EXPERIMENTAL STUDIES

THE STUDY OF THE AZITHROMYCIN EFFECT ON GENE EXPRESSION OF THE TOLL-LIKE RECEPTOR SYSTEM IN THE BRAIN NUCLEUS ACCUMBENS OF RATS DURING ETHANOL WITHDRAWAL AND SEARCH FOR POSSIBLE MOLECULAR TARGETS BY AN *IN SILICO* METHOD

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The brain's nucleus accumbens (NAc) is a key link in the internal reinforcement system, which mediates manifestations of various components of addiction, including ethanol. The neuroinflammatory theory of alcoholism development suggests that changes in the molecular mechanisms of the innate immune system may be involved in the development of this pathology. The aim of our study was to investigate the effect of azithromycin (AZM) on expression of toll-like receptor system genes in the NAc during experimental alcoholization of rats. The objectives of the study also included an *in silico* search for possible molecular targets for AZM that could be associated with the toll-like receptor system. AZM corrected the changes observed in the expression of toll-like receptor system genes under conditions of alcohol withdrawal after long-term ethanol exposure in the NAc of the brain. The *in silico* analysis revealed the most probable proteins which could be involved in the interaction with AZM. Based on results of these predictions a number of assumptions about possible ways of implementing the observed pharmacological effect of AZM in the experiment have been made.

Keywords: nucleus accumbens; ethanol; neuroinflammation; toll-like receptors; azithromycin; *in silico*

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INTRODUCTION

The nucleus accumbens (NAc) is considered as of the key brain structures involved in the mechanisms of addictive behavior formation [1–5]. Many studies are aimed at investigating the biochemical and molecular changes that may be involved in the implementation of such mechanisms [1–5]. The special attention is paid to the study of neurochemical features of mediator systems in this region of the brain, especially on dopaminergic neurotransmission [1–5]. One recent paper reports that experimental exposure to ethanol significantly influenced dopamine levels: low doses of ethanol administered intraperitoneally, intravenously, orally, or directly into the NAc increased dopamine levels in this brain structure; chronic ethanol administration to rats caused minor changes in the dopamine levels, while ethanol withdrawal after chronic ethanol exposure decreased the dopamine level [6].

Over the past few years, we have been conducting research into the neuroinflammatory theory of alcoholism [7, 8]. Accepting the importance of studying and searching for changes in other areas, we suggest that one of the important components of the pathogenesis of alcoholism is an increase in the activity of neuroglial interactions in response to prolonged ethanol intake. This leads to the development of neuroinflammation, which could be the cause of neurodegeneration, the cell death in the most vulnerable structures of the brain [7, 8]. Neuroimaging data provide good evidence that pathomorphological changes develop in the brain with chronic ethanol intake, but this does not affect all brain structures equally [9]. Such changes are observed in the NAc [9], but their exact pathophysiological mechanisms have not yet been determined. Changes in the functioning of the toll-like receptor (TLR) system are considered one of the possible events in the development of this pathological condition [7, 8, 10].

The aim of this study was to model long-term exposure to ethanol followed by its withdrawal to investigate the expression of genes associated with neuroinflammation, as well as to assess the effect of azithromycin (AZM) on the state of these genes. AZM acts as an effective neuroprotector in experiments on modeling various pathological conditions of the nervous system associated with the development of neuroinflammation [11]. The exact molecular target has not been determined yet, but the mechanism of action is most likely realized by suppressing increased microglial activity, which reduces the manifestation of neuroinflammation and delays

the neurodegeneration process [11]. The neuroprotective properties of AZM have not been previously studied in the model of long-term exposure to ethanol.

MATERIALS AND METHODS

Animals

Forty adult (three-months-old) male Wistar rats (average body weight 250–300 g), purchased from the Rappolovo nursery (Russia) were used in the experiment. Before the experiment, the animals were divided into groups of 8 animals in each group (control group, long-term ethanol exposure group, and three groups of ethanol withdrawal rats after long-term ethanol exposure). The animals had free access to standard rat food.

Modeling of Long-Term Exposure to Ethanol

Long-term exposure to ethanol was modeled by intragastric administration of 20% ethanol via a gastric tube at a dose of 4 g/kg of pure ethanol from Monday to Friday for 4 weeks (20 administrations in total). Control animals received equivalent volumes of water according to the same scheme. All animals had free access to water.

Injections of Pharmacological Agents

After the end of prolonged ethanol exposure, the animals were treated for three days with intragastric administration of AZM (40 mg/kg, 160 mg/kg; Hemomycin, powder for preparation of oral suspension, Hemofarm, Serbia). The rats of the control group were given an equivalent volume of water.

Biomaterial Sampling

After the end of the experiment, the rats were decapitated on the last day of prolonged ethanol exposure and on day 7 of ethanol withdrawal, and the selected brain structures were collected. The NAc borders were defined according to the rat brain atlas [12]. Brain samples were immediately frozen and stored at -80°C.

RNA Isolation

Total RNA was isolated using the ExtractRNA reagent (Eurogen, Russia) in full accordance with the manufacturer's instructions. The concentration of the obtained RNA was measured using an Implen NanoPhotometer P330 spectrophotometer (Implen, Germany), the purity of the isolated product was assessed by the A260/A280 ratio (normally ≥1.8).

RT-PCR

cDNA was synthesized by reverse transcription (RT) in 20 μl of the reaction mixture using the MMLV RT kit (Eurogen) in full accordance with the manufacturer's instructions. Polymerase chain reaction (PCR) with real-time detection was carried out in an Mx3005P amplifier (Stratagene, USA) in 10 μl of the reaction mixture containing SYBR Green (Eurogen) and a mixture of specific forward and reverse primers (Table 1) (Beagle, Russia). The relative level of the mRNA content, calculated by the $2^{\Delta\Delta Ct}$ method, was normalized to the level of *Gapdh* gene expression.

Statistical Data Processing

Statistical processing of the obtained data was carried out using the Graph Pad Prizm v.6 software. The Mann-Whitney U test for independent samples was used to compare groups. Differences were considered statistically significant at $p \leq 0.05$. Since we did not plan to elucidate a dose-dependent effect of the drug, two-factor analysis of variance was not used.

In Silico Analysis

In order to search for potential molecular targets for AZM, an *in silico* analysis was performed using the Way2drug software [13]. Using this software it is possible to determine the probability of interaction of AZM with a particular potential molecular target in the body.

RESULTS AND DISCUSSION

The Level of TLR4 System Gene Expression during Long-Term Ethanol Exposure and its Withdrawal

We have analyzed expression of the following genes of the TLR4 signaling pathway system: *Hmgb1*, *Tlr4*, *Il1β*, *Ccl2*, *Irf3*. The relative level of mRNA for all these genes demonstrated several-fold increase in the NAc in the group of animals exposed

Table 1. Sequences of primers used in this study

Gene	Primers	
	Forward (5′-3′)	Reversed (5′-3′)
Tlr4	ACTCTGATCATGGCATTGTT	GTCTCAATTTCACACCTGGA
Hmgb1	CTCTGATGCAGCTTATACGA	AAAAGACTAGCTTCCCCTTG
Nfκb1	ATACTGCTTTGACTCACTCC	AGGTATGGGCCATCTGTT
Irf3	AATTCCTCCCCTGGCTC	CATGGGATCCTGAACTTTGT
Il1β	TGTCTGACCCATGTGAGCTG	TTTGGGATCCACACTCTCCAG
Ccl2	AAGATGATCCCAATGAGTCG	TGGTGACAAATACTACAGCTT
Gapdh	GCCAGCCTCGTCTCATA	GTGGGTAGAGTCATACTGGA

to long-term ethanol intake as compared to the group of intact animals. The level of HMGB1 mRNA increased by 4.0 times, TLR4 mRNA increased by 2.2 times, IL1β mRNA increased by 4.1 times, CCL2 mRNA increased by 3.9 times, and IRF3 mRNA increased by 12.0 times. In the group of ethanol withdrawal animals the relative levels of HMGB1, TLR4, and CCL2 mRNAs changed insignificantly, while the levels of IRF3 and IL1β mRNAs increased relative to the control (by 12.2 times and 3.2 times, respectively). AZM administration corrected the mRNA changes observed during ethanol withdrawal. The use of AZM decreased the levels of IRF3 and IL1β mRNAs. Despite the fact that the expression of the *Hmgb1*, *Tlr4*, *Ccl2* genes was not changed during the period of ethanol withdrawal, the AZM administration reduced the expression of these genes below lower values found in the group of intact animals.

Our data suggest that the development of elements of neuroinflammation in the NAc occurs due to prolonged ethanol intake. In the group of animals with prolonged ethanol exposure (see Figs. 1–2 "ethanol"), we found increased expression of the genes encoding key proinflammatory cytokines *Il1β* and *Ccl2*. One of the mechanisms causing the development of this condition may be an increase in the activity of the TLR4-dependent signaling pathway, which is responsible for regulating the expression of proinflammatory cytokine genes [8, 10]. Activation of these mechanisms during prolonged ethanol intake in the cerebral cortex has been well demonstrated by Crews et al. [14–16]. However, the expression state of these genes in the subcortical structures of the brain has been insufficiently studied. In our work, we have focused on one of the key structures responsible for the manifestation of addictive behavior and obtained information that the expression of the genes of the TLR4-signaling pathway system could be affected in this brain structure. It is possible that these changes may also contribute to the pathogenesis of alcohol dependence. In the present study, we assessed the expression status of the TLR4 system genes on day 7 after the withdrawal of long-term ethanol exposure. We did not observe significant changes in the expression of the *Tlr4* and *Hmgb1* genes, but an increased expression of the *Il1β* gene was found. It should be noted that in our earlier work, we already reported the expression status of these genes in the NAc on day 7 after the withdrawal of long-term ethanol exposure [10]. However in that study, an increased mRNA level was detected for the *Tlr4* and *Hmgb1* genes after ethanol withdrawal. One explanation for these differences consists in different ethanol exposure protocols: in the previous study we administered 20% ethanol solution to rats at a dose 2 g/kg for 2 months, whereas in the present study we used the same dosages but for a shorter time period. The literature data analysis shows that the use of different protocols of ethanol exposure often leads to contradictive results. This also complicates comparison of results obtained by different research groups employing different protocols of the long-term ethanol exposure.

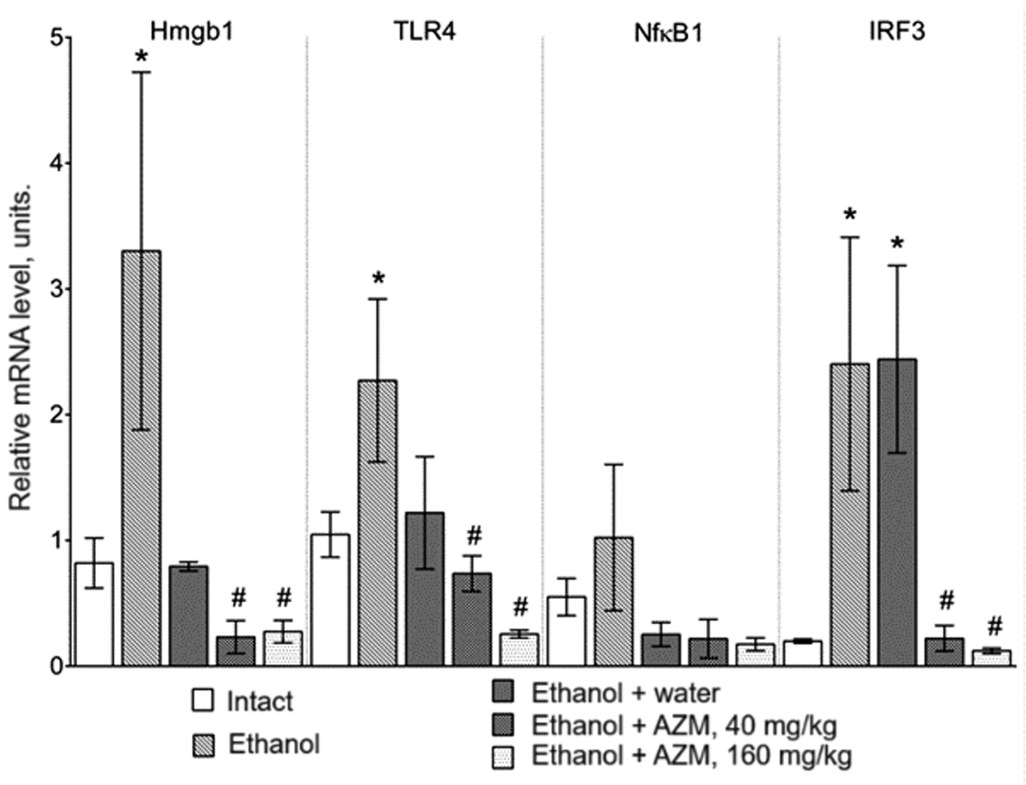

Figure 1. The level of the TLR4 system genes expression in the NAc. *$p \leq 0.05$ as compared to the group of intact animals, #$p \leq 0.05$ as compared to the group "ethanol + water".

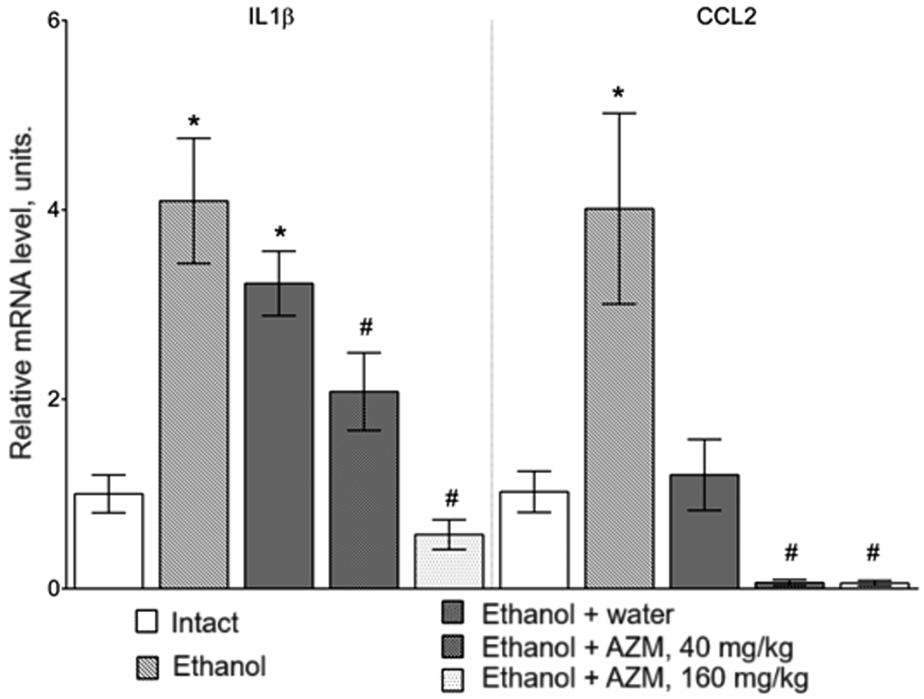

Figure 2. The level of the proinflammatory cytokine gene expression in the NAc. *$p \leq 0.05$ as compared to the group of intact animals, #$p \leq 0.05$ as compared to the group "ethanol + water".

The Effect of AZM on the TLR4 System Gene Expression

According to the data obtained, AZM reduced the mRNA level of neuroinflammation-related genes in the NAc.

Previously, the ability of AZM to reduce the neuroinflammation level was shown in several studies in various pathological conditions. Spinal cord injury modeling in mice is accompanied by the increase in the level of proinflammatory macrophages (M1 macrophages) at the site of injury, which is one of the reasons aggravating the course of regeneration. The use of AZM (160 mg/kg, during three consecutive days) caused a decrease in the level of M1 macrophages and accelerated the process of tissue regeneration [17]. Another study showed a decrease in the level of M1 macrophages in a spinal cord injury model by administering AZM at doses of 10 mg/kg, 40 mg/kg or 160 mg/kg for 7 days; the lowest (10 mg/kg) and highest (160 mg/kg) doses increased the expression of marker genes of anti-inflammatory macrophages (M2 macrophages) [18]. Administration of AZM to mice after spinal cord injury (at 30 min, 3 h, and 24 h, and then daily for 7 days) showed that the drug was effective at 30 min and 3 h after injury, but not at 24 h [19]. However, regardless of the time of initial treatment, AZM did not reduce the development of neuropathic pain or increase neuronal survival [19]. A single intraperitoneal administration of AZM (150 mg/kg) to mice subjected to transient middle cerebral artery occlusion reduced the level of cerebral edema in the ischemic hemisphere [20]. A single administration of AZM (150 mg/kg) prevented retinal ganglion cell death. This effect was accompanied by a decrease in the activity of calpain, MMP-2/-9 and the level of ERK1/2 phosphorylation [21]. In another study, a single dose of AZM (150 mg/kg) after modeling ischemia resulted in a decrease in ischemic brain damage and an increase in STAT3 phosphorylation in astrocytes and neurons of the peri-ischemic motor cortex and central striatum [22]. However, the molecular targets of the neuroprotective effects of AZM remain unclear.

In one recent study, lipopolysaccharide addition (LPS; 2 μg/ml) to the culture of rat alveolar macrophage cells NR8383 increased TLR4 protein expression, but when cells were co-cultured with LPS (2 μg/ml) and AZM (8 μg/ml), the level of TLR4 protein expression did not differ significantly from the control group. In this study AZM attenuated the overexpression of NF-κB (P65), EZH2, and H3K27me3 and normalized the reduced level of the anti-inflammatory cytokine IL-10. AZM also inhibited LPS-induced nuclear translocation of NF-κB (P65) [23].

Taking into consideration the available data on the contribution of increased TLR4 expression and microglia activation into a proinflammatory phenotype during long-term ethanol intake [8, 10], we suggested that the use of AZM would be able to make changes in these mechanisms. Our results confirmed this suggestion. Treatment with AZM had a positive impact on the expression of genes encoding key proinflammatory cytokines *Il1β* and *Ccl2* (their expression increased in activated microglia). In addition, we have found that AZM decreased expression of both *Tlr4* and also *Hmgb1*, which

encodes an endogenous TLR4 agonist protein. An increase in the content of the TLR4 mRNA and the protein product is considered as one of the possible prognostic markers of the development of neuroinflammation in the nervous tissue after long-term ethanol intake [7, 8]. AZM did not affect *Nfκb1* expression (P50), but reduced the expression of the interferon transcription factor 3 gene (*Irf3*). At the same time, certain evidence exists that microglia with IRF3 overexpression upregulates key anti-inflammatory cytokines (IL-1, IL-10, and IFNβ receptor antagonist) and suppresses pro-inflammatory cytokines (IL-1α, IL-1β, TNFα, IL-6, IL-8, and CXCL1) [24]. Thus, it appears that upregulation of neuroinflammation-related genes, triggers a regulatory pathway that would restrain excessive expression of pro-inflammatory cytokines. It is possible that the increased level of *Irf3* expression observed in the experiment in the group of animals with long-term exposure to alcohol and with alcohol withdrawal on day 7 serves to restrain high expression of pro-inflammatory cytokines. This speculation requires further research.

In Silico Analysis

Based on the results of the *in silico* analysis, among all the potential biomolecules characterized by a high score probability (>0.8) of their interaction with AZM we have selected molecular targets that are related to the genes that we have investigated in our experiments (Table 2). The *in silico* analysis partially explains the pharmacological effects of the drug we used.

Table 2. Results of *in silico* analysis of the interaction of AZM with molecular targets in the body and their localization

Target of interaction[a]	**Interaction probability (p)**[a]	**Localization in NAc**[b]
NUAK2, NUAK family SNF1-like kinase 2	0.87	Predominantly expressed by microglial cells
NTF3, NT-3 growth factor receptor (TrkC)	0.86	Highly expressed by neurons and glial cells
MAP3K1, Mitogen-activated protein kinase kinase kinase 1	0.84	Expressed by neurons and glial cells
MAP3K3, Mitogen-activated protein kinase kinase kinase 3	0.84	Expressed by neurons and glial cells
TNK1, Non-receptor tyrosine-protein kinase TNK1	0.84	Expressed by neurons
PHKG1, Phosphorylase kinase catalytic subunit gamma 1	0.83	Expressed by neurons and glial cells
CILK1, Serine/threonine-protein kinase ICK	0.83	Highly expressed by neurons and glial cells
LATS2, Serine/threonine-protein kinase LATS2	0.82	Predominantly expressed by glial cells
IRAK3, Interleukin-1 receptor-associated kinase 3	0.81	Predominantly expressed by microglial cells
CAMK2A, CaM-kinase alpha	0.81	Predominantly expressed by neurons
RIOK1, Serine/threonine-protein kinase RIO1	0.81	Expressed by neurons and glial cells
MAP2K4, Dual specificity mitogen-activated protein kinase kinase 4	0.81	Expressed by neurons and glial cells
SIK2, Serine/threonine-protein kinase SIK2	0.81	Expressed by neurons and glial cells
RIOK3, Serine/threonine-protein kinase RIO3	0.80	Expressed by neurons and glial cells
PRKAA1, AMP-activated protein kinase alpha-1 subunit	0.80	Expressed by neurons and glial cells
PAK2, Serine/threonine-protein kinase PAK2	0.80	Expressed by neurons and glial cells
MAP3K5, Mitogen-activated protein kinase kinase kinase 5	0.80	Expressed by neurons and glial cells

The information source: a Way2drug [13]; b The Human Protein Atlas [33].

In order to explain the effects of AZM on the studied genes we have analyzed existing literature data regarding the molecular targets identified by the *in silico* method. Among potential molecular targets the effects we observed in the experiment could be explained by the influence of AZM, primarily on NTF3 (neurotrophin receptor 3 or TrkC, NT-3 growth factor receptor), IRAK3 (interleukin-1 receptor-associated kinase 3), and PAK2 (serine/threonine-protein kinase PAK2).

The increase in MCP-1 (CCL2) and fractalin levels is attributed to activation of the TrkC receptor by the neurotrophic factor PDNF [25]. Intravenous administration of PDNF increased serum MCP-1 and fractalin levels in *Myd88* knockout mice (deficient in TLR signaling) thus highlighting the MyD88-independent action of PDNF [25].

There is evidence that the IRAK3-mediated pathway suppresses proinflammatory signaling. According to results of a recent study, IRAK3 is expressed in macrophages as well as in pancreatic acinar cells, where it inhibits NF-κB activation [26]. Deletion of the *Irak3* gene increased monocytes migration into the pancreas of mice and caused type 1 proinflammatory immune response, characterized by a significant increase in serum levels of TNFα, IL-6, and IL-12p70 [26].

Regarding the PAK2 kinase, certain evidence exists that modeling a high-calorie diet in mice with cardiac-specific deletion of PAK2 ($Pak2^{cKO}$) was accompanied by an increase in the mRNA levels of proinflammatory cytokines *Il1β*, *Crp*, *Mcp1*, *Ccl24*, and *Hmgb1*, while the mRNA levels of genes encoding anti-inflammatory cytokines (*Il10*, *Mmp9*, and *Gdf15*) were reduced [27]. In another study, a decrease in PAK2 was associated with oxidative stress and activation of apoptosis in N2a neuroblastoma cells under hypoxia [28]. Taking into consideration our *in silico* data on possible binding of AZM to PAK2, this kinase may be one of the targets mediating AZM effects.

Since TNK1 protein expression is shown predominantly in neurons, this makes it difficult to assume that AZM may influence neuroinflammatory pathways via the TNK1 protein. The same should be noted for the CAMK2A protein, which is also expressed predominantly by neurons in the brain.

Limited information currently exists on the functional interaction of other target genes (and corresponding protein products) used for *in silico* evaluation of their putative interaction with AZM. We can only speculate that MAP kinase cascades of reactions are also involved in the signal transduction from innate immunity receptors to innate immunity genes [29], in particular, to pro- and anti-inflammatory cytokines. In this context AZM, targeting one of its possible targets among MAP kinases (MAP3K1, MAP3K3, MAP2K4, MAP3K5), can also induce changes in these regulatory mechanisms.

It should be also noted that our *in silico* modeling did not reveal any possible interactions between AZM and subunits of the NF-κB complex, discussed in the literature [30–32]. This may be due to the fact that the software we used has some limitations and is not able to identify a larger number of possible molecular targets, or the hypothesis of an anti-inflammatory effect of AZM through NF-κB itself requires more thorough experimental verification.

CONCLUSIONS

The results of this study have demonstrated the effect of AZM (40 mg/kg, 160 mg/kg) on the expression of TLR4-signaling system genes (*Hmgb1*, *Tlr4*, *Irf3*, *Il1β*, *Ccl2*) on in the NAc of rats on day 7 after the withdrawal of long-term ethanol exposure. Being administered intragastrically, both doses were effective during the first three days after ethanol withdrawal. Our *in silico* analysis revealed a number of proteins with a high probability of interaction with AZM. Considering that some of these proteins are expressed by nervous tissue cells at a high level and are associated with mechanisms regulating neuroinflammation signaling cascades, we assume that these molecular targets are associated with the observed effects of AZM in our experiment.

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COMPLIANCE WITH ETHICAL STANDARDS

The study was reviewed and approved by the Ethics Committee for Animal Research of St. Petersburg State University, protocol No. 131-03-1 dated January 10, 2025.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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ИЗУЧЕНИЕ ВОЗДЕЙСТВИЯ АЗИТРОМИЦИНА НА ЭКСПРЕССИЮ ГЕНОВ СИСТЕМЫ TOLL-ПОДОБНЫХ РЕЦЕПТОРОВ В ПРИЛЕЖАЩЕМ ЯДРЕ ГОЛОВНОГО МОЗГА КРЫС ПРИ ОТМЕНЕ ДЛИТЕЛЬНОГО ВОЗДЕЙСТВИЯ ЭТАНОЛА И ПОИСК ВОЗМОЖНЫХ МОЛЕКУЛЯРНЫХ МИШЕНЕЙ МЕТОДОМ *IN SILICO*

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Прилежащее ядро (NAc) головного мозга — ключевое звено в системе внутреннего подкрепления, которое опосредует проявление различных компонентов зависимости, в том числе от этанола. Нейровоспалительная теория развития алкоголизма предполагает, что изменения в молекулярных механизмах врождённой иммунной системы могут быть вовлечены в развитии патологии. Цель нашего исследования заключалась в изучении влияния азитромицина (АЗМ) на состояние экспрессии генов системы toll-подобных рецепторов в NAc головного мозга крыс при экспериментальной алкоголизации. Также в задачи исследования входил поиск методом *in silico* возможных молекулярных мишеней для АЗМ, которые могли бы быть связаны с системой toll-подобных рецепторов. АЗМ корректировал изменения, наблюдаемые в экспрессии генов системы системой toll-подобных рецепторов в условиях отмены длительной алкоголизации в NAc головного мозга. Выполненный анализ *in silico* выявил ряд белков, с которыми была обнаружена высокая вероятность взаимодействия АЗМ, что позволило сделать ряд предположений о возможных путях реализации наблюдаемого фармакологического эффекта АЗМ в эксперименте.

Полный текст статьи на русском языке доступен на сайте журнала (http://pbmc.ibmc.msk.ru).

Ключевые слова: прилежащее ядро; этанол; нейровоспаление; toll-подобные рецепторы; азитромицин; *in silico*

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