-1β in the dorsal hippocampus of rats. As a result, reduced neurogenesis in the hippocampus was observed together with the decreased level of neurite branching on neurons [45]. Data are available indicating that proinflammatory cytokines suppress long-term potentiation in the hippocampus, thus triggering changes in the key mechanisms of memory formation. The researchers revealed using synaptosomes and neuronal cell cultures that IL-1β directly suppresses plasticity via neuron-specific mechanisms. It has also been reported that IL-1β could suppress long-term potentiation directly in the mouse synapses [46]. Furthermore, it is known that the hippocampal neurons express high levels of IL-1R1 [46]. These data indicate involvement of the cytokine in the disruption of memory formation mechanisms. There is a number of studies supporting these suggestions, which were conducted using various animal models [46].

The results of our study showed that the animals in the group with long-term exposure to ethanol and in the group 7 days after ethanol abstinence have elevated levels of the IL-1β protein (Fig. 6). Hence, it could be concluded based on the available information on IL-1β that the observed increase of the level of this protein in hippocampus (more than 2-fold increase) during the long-term exposure of an organism to ethanol could be the cause of development of emotional and cognitive disorders, as well as of disruption of the mechanisms of memory formation.

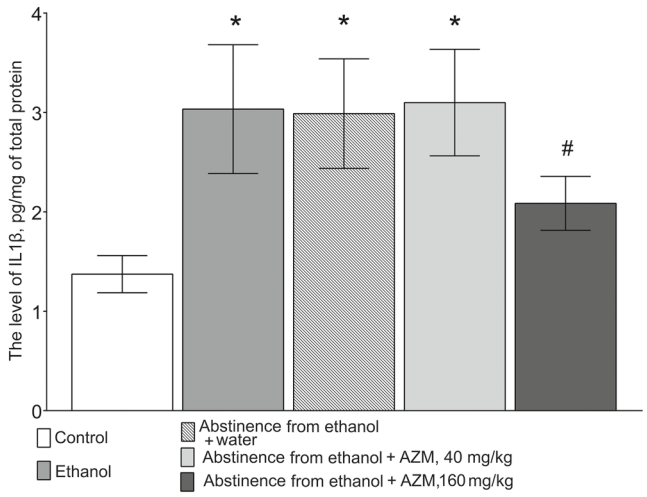

Fig. 6. Level of IL-1β in the rat hippocampus; * $p < 0.05$ in comparison with the control group; # $p < 0.05$ in comparison with the "Abstinence from ethanol + water" group.

In the experiment we used two doses of azithromycin to test its efficiency with respect to correction of the IL-1β protein content. Use of azithromycin at the dose 40 mg/kg did not produce any significant effect, but using the dose of 160 mg/kg resulted in the decrease of the level of the protein (to almost control levels) both in comparison with the group after ethanol abstinence and with the group of animals subjected to long-term exposure to ethanol (Fig. 6). At the same time, analysis at the mRNA level demonstrated efficiency of both doses of AZM used (40 mg/kg and 160 mg/kg) (Fig. 5). Hence, the dose of 40 mg/kg, which is sufficient for reduction of the level of expression of the *Il1β* gene, is insufficient to decrease to the level of the protein product. The observed effect could be mediated by the effects of azithromycin on mechanisms of maturation or degradation of the IL-1β product. The IL-1β protein is formed in the course of proteolysis from its precursor pro-IL-1β, and, despite the observed decrease of the level of products of transcription of this gene at the particular time point, the increased level of the IL-1β protein could be maintained due to formation of the protein from the pro-IL-1β molecule in the course of posttranslational mechanisms. Moreover, it is known that degradation of the IL-1β protein also occurs through a complex pathway, which is mediated, in particular, by matrix metalloproteinases [47, 48]. It would be interesting to investigate in future possible contribution of azithromycin to regulation of the pathways regulating the rate of IL-1β protein degradation. It could be suggested that the observed persistent increase of the levels of IL-1β protein with the decreased levels of the level of expression could be explained by activation of the pathways leading to the decrease of the rate of protein degradation. The available dynamic

correlations also show existence of deviation in time between the levels of IL-1β mRNA and protein – decrease in the levels of mRNA is accompanied with the increasing trend of the protein level [49].

Summarizing the obtained results about the content of the key proinflammatory cytokine protein IL-1β, it could be stated that there are signs of neuroinflammation in the hippocampus of the rats subjected to long-term exposure to ethanol and ethanol abstinence. The tested compound, azithromycin, was shown to exert positive effect at certain doses with regard to decrease of the level of neuroinflammatory mediator IL-1β, which is in agreement with the results of other studies.

CONCLUSIONS

Modeling of the long-term exposure to moderate doses of ethanol conducted in this study revealed several changes both in biochemical (increase of the levels of ALT and albumin) and clinical (increase of the level of monocytes and decrease of the level of lymphocytes) parameters of the blood, and in the system of neuroinflammation in the rat brain hippocampus. We analyzed the levels of activity of the genes of the Toll-like receptors system, as well as of the molecules functionally associated with this system including microRNAs (miRlet7b, miR96, miR155, miR182) and cytokines. Long-term exposure to ethanol caused increase in the *IL-1β* mRNA level and the level of IL-1β cytokine protein, while the levels of *Tnfα*, *Il11*, *Tlr3*, and *Tlr7* mRNAs were found to be decreased. Seven days after ethanol abstinence the increased levels of *IL-1β* mRNA and protein were preserved, as well as of the *Hmgb1* mRNA. On the contrary, the level of the *Tlr3* mRNA was found to be increased after the ethanol abstinence. Peroral administration of azithromycin facilitated correction of some biochemical changes developed during ethanol abstinence – the increased levels of *Il1β*, *Tlr3*, and *Hmgb1* mRNAs were decreased, and higher doses facilitated decrease of the increased levels of the IL-1β protein in the rat hippocampus. Contents of miRlet7b, miR96, miR155 were decreased in the hippocamp as a result of long-term exposure to alcohol. These molecules are functionally associated with the system of TLR, and changes in their content could reflect or mediate (not known at the moment) dysfunctions in molecular mechanisms of functioning of Toll-like receptors.

Investigation of biochemical changes in the rat hippocampus during modeling of long-term exposure to ethanol and its abstinence is of significant interest, because patients with alcohol addiction exhibit disruption in memory formation and in emotional sphere. Hippocampus plays an important role in these processes, and evaluation of biochemical changes could help to obtain new information expanding our understanding of pathophysiological mechanisms developing in this brain region during long-term exposure of an organism to ethanol.

Contributions. All authors contributed significantly to the concept development, conducting research, and preparation of the manuscript, all authors read and approved final version of the manuscript. M.I.A., S.O.E., S.A.S., and E.R.B. acquiring and analysis of the data; A.A.L. and P.D.S. development of general concept of the study, preparation of the final version of the manuscript.

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Ethics declarations. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures in the animal studies were approved by Ethics Committee of the Saint-Petersburg State University (protocol no. 131-03-8, 29.04.2024). The authors of this work declare that they have no conflict of interest.

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