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Neurochemical and pharmacological analysis of toll-like receptor system of rat brain under the action of ethanol

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Introduction

Relevance of the research topic

Systematic abuse of ethanol-containing products by the population is a serious social problem, the solution of which is an important task of the health care system (Shabanov P.D., 1998, 2002, 2012; Eryshev O.F., 2002).

The scale of this problem persists today in many countries of the world (Burov Y.V., 1985; Eryshev O.F., 2002; Shabanov P.D., 2002, 2012; Vetlugina, T.P., 2023). In this regard, there is a need for studies aimed at obtaining new information about the peculiarities of the development of metabolic changes in the body during chronic ingestion of high doses of ethanol. Nervous tissue is one of the most susceptible to ethanol intake in the body due to its high content of lipids and water (Burov Y.V., 1985; Shabanov P.D., 1998, 2002, 2012). For this reason, it seems important to conduct studies aimed at neurochemical analysis of the changes developing in the brain during systematic ethanol ingestion (Burov Y.V., 1985; Shabanov P.D., 1998, 2002, 2012). The second reason why the nervous tissue is of the greatest interest for studying the influence of ethanol is an extremely complex system of regulation of biochemical processes occurring here, and ethanol intake into the body serves as a cause of their dysregulation, which in turn leads to the development of various dysfunctions in the brain structures (Burov Y.V., 1985; Shabanov P.D., 1998, 2001, 2002, 2007, 2008, 2012; Droblenkov, A.V., 2008, 2011).

In recent years, researchers have focused on studying changes in neuroimmune mechanisms that are realized in neural tissue among different parts of the brain upon systematic/long-term consumption of products containing ethanol (Coleman L.G. et al., 2018; Crews F.T. et al., 2013, 2017; Orio L. et al., 2019; Sanchez-Alavez M. et al., 2018; Melbourne, J.K., 2019). Recent studies have concluded that ethanol ingestion leads to increased gene activity of some toll-like receptor (TLR) subtypes in the brain, as well as increased gene expression of their endogenous agonists (Antón M. et al., 2017; Crews F.T. et al., 2013; Pascual M. et al., 2015; Whitman B.A. Et al., 2013).

In rodent experiments and in studies of postmortem brain samples from humans with alcoholism, ethanol has been shown to increase TLR (TLR2, TLR3, TLR4, TLR7) expression at the protein level in the brain, which persists over time (Vetreno R.P. et al., 2012, 2013).

TLR activation by specific agonists serves as a signal to trigger intracellular reaction cascades that lead to the activation of many innate immune system genes (Crews F.T. et al., 2017; Hanke M.L. et al., 2011; Okun E. et al, 2009), as well as to the development of neuroinflammatory

process in the CNS, which may result in increased levels of neurotoxicity with subsequent death of neurons and neuroglia cells in the brain (Blednov et al., 2011, 2013; Qin et al., 2008, 2013).

In addition to the participation of TLRs in the realization of the neuroinflammatory process, TLRs may also play a role in the regulation of other molecular and cellular mechanisms that may also be involved in the development of pathological attraction to alcohol (Warden A.S. et al., 2018; Randall P.A., et al., 2018; Liu J. et al., 2011).

In the available works, researchers have mainly focused their attention on studying the activity level of TLRs and mRNA levels of various proinflammatory factors in rat and mouse models of alcoholization (Lawrimore C.J. et al., 2019; Blednov Y.A. et al., 2017; Qin L et al., 2007, 2008, 2012). At the same time, it is of interest to analyze the dynamics of changes in the level of TLRs activity during alcohol withdrawal and to study the duration of the neuroinflammatory process in the CNS mediated by TLRs.

It is also important to understand how the level of TLRs activity changes in various brain structures that are primarily affected by alcohol intoxication and that are part of the reinforcement system. Such brain structures are considered key in studying the mechanisms of various forms of addiction, including alcohol addiction (Nozdrachev, A.D., 2000; Shabanov P.D., 1998, 2001, 2002, 2007, 2008, 2012; Nestler E.J., 2005) Droblenkov, A.V., 2008, 2011; Koob, G.F., 2014; Coleman L.G., 2018; Crews F.T., 2013, 2017). Of particular interest here are brain structures such as the medial prefrontal cortex (mPFC), hippocampus, striatum, amygdala, and entorhinal cortex (EC). More research is needed to answer these questions and move a little closer to understanding how neuroimmune signaling functions in the pathogenesis of alcoholism.

Studying the mechanisms of activation of the inflammatory process through TLR-dependent signaling may reveal new targets for pharmacological action (Crews F.T. et al., 2017), which can be used in the complex therapy of alcoholism, as well as for the prevention of the development of severe forms of alcoholic encephalopathy (Tsygan, V.N., 2007; Gazatova N.D., 2018; Crews F.T. et al., 2017; Vetlugina, T.P., 2023).

Background of research topic

The available literature strongly suggests that prolonged ethanol exposure activates mechanisms of the innate immune system by increasing mRNA and protein expression of components of TLR-dependent signaling pathways. Exposure to toll-like receptor agonists triggers ethanol-like molecular signaling pathways in neural tissue cells that lead to increased activity of pro-inflammatory genes, whereas, on the contrary, the use of toll-like receptor antagonists (before/after ethanol exposure) decreases the expression of neuroinflammatory genes. Thus, at this

point, the neuroinflammatory theory of alcoholism development has been developed, the essence of which is that the pathogenesis of alcoholism is inextricably linked to the activation of genes of the innate immunity system. Despite this, most of the data for the formation of this hypothesis have been obtained on cortical structures and in vitro experiments, while other brain structures associated with the development of alcoholism have not been studied sufficiently, sometimes there is only brief information. This area of research has been actively developed in the last 10-15 years, but there are not many studies aimed at pharmacological correction of the observed changes at the molecular level by potential pharmacological agents. Thus, there is a need for studies aimed at analyzing the toll-like receptor system among various brain structures associated with the development of alcoholism, as well as in the application of potential pharmacological agents aimed at correcting changes at the molecular level. In particular, such compounds may include ginseng ginsenosides and rifampicin, which, according to the literature, have established themselves as agents for the correction of the neuroinflammation system in various pathological conditions of the nervous system. These compounds have the ability to penetrate through the GEB and reduced the level of expression of proinflammatory mediators. There is an assumption that the mechanism of action of ginsenosides may be mediated by their influence on the toll-like receptor system, and for rifampicin its direct binding to MD2, a TLR4 correceptor, has been shown to cause inhibition of TLR-signaling intracellular signal transduction pathways.

Purpose of the study

To study the dynamics of toll-like receptor system gene expression in different structures of the rat brain under conditions of chronic alcoholization, ethanol withdrawal, and against the background of pharmacotherapy.

Research Objectives

1. to study the dynamics of toll-like receptor system gene expression in different brain structures (hippocampus, entorhinal cortex, striatum, amygdala) of rats under chronic alcoholization and under conditions of ethanol withdrawal.

2. to study the dynamics of cytokine gene expression (*Il-1b, Ccl2*) in different structures of the rat brain under chronic alcoholization and under conditions of ethanol withdrawal. Identify functional relationships between TLR3/4/7 expression and Il-1b and Ccl2 expression in the hippocampus, entorhinal cortex, striatum and amygdala.

3. To study the expression of TLRs ligands (Hmgb1, microRNA) in different structures of rat brain under chronic alcoholization and under conditions of ethanol withdrawal.

4. To study the effects of rifampicin and ginzenosides on the expression of genes of toll-like receptor system in the nucleus accumbens and hippocampus of the brain of alcoholized rats subjected to ethanol withdrawal.

Scientific novelty of the research

The novelty of the performed study lies in the fact that for the first time the analysis of gene expression of toll-like receptor system in different brain structures in long-term alcoholized rats, as well as at different withdrawal periods of long-term ethanol exposure was performed. The results of the study showed that the level of *Tlr3* gene expression was not subject to changes in most of the brain structures studied (striatum, nucleus accumbens, amygdala, entorhinal cortex), except for a slight decrease in the hippocampus; the expression of *Tlr4* and *Tlr7* genes was not altered in any of the brain structures studied. However, there was evidence of multidirectional changes in *Tlr3, Tlr4*, and *Tlr7* gene expression upon withdrawal of long-term ethanol exposure at different time points. The degree of changes in gene expression depended not only on the time after ethanol withdrawal and brain region, but also on the modeling of ethanol exposure, i.e., on the duration and concentration of administered doses of ethanol solution. Thus, different variants of modeling long-term alcoholization have different effects on toll-like receptor system gene expression. Evaluation of the expression level of other genes from the toll-like receptor system (genes of adaptor proteins, transcription factors, pro- and anti-inflammatory cytokines) also showed the presence of changes in the studied rat brain structures both in the groups of long-term ethanol exposure and at ethanol withdrawal for different periods. The obtained data are new for both domestic and world literature, as the analysis of expression of a series of genes from the toll-like receptor system in the studied brain structures has not been previously performed.

Original in the work is the pharmacological approach aimed at correction of altered molecular mechanisms by prolonged exposure to ethanol in such brain structures as the hippocampus and the nucleus accumbens. These brain structures are the most susceptible to various changes during prolonged ethanol exposure. There is speculation that changes in these structures may be key in the formation of alcohol dependence. The results of the study showed that ginseng ginsenosides and rifampicin have an effect aimed at restoring the level of expression of a number of genes from the toll-like receptor system.

Theoretical and practical significance of the work

The scientific and theoretical significance of the work is determined by the evidence of involvement of genes of toll-like receptor system in pathogenetic molecular mechanisms that develop under long-term ethanol exposure and ethanol withdrawal in a number of motor and emotionogenic brain structures, such as striatum, nucleus accumbens, hippocampus, amygdala, and entorhinal cortex. The work shows that different models of long-term ethanol exposure change gene expression of toll-like receptor system in different ways, as well as that different brain structures react differently to long-term ethanol intake into the organism, as well as when its intake is canceled. Theoretical significance of the dissertation work also lies in the fact that in this work we obtained information about the ability of ginseng ginsenosides and rifampicin to make changes in gene expression of TLR-system, which once again indicates that the long-known immunomodulatory effect of these compounds may be mediated by their influence on the molecular mechanisms of the system of innate immunity, including the system of toll-like receptors.

The practical significance of the work stems from the understanding that the obtained data on changes in gene expression of TLR-system reveal one of the molecular pathogenetic pathways that develop in the studied brain structures, which are key in the mechanisms of alcoholism development, which creates prerequisites for the use of these pathways as pharmacological targets for the correction of existing changes. Another practical result of the work is that the pharmacological substances used in the work, such as ginseng ginsenosides and the antibiotic rifampicin, have a corrective effect on the altered gene expression of the toll-like receptor system. In addition, the practical result is that the existing pathogenetic molecular mechanism can be regulated up to the recovery of a number of indicators to the level of control values with the use of pharmacological substances, which opens new opportunities for studying the ways of pharmacological correction of the observed changes in the studied brain structures under prolonged exposure to ethanol by affecting the toll-like receptor system.

Study methodology and methods

The methodology of the study consisted of prolonged exposure of male Wistar rats to ethanol using two models - semi-induced prolonged exposure to ethanol solution as the sole source of fluid, and by intragastric administration of ethanol solution through a gastric tube. Summa ginsenosides as well as the antibiotic rifampicin were administered to the animals intraperitoneally during the first 7 days of withdrawal of prolonged ethanol exposure. Ginsenosides of ginseng were

obtained by their extraction from the culture of Japanese ginseng, which was previously cultivated under in vitro conditions (Lomonosov Moscow State University, Department of Plant Physiology). According to the results of extraction, the sum of ginsenosides was obtained and its chemical composition was mass spectrometrically evaluated for quantitative assessment of the content of different groups of ginsenoside compounds. At the end of the experiment, rat brain structures (striatum, nucleus accumbens, hippocampus, amygdala, entorhinal cortex) were collected, total RNA molecules were isolated from brain structures, cDNAs were obtained by reverse transcription reactions, and the level of relative gene expression was determined by real-time PCR. The health status of the animals was evaluated throughout the experiment.

Main scientific results

1. The data on the state of gene expression of toll-like receptor system in different brain structures in long-term alcoholized rats, as well as on different terms of withdrawal of long-term ethanol exposure were obtained (Airapetov M.I. et al., 2020). The results of the study showed that the level of *Tlr3* gene expression was not subject to changes in most of the studied brain structures (striatum, nucleus accumbens, amygdala, entorhinal cortex), except for a slight decrease in the hippocampus, the expression of *Tlr4* and *Tlr7* genes was not altered in any of the studied brain structures. However, new data were obtained on the presence of pronounced multidirectional changes in the expression of *Tlr3*, *Tlr4* and *Tlr7* genes under conditions of abolition of long-term ethanol exposure at different time periods in dynamics; see 3.1.1, 3.1.2, 3.1.3, 3.3.1, 3.4.2 (Ayrapetov M.I. et al., 2020) (personal contribution is at least 80%).

2. It was revealed that not only the expression level of Tlr genes is subjected to pronounced changes under abolition of long-term ethanol exposure, but the whole system of toll-like receptor genes (endogenous agonists, genes of adaptor proteins, transcription factors, pro- and anti-inflammatory cytokines) is changed to a greater or lesser extent in the brain structures of experimental animals; see 3.1.4, 3.2, 3.3.2, 3.3.3, 3.3.3, 3.3.4, 3.4.3, 3.4.4, 3.5 (M.I. Airapetov et al, 2020; 2022; Airapetov M.I. et al., 2024) (personal contribution is at least 80%).

3. It was shown that correction of altered molecular mechanisms by long-term ethanol exposure in the hippocampus and adjacent nucleus of the rat brain using potential anti-neuroinflammatory compounds rifampicin and ginsenosids allowed to influence the expression of a series of genes from the toll-like receptor system. The selected compounds in this work have proved themselves, according to the literature, as potential neuroprotectors, which are able to eliminate the signs of neuroinflammatory response in various pathological conditions of the central nervous system, as well as modulate the level of expression of the studied genes and their

translation products. The obtained data on the restoration of a number of altered molecular mechanisms with the help of selected pharmacological compounds are very interesting and open the prospect of a new direction in this field of research for further work; see 3.3, 3.4 (Airapetov M.I. et al., 2022; Airapetov M.I. et al., 2024) (personal contribution is at least 80%).

4. The obtained data on the state of gene expression of the toll-like receptor system indicate the involvement of this system in the pathogenetic mechanisms that develop during long-term ethanol exposure, in the emotiogenic and motor structures of the brain. This expands the existing theoretical understanding of the involvement of toll-like receptors in the neurobiology of alcoholism; see 1.3 (Airapetov M.I. et al., 2021) (personal contribution is at least 80%).

Statements to be defended

1. Alcoholization (1 month) does not cause significant disturbances of TLR3/4/7 expression in emotiogenic structures of rat brain. At the same time, ethanol withdrawal stimulates a dramatic imbalance of TLR3/4/7 gene expression and pro-inflammatory cytokines (IL-1β and CCL2) during two weeks of observation.

2. The positive correlation between TLR3/4/7 gene expression level and gene expression level of pro-inflammatory cytokines (IL-1β and CCL2) indicates an important role of TLR3/4/7 in the development of neuroinflammation in chronic and unsystematic ethanol consumption.

3. One of the mechanisms of neuroinflammation during long-term alcoholization may be the increased expression of endogenous activator of TLRs - secretory protein Hmgb1 and changes in the content of microRNAs (miR-155, miR-let7b, miR-96, and miR-182) in emotiogenic brain structures.

4. In chronically alcoholized rats subjected to ethanol withdrawal, rifampicin will reduce the expression of neuroinflammatory genes and activate the anti-inflammatory cytokine genes *Il10* and *Il11* in the contiguous brain nucleus.

5. In chronically alcoholized rats subjected to ethanol withdrawal, ginsenosides correct the imbalance of toll-like receptor system gene expression in the nucleus accumbens and hippocampus of the brain.

Degree of reliability and approbation of the work

The thesis materials were reported at the VIII International Youth Medical Congress St. Petersburg Scientific Readings-2019" (St. Petersburg, 2019); XXXII Winter Youth Scientific

School "Perspective directions of physicochemical biology and biotechnology" (Moscow, 2020); XXIV International Medical and Biological Conference of Young Researchers "Fundamental Science and Clinical Medicine - Man and His Health" (St. Petersburg, 2021); XII All-Russian Scientific Conference of Students and Postgraduates with International Participation "Young Pharmacy - Potential" (St. Petersburg, 2021). S.V. Anichkov FGBNU "Institute of Experimental Medicine".

The work has been reviewed and approved by the Ethical Committee in the field of animal research of SPbSU, conclusion № 131-03-8 dated April 29, 2024.

Publications

On the subject of the thesis 19 scientific papers, including 7 journal articles (including 5 articles in journals by Scopus) and 11 theses have been published.

Personal contribution of the author to the research

The contribution of the author of the completed dissertation research was made at all stages of the work and consisted in the planning and preparation of the experimental part, in the direct performance of experiments, in obtaining and analyzing the obtained results, in their discussion and writing scientific publications (articles and theses), writing the manuscript of the text of the dissertation.

CHAPTER 1. LITERATURE REVIEW

1.1 The toll-like receptor system

The last decade has been marked by the discovery of a large number of pattern recognition receptors (PRRs) and their intensive study. They have been identified in all multicellular organisms, from invertebrates (e.g., sponges) to mammals and humans (Kokryakov V.N., 2006; Ketlinsky S.A., 2008; Lebedev K.A., Ponyakina I.D., 2017). To date, 5 families of signaling PRRs are known: Toll-like receptors (TLRs), C-type lectin receptors, scavenger receptors, NOD-like receptors and CARD chilicases (Lebedev K.A., Ponyakina I.D., 2017). All PRRs specifically bind to various molecular structures of microorganisms, including bacteria, fungi, viruses, and unicellular protozoa. PRRs also respond specifically to a number of plant-derived substances and complex synthetic molecules. All these compounds serve as their exogenous ligands. PRRs are also able to specifically respond to a number of substances of their own organism - endogenous ligands (Lebedev K.A., Ponyakina I.D., 2017).

The TLRs family is the most well studied. There are 10 representatives of this type (TLR1-TLR13) in human and 13 in mouse (Lebedev K.A., Ponyakina I.D., 2017, Yamamoto M., et al., 2002). All TLRs have a similar structure and are integral transmembrane proteins consisting of 3 parts that differ in their functions. The extracellular N-terminal region, responsible for ligand binding, has 19-25 leucine-rich repeats. This is followed by a cysteine-rich transition region, which is responsible for the attachment of the receptor to membrane proteins. Finally, the cytoplasmic site, represented by the TIR domain (Toll/IL-1 receptor), carries out interaction between TLRs and adaptor proteins, triggering intracellular signaling cascades of reactions (Lebedev K.A., Ponyakina I.D., 2017) (Table 1).

Table 1 - TLR adaptor proteins (Murphy K., et al., 2017)

Receptor	Adaptor protein
TLR1/TLR2	Myd88/TIRAP
TLR3	TRIF
TLR4	Myd88/ TIRAP; TRIF/TRAM
TLR2/TLR6	Myd88/ TIRAP
TLR7	Myd88
TLR8	Myd88
TLR9	Myd88
TLR11/TLR12	Myd88
TLR13	Myd88

All subtypes of TLRs are expressed in the CNS and are distributed as follows: TLR1-TLR9 are expressed by microglia cells, TLR3 and TLR7-9 are expressed by neurons, TLR2-3 and TLR9 by astrocytes, TLR2-3 by oligodendrocytes (Hanke M.L., et al., 2011; Esen N., et al., 2009; Alexeeva O.S., 2019) (Figure 1).

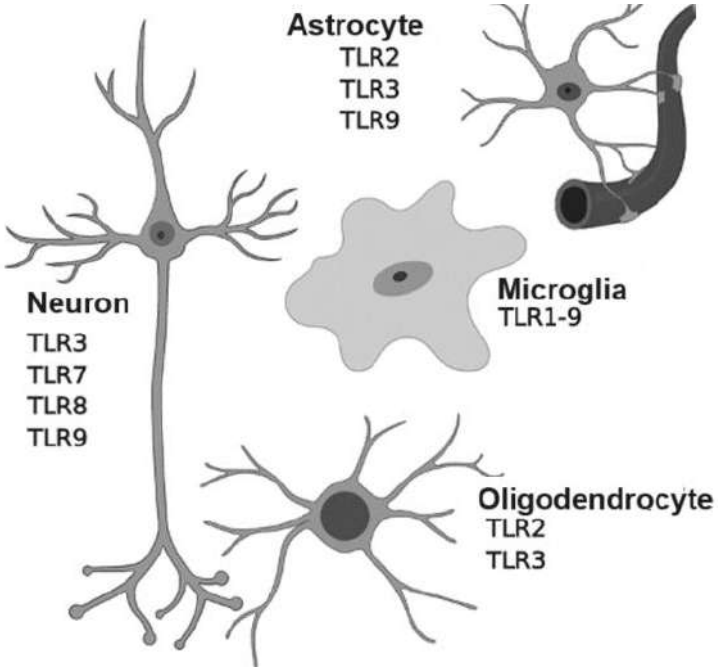

Figure 1 - TLR expression in the CNS (Airapetov M.I. et al., 2021)

It is important to note that TLR1-2 and TLR4-6 are expressed on the cytoplasmic membrane surface, while TLR 3, 7-13 are expressed on endosomes within the cell (Leifer C.A., et al., 2016; Nie L., et al., 2018).

1.2 Signaling cascades of TLR reactions

All TLRs function as dimers. TLR2 forms heterodimers with TLR1 or TLR6, and TLR11 with TLR12. TLR3-5, 7-9 and TLR13 form homodimers (Akira S., et al., 2004). After ligand recognition (Table 2), TLR dimerization occurs to form a heterodimer or homodimer, followed by conformational changes in the receptor necessary for interaction of the cytoplasmic TIR domain with intracellular adaptor proteins and subsequent activation of a cascade of intracellular signaling reactions (Akira S., et al., 2004).

The most common case is when the TIR domain of the TLR receptor binds to the adaptor protein MyD88 (Myeloid differentiation primary response 88). MyD88 interacts with kinases of the IRAK (IL-1 receptor associated kinase) family; the family consists of several enzymes: IRAK1-4 and IRAK-M. IRAK4 kinase is activated first, after IRAK1. Activation of the latter leads

to its interaction with TRAF6 (TNF receptor-associated factor 6). This factor can trigger two signal transduction pathways: 1) activation of AP-1 transcription factor by MAP-kinases (mitogen-activated protein kinase), JNK-kinases (c-Jun N-terminal kinases) and p38; 2) activation of TAK1/TAB complex and IKK (IκB kinase). After IKK activation, phosphorylation and degradation of the inhibitor protein IkB occurs, which leads to the release of the NF-kB dimer and its subsequent translocation to the nucleus, where NF-kB binds to the promoter regions of genes that activate and regulate the development of the inflammatory response. Such an intracellular signaling mechanism functions in the activation of almost all known TLRs, with the exception of TLR3. This indicates that different pathogens activating different TLRs initiate in principle a single universal pathway of inflammatory response activation (Lebedev K.A., Ponyakina I.D. (2017)). The TIR-domain of TLR3 and TLR4 can interact with the adaptor protein TRIF (TIR-domain containing adaptor inducing IFNb) (Smith K.M., et al., 2000). TRIF protein activates TRAF6 and TRAF3 (TNF receptor-associated factor 3). This results in the activation of intracellular factor TANK1 (TANK-binding kinase 1) and then IRF3 (Interferon regulatory factor 3). Activated IRF3 triggers the expression of IFNb and IFNa genes necessary for the development of antiviral response (Lebedev K.A., Ponyakina I.D., 2017).

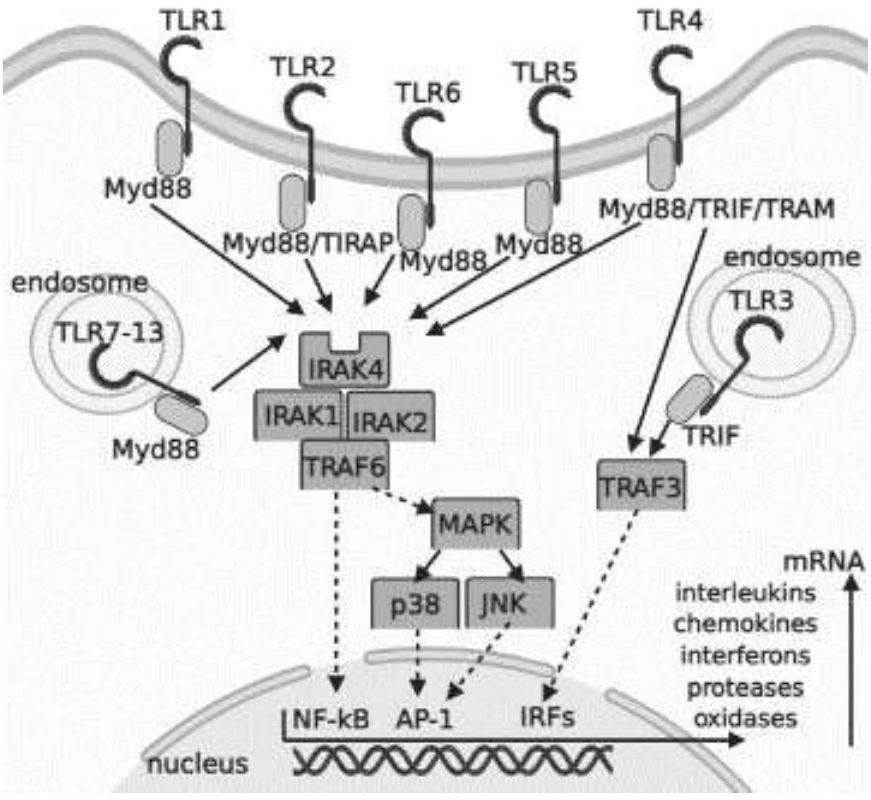

Figure 2 - Signaling cascades of TLR reactions response

(Airapetov M.I. et al., 2021, Lebedev K.A., Ponyakina I.D., 2017).

Table 2 - Ligands of TLRs (Volkov M.Y., 2016; Takagi M., et al., 2017; Jang T.H., et al., 2015; Murphy K et al., 2017)

Receptor	Exogenous ligands	Endogenous ligands
TLR1	Triacetylated peptides	Неизвестны
TLR2	Zymosan, diacetylated peptides, triacetylated peptides, lipoteichoic acid	rHSP70, gp96, HMGB1, uric acid, hyaluronic acid, α-synuclein
TLR3	Double-stranded RNA (dsRNA), poly (I:C)	mRNA, stathmin
TLR4	Lipopolysaccharide (LPS)	HMGB1, HSP60, HSP70, HSP72, hyaluronic acid, fibrinogen, protein S100, uric acid, heparan sulfate fragments, tenascin-c
TLR5	Flagellin	Unknown
TLR6	Zymosan, diacetylated peptides, triacetylated peptides, lipoteichoic acid, lipoarabinomannan	Unknown
TLR7	Imiquimod, gardiquimod, single-stranded RNA (ssRNA), miRNAs let-7, microRNA-21, imidazoquinoline, loxoribine, bropyrimine	Unknown
TLR8	Single-stranded RNA (ssRNA), ssRNA40/Lyovec, gardiquimod	Unknown
TLR9	DNA with unmethylated CpG oligodeoxynucleotides	Chromatin-IgG complexes
TLR10*	Double-stranded RNA (dsRNA)	Unknown
TLR11**	Profilin and profilin-like proteins	Unknown
TLR12**	Profilin	Unknown
TLR13**	Single-stranded RNA (ssRNA)	Unknown

* - found only in humans, ** - found only in mice

In addition to the above, a number of other adaptor proteins necessary for signaling from specific TLRs have been identified. TIRAP (TIR-domain-containing adaptor protein) participates together with MyD88 in signaling from TLR2 and TLR4, but not from other TLRs (Sinch-Jasuja

H., et al., 2001). TRIF-related adaptor molecule (TRAM) interacting with TRIF is used for signaling from TLR4 (Fitzgerald K., et al., 2003).

Moreover, activation of TLRs triggers several intracellular signaling pathways. This results in complex intracellular cascades of reactions that can cause both enhancement and inhibition of the final effect of cytokine expression. For example, activation of TLR3 and TLR4 can enhance TLR2 expression on the surface of macrophages in a Myd88-independent manner, whereas excitation of TLR7 and TLR9 induce its expression in a Myd88-dependent manner (Nilsen N., et al., 2004). TLR4 activation can positively regulate TLR2, TLR4 and TLR9 (Fitzgerald K., et al., 2003). Such regulation (TLR-TLR) often leads to an enhanced immune response by recruiting more TLRs, but the initial stimulatory dose and the timing of activation of the second TLR involved in the process can have a significant impact on the immune response (Lebedev K.A., Ponyakina I.D., 2017; Nilsen N., et al., 2004; An H., et al., 2006; Lee M.C., et al., 2007; Napolitani G., et al., 2005). In addition, different subtypes of TLRs may cooperatively form synergistic effects or form opposite responses to another TLR. Stimulation of the dendritic cell by TLR2 agonist counteracts the expression of IL10 and IL12, which is initiated by TLR3 and TLR4 agonists. TLR8 inhibits TLR7 and TLR9, and TLR9 inhibits TLR7 as a result of direct or mediated interactions between them (Wang J., et al., 2006).

1.3 Involvement of toll-like receptors in neuroimmune mechanisms during ethanol exposure

Ethanol consumption contributes to the activation of the innate immunity system via TLRs (Figure 3), which is expressed as an increase in the level of a number of pro-inflammatory signaling molecules (Tsygan, V.N., 2007; Gazatova, N.D., 2018; Crews F.T., et al., 2017; Vetlugina, T.P., 2023). Of TLRs in the context of alcoholism pathogenesis, TLR3, TLR4, TL7 have received the most attention in the literature (Crews F.T., et al., 2011, 2013, 2014, 2015, 2017; Hanke M.L., et al., 2011; Qin L., et al., 2012; Okun E., et al., 2009; Coleman L.G., et al., 2017; Pascual M., et al., 2015; Qin L., et al., 2007).

Elevated levels of pro-inflammatory cytokines play an important role in the development of a state of neurotoxicity with subsequent death of multiple cells in the CNS. While microglia are considered a major source of proinflammatory cytokines in the CNS, it has not been definitively elucidated what role neurons play in the ethanol-induced neuroimmune signaling chain (Lawrimore C.J., et al., 2017; Alexeeva O.S., 2019). The activity of TLRs depends on the level of exogenous and endogenous ligands by which they are activated. Ethanol consumption is accompanied by an increase in the level of endogenous ligands, in particular HMGB1 (Crews F.T., et al., 2017), heat shock proteins (Vabulas R.M., et al., 2002), extracellular matrix breakdown

proteins (Wang J., et al., 2006), and various microRNA variants (Volkov M.Y., 2016; Takagi M., et al., 2017; Jang T.H., et al., 2015; Murphy K., et al., 2017). Endogenous ligands are released in response to inflammatory activation in the brain and during apoptotic cell damage in the CNS (Crews et al., 2011, 2013, 2014, 2014, 2015, 2017). Exogenous ligands and various cytokines can be transported with the bloodstream into the CNS from the periphery (Crews F.T., et al., 2015).

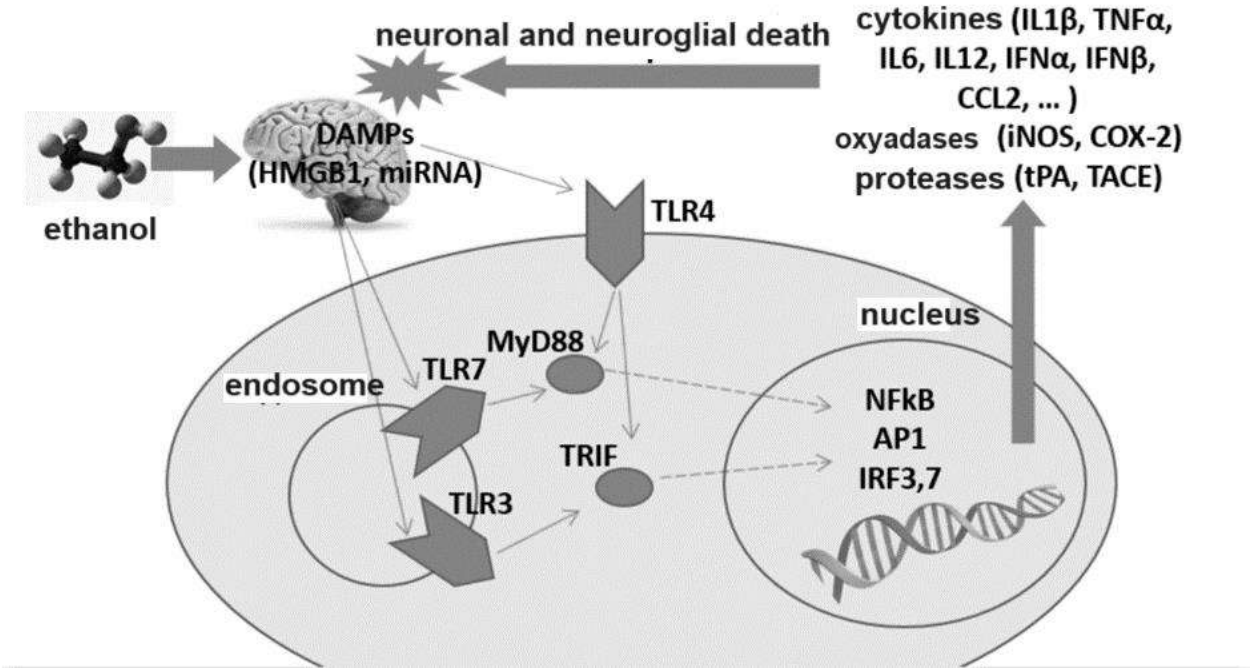

Figure 3 - Hypothesized views of the effects of ethanol on the toll-like receptor system in the brain

1.3.1 The role of TLR3 in the pathogenesis of alcoholism

Increased levels of TLR3 mRNA have also been reported in postmortem samples of the orbifrontal cortex of alcoholic individuals (Crews F.T., 2013). There is evidence to suggest that TLR3-dependent signaling influences voluntary ethanol consumption in rodent experiments (McCarthy G.M., et al., 2018; Warden A.S., et al., 2019). Alcoholization of mice for 10 days results in an increase in TLR3 mRNA levels in the mouse brain, as well as an increase in TLR3 protein expression levels in the orbifrontal and entorhinal cortex (Crews F.T. et al., 2013).

A single intraperitoneal injection of the TLR3 agonist, poly (I:C) (poly-inosine-poly-poly-cytidylic acid, a synthetic analog of double-stranded RNA from viruses), into mice results in increased levels of voluntary ethanol consumption in a two-tailed test, with the increased ethanol consumption developing over several days (Warden A.S., et al., 2019).

By investigating the effects of poly (I: C) on gene expression in the nucleus accumbens (nucleus accumbens, NA) of the rat brain, it has been shown that activation by a TLR3 agonist leads to increased mRNA levels of TLR3, COX2 (cyclooxygenase 2) and glutamatergic system genes (mGluR2 - metabotropic glutamate receptor 2; mGluR3; GLT1 - glutamate transporter 1), as well as the BDNF (brain-derived neurotrophic factor) gene. Moreover, the increase in mRNA of each of these genes correlated with an increase in TLR3 mRNA (Randall P.A., et al., 2019).

Application of poly (I:C) results in increased expression of a number of pro-inflammatory genes (CCL5, CCL2, IL-1b, IL-6, etc.) in the prefrontal cortex of mouse brain. Moreover, when free access to ethanol was provided to mice during peak activation of the proinflammatory response, the level of voluntary ethanol consumption by mice decreased and did not change when ethanol access was provided to mice in the descending pathway of activation of the innate immune system. These results suggest that the gradual increase in the inflammatory response may contribute indirectly to the increased level of alcohol craving in mice. The authors thus conclude that specific pathways and the balance between cytokines may regulate the level of craving for alcohol (Warden A.S., et al., 2019).

In a 10-day ethanol alcoholization model of mice, a single administration of the TLR3 agonist poly (I:C) resulted in an increase in TRAIL mRNA levels in the orbitofrontal and entorhinal cortex of mice (Lawrimore C.J., et al., 2019). In cell culture work, it was shown that ethanol supplementation induces TLR3 activation, which promotes the release of IFNβ (interferon-beta) and IFNγ (interferon-gamma) by neurons and astrocytes. Moreover, increased activity of TRAIL (TNF-related apoptosis-inducing ligand) gene was observed after the introduction of poly (I:C) into cell culture. Blocking the TRAIL gene resulted in decreased levels of IFNβ and IFNγ in both astrocytes and neurons. In contrast, investigating the combined effect of ethanol and TLR3 agonist (poly (I:C)), an increase in TNF-α, IL-1β and IL-6 mRNA levels, as well as an increase in p38 and IRF3 protein levels were shown in microglia and neuronal cell cultures (Lawrimore C.J., et al., 2019).

One study showed that TLR3 mRNA and components of the TRIF-dependent pathway were upregulated in the prefrontal cortex of mouse brains 24 hours after ethanol withdrawal. Expression of TLR3-related and TRIF-dependent pathway components increased in the nucleus accumbens but decreased in the amygdala. In addition, Amlexanox, an inhibitor of the IKKKε/TBK1 complex, reduced immune activation of the TRIF-dependent pathway in the brain and decreased ethanol consumption. Thus, it has been hypothesized that the TRIF-dependent pathway regulates ethanol consumption (Liu J., et al., 2011).

Decreased activity of the MyD88-dependent pathway correlates with decreased ethanol consumption and increased levels of the TRIF-dependent pathway. In order to test whether the

effects of poly (I:C) mediated through MyD88, female Myd88 gene knockout mice were used and showed that administration of poly (I:C) did not alter the level of alcohol consumption in Myd88 knockouts, indicating that poly (I:C)-induced changes in alcohol consumption are dependent on the MyD88-dependent pathway (Warden A.S., et al., 2019).

Based on experimental data performed on various model subjects, we can conclude that TLR3 plays an important role in the pathogenesis of alcoholism, but the exact mechanisms of TLR3-dependent signaling remain still definitively unidentified.

1.3.2 The role of TLR4 in the pathogenesis of alcoholism

The greatest amount of work has focused on the involvement of TLR4 in the mechanisms of activation of proinflammatory signaling as a result of ethanol consumption (Pascual M., et al., 2015; Blednov Y.A., et al., 2017; Harris R.A., et al., 2016; June H.L., et al., 2015) (Figure 4).

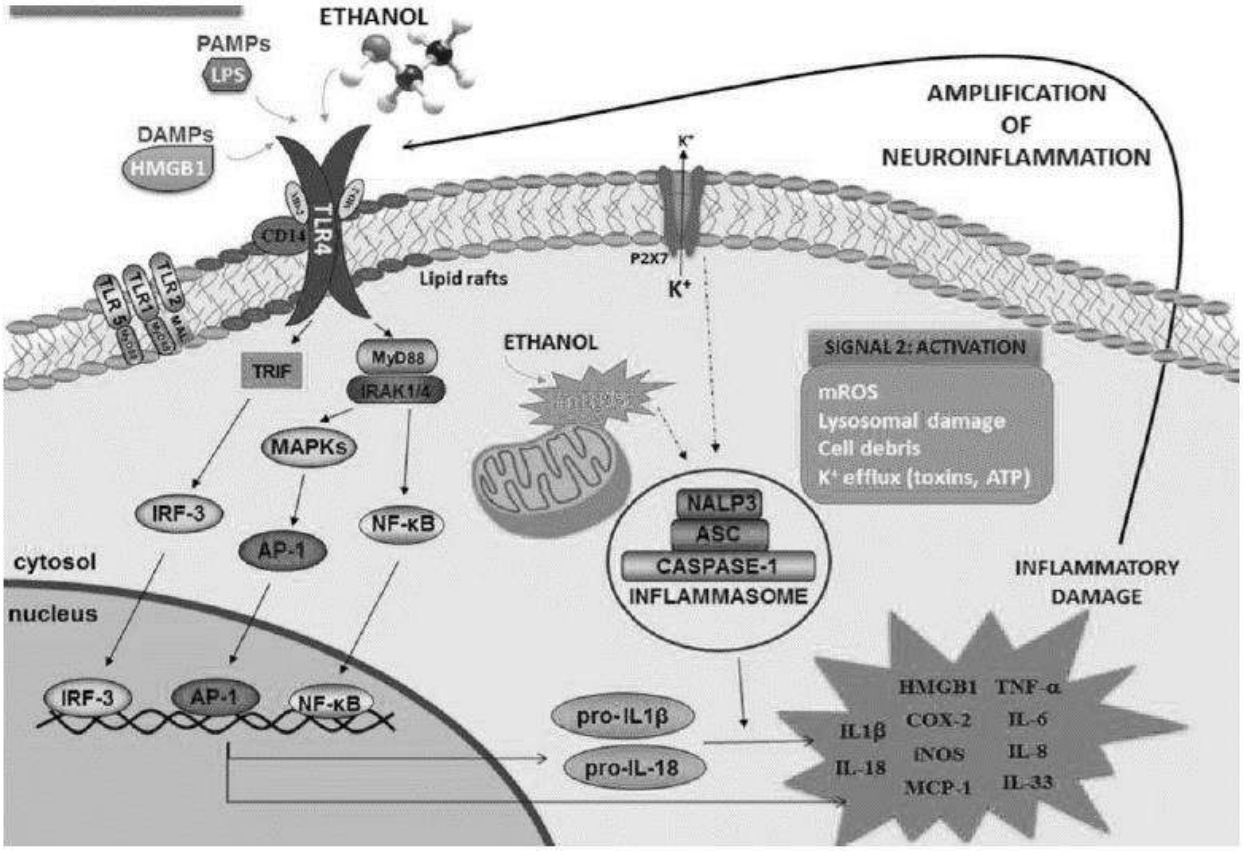

Figure 4 - Activation of the neuroinflammatory process by TLR4 (Montesinos J., 2016).

A large amount of data has been generated in rats and mice using genetic and pharmacological manipulations (TLR4 knockout and antagonist application), which showed that

TLR4 activity does not regulate ethanol consumption, but changes in TLR4-mediated signaling subsequently consumed alcohol are observed (Blednov Y.A., et al., 2017; Harris R.A., et al., 2016).

A study on mice showed that ethanol consumption by mice for 2 weeks resulted in activation of TLR4-dependent pro-inflammatory processes, which were characterized by activation of MAP-kinases and NF-kB with subsequent release of COX-2 (Cyclooxygenase 2) iNOS (Inducible nitric oxide synthase), HMGB1 (high-mobility group protein B1). The development of the inflammatory process against the background of increased activity of these pro-inflammatory signaling molecules led in the experiment to demyelination of axons and to structural synaptic changes as a result of damage to myelin and synaptic proteins, which subsequently affected in mice the deterioration of cognitive parameters in tests of object recognition, passive avoidance and olfactory behavior (Montesinos J., et al., 2015). Knockdown of the TLR4 gene, on the other hand, inhibits the production of pro-inflammatory mediators by blocking the activation of MAP kinases and NF-kB pathways in astrocytes (Alfonso-Loeches S., et al., 2010). In TLR4 gene knockout mice, it has been shown that such mice are protected from ethanol-induced increases in brain cytokine and chemokine concentrations for 5 months, whereas the presence of the TLR4 gene leads to increased concentrations of cytokines (IL-1β, IL-17, TNF-α) and chemokines (MCP-1, MIP-1α, CX3CL1) in the blood and in the striatum of the mouse brain (Pascual M., et al., 2015).

Ethanol has been shown to lead to the accumulation of polyubiquitinated forms of proteins in the cortex and to promote the activation of immunoproteasomes and autophagolysosomes (Sinch-Jasuja H., et al., 2001). Mice lacking TLR4 receptors were also protected from ethanol-induced such changes (Pla A., et al., 2014).

There is evidence that TLR4/MCP-1-mediated signaling in the tt 5mindbrain and ventral tegmental area (VTA) makes rats predisposed to increased ethanol consumption. Such signaling is supported by increased expression of corticotropin-releasing factor (CRF), which is able to exert inverse regulation of TLR4 (June H.L., et al., 2015). In addition, there is evidence that MCP-1 levels with a concomitant increase in microglia activity are increased in the VTA, amygdala, substantia nigra, and hippocampus in people with alcoholism (He J., et al., 2008).

TLR4-MyD88-dependent signaling is thought to mediate acute depressive disorders that develop after ethanol consumption and may also be involved in the regulation of GABAergic transmission in the CNS (Blednov Y.A., et al., 2017). In one of the works, rats knocked out on TLR4 gene decreased the level of voluntary alcohol consumption. The authors of this work suggest that this was associated with a decrease in the expression of the GABAA a2 subunit in the amygdala body of rats (Liu J., et al., 2011). Intraperitoneal injection of the TLR4 ligand LPS (lipopolysaccharide, lipopolysaccharide) accelerates the development of anxiety-like behavior in animals subsequently exposed to ethanol (Breese G.R., et al., 2008). Mice lacking TLR4 or

MyD88 became less sensitive to the sedative and intoxicating effects of ethanol, whereas mice lacking TLR2 did not differ from control mice in these tests (Breese G.R., et al., 2008).

All this suggests that TLR4 may possibly mediate interactions with neurotransmitter receptors (or other targets) to regulate ethanol consumption indirectly.

1.3.3 The role of TLR7 in the pathogenesis of alcoholism

In addition to TLR3 and TLR4 already described, there are a small number of studies investigating TLR7 in the pathogenesis of alcoholism. For example, TLR7 expression is upregulated in the human hippocampus in postmortem brain samples (Coleman L.G., et al., 2017). There is evidence that ethanol induces TLR7 agonist (miRNA let-7b) secretion, leading to TLR7-mediated activation of neurodegenerative processes in the CNS (Coleman L.G., et al., 2017). A slice culture of rat hippocampo-entorhinal cortex (HEC) was used to evaluate the effects of ethanol on TLR7 and let-7b. The study reported that alcoholized hippocampal tissue has increased expression of TLR7 (Coleman L.G., et al., 2017). Ethanol was found to induce the formation of HMGB1-miR-let-7 complexes in microvesicles, which cause the development of neurotoxic effects through TLR7 activation (Coleman L.G., et al., 2017). Ethanol causes an increase in TLR7 expression and release of let-7b and HMGB1 from microglia. Inhibition of HMGB1 by glycyrrhizin prevented the development of neurotoxicity (Coleman L.G., et al., 2017).

1.4 Involvement of HMGB1 in neuroimmune mechanisms during alcoholization

A major role has been given to HMGB1 protein as a critical factor involved in the mechanisms of innate immune system gene induction following ethanol ingestion, which may contribute not only to the inflammatory process in the brain, but also alter the functionality of brain neurotransmitter systems (Lewohl J.M., et al., 2000; Valles S.L., et al., 2004; Crews F.T., et al., 2011, 2013, 2014, 2017;, Esen N., et al., 2009; Okun E., et al., 2009), lead to neurotoxic effects with subsequent neurodegeneration (Airapetov M.I., et al., 2020, 2021; Becker H.C., 2014; Blednov Y.A et al., 2011; Qin L., et al., 2013; Lawrimore C.J., et al., 2017; Fernandez-Lizarbe S., et al., 2009) (Figure 5).

Studies show that ethanol consumption is responsible for increased expression levels of HMGB1 and TLRs in the brain (Leifer C.A., et al., 2016).

HMGB1 is secreted by most brain cells (Pascual M., et al., 2011). Neurons and glia cells have been shown to release HMGB1 during glutamate receptor excitation (Zou J., et al., 2012). Long-term alcohol consumption is known to be accompanied by an excitotoxic effect of glutamate

(Crews F.T. et al., 2013, 2015; Vetreno R.P., et al., 2014). Perhaps, it is the excitotoxicity that is one of the reasons for the increased expression level of HMGB1 during prolonged ethanol exposure.

A study on hippocampal-entorhinal cortex cell cultures by cytometric method showed that HMGB1 is released mainly by microglia cells and to a lesser extent by astroglia cells and neurons. When released into the extracellular space, HMGB1 is able to bind to microglia membrane receptors such as TLR-2, TLR-4, TLR-5, and TLR-2 of astro- and oligodendrocytes. Activation of these receptors triggers intracellular reaction cascades, resulting in activation of transcription factors such as NF-kB, IRF-3 and IRF7, AP1 with subsequent expression of a number of cytokines, chemokines, oxidases and proteases (see figure) (Yamamoto M., et al., 2002; Shabanov P.D., et al., 2008; Walter T.J., et al., 2017; Crews F.T., et al., 2000; Qin L., et al., 2013; Lawrimore C.J., et al., 2017; Alfonso-Loeches S., et al., 2011; Fernandez-Lizarbe S., et al., 2009; Yamamoto M., et al., 2002; Vabulas R.M., et al., 2002).

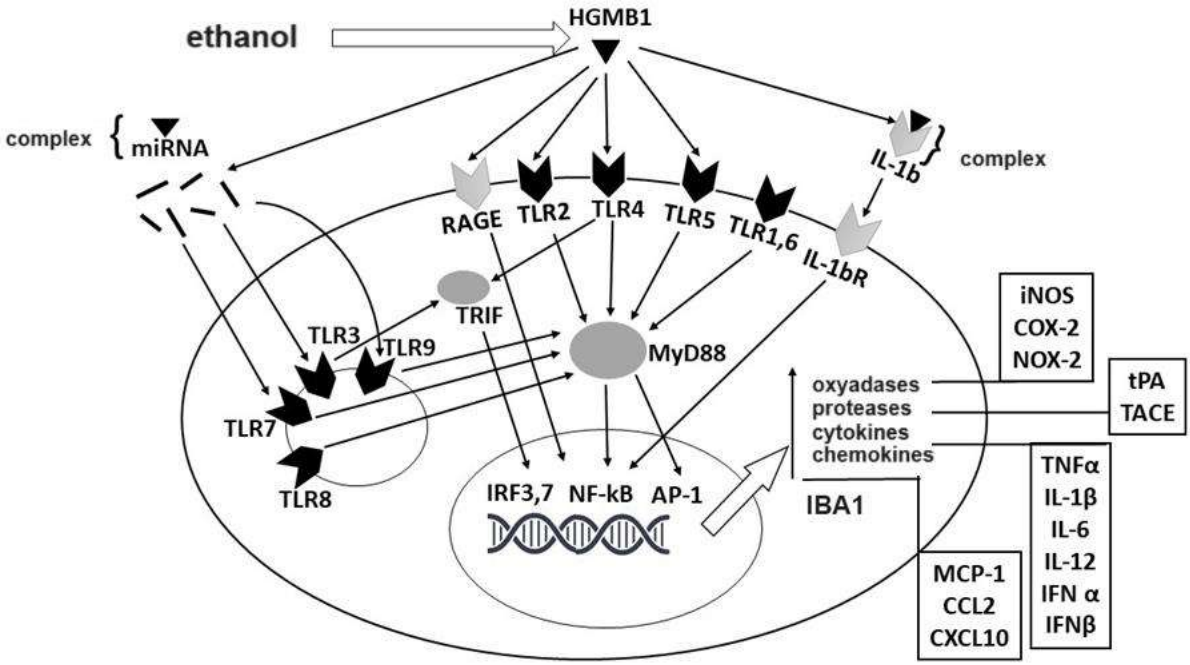

Figure 5 - The role of HMGB1 protein in intracellular signaling
(Airapetov, M.I., 2019)

It is known that long-term alcohol consumption activates microglia and astrocytes (Fernandez-Lizarbe S., et al., 2009), increases the expression of cytokines, chemokines, oxidases and other genes of the innate immune system in the brains of mice (Alfonso-Loeches S., et al., 2011; Fernandez-Lizarbe S., et al., 2009). An experiment in mice using the TLR4 agonist

lipopolysaccharide showed a sustained increase in TNFa protein and mRNA expression in the brain that persisted for 10 months after alcohol withdrawal (Lawrimore C.J., et al., 2017).

Similarly, in an experiment, 10-day ethanol alcoholization of mice steadily increased the levels of the cytokine MCP-1 (aka CCL2) in the brain for at least 7 days after ethanol withdrawal. MCP-1 is the most potent monocyte chemotaxis factor in the mammalian body, controls the exit of cells from hematopoietic organs, their transport to the site of inflammation (Alfonso-Loeches S., et al., 2011).

Results on rats showed that ethanol leads to prolonged activation of innate immune signaling mechanisms in the prefrontal cortex, which correlates with neurocognitive dysfunction (Leifer C.A., et al., 2016).

Studies performed on rodents under ethanol alcoholization conditions and on postmortem brain samples from human alcoholics show increased expression of HMGB1 and toll-like receptors in the prefrontal cortex (Leifer C.A., et al., 2016; Yamamoto M., et al., 2002), hippocampus (Airapetov M.I., et al., 2020; Shabanov P.D. Kalishevich S.Y., 1998; Yamamoto M., et al., 2002) and cerebellum (Zou J., et al., 2010; Yamamoto M., et al., 2002).

Studies performed on postmortem prefrontal cortex samples from the brains of people with alcoholism have shown elevated levels of HMGB1, as well as TLR2, TLR3, and TLR4 receptors (Shabanov P.D., et al. 2008) and the RAGE receptor (Nie L., et al., 2018).

In an experiment on adolescent and adult rats it was shown that ethanol exposure in adolescence leads to a prolonged increase in the level of TLR4 and TLR3 expression in the prefrontal cortex of the rat brain, while persisting in the adult brain. It is also worth noting that TLR3 were localized in cortical layers 2 and 3 of the prefrontal cortex. Increased expression levels of TLR4 and TLR3 correlated in the prefrontal cortex of animals with the development of cognitive dysfunction, when studying learning and memory processes using the Barnes test (Leifer C.A., et al., 2016).

Ethanol exposure during adolescence increases the expression of TLR4 and HMGB1 in the orbitofrontal cortex, which is long-lasting and observed subsequently in the adult rat brain. Increased expression of pro-inflammatory cytokines, oxidases, and other innate immune genes in adult orbitofrontal cortex 25 days after the last ethanol exposure such as TNFα, MCP-1, NOX2, COX2, fibronectin, S100ß, and MyD88 was also observed. The levels of iNOS remained unchanged. Meanwhile, HMGB1 and TLR4 expression levels in the orbitofrontal frontal cortex (OFC) correlated with alcohol consumption. Studies on postmortem brain samples from people with alcoholism also find increased expression of HMGB1, TLR2, TLR3, and TLR4 (Leifer C.A., et al., 2016).

It has also been shown that young women who regularly consume alcohol have elevated plasma HMGB1 levels (Airapetov M.I., et al., 2018).

The study of mRNA levels of TLR2, TLR4, TLR9 and RAGE receptors in the cerebellum of mice alcoholized for 5 months showed a significant increase in receptor expression, there was also an increased level of HMGB1 mRNA compared to the control group (Zou J., et al., 2010).

Alcohol exposure for 5 weeks increases allograft inflammatory factor 1 (AIF-1) immunoreactivity in frontal cortex, amygdala, hippocampus, substantia nigra, and cerebellum. AIF-1 levels remain elevated 28 days after alcohol withdrawal in frontal cortex, amygdala and substantia nigra (Yamamoto M., et al., 2002).

Recent work performed by RNA immunoprecipitation showed that HMGB1 protein is able to form complexes with microRNA let-7b molecules (HMGB1-let-7b) in microvesicles isolated from brain slices (Airapetov M.I. et al., 2020). Performed co-immunoprecipitation for HMGB1 and IL-1b on postmortem hippocampal samples of alcoholic people, similar complexes were detected. HMGB1 is known to increase in the liver during prolonged alcoholization, but HMGB1 - IL-1b complexes were not found there. At the moment it is difficult to make a conclusion about the physiological role of these complexes, but probably they contribute to neuroimmune signaling of the brain (Shabanov P.D. Kalishevich S.Y., 1998).

It should be noted that in all the experiments, the researchers took into account mainly only the structures of the prefrontal cortex, with a few studies focused on the hippocampus and cerebellum. However, the role of HMGB1 in other brain structures remains unexplored.

1.5 Participation of microRNA molecules in the implementation of neuroimmune mechanisms during alcoholization

A number of researchers have noted changes in the content of microRNA (miR) molecules in blood plasma and in a number of brain structures during long-term alcohol exposure (Lim Y., et al., 2021; Peregud D.I., et al., 2022). MicroRNAs are a class of small non-coding RNAs that, most often by targeting mRNAs, can participate in the mechanisms of protein synthesis in the cell. In addition to this well-known mediated regulatory role, miR molecules can also act as physiologically specific ligands to toll-like receptors (TLRs) and initiate immune response signaling cascades (Bayraktar R., et al., 2019).

There is evidence that many of the miR molecules have the potential to control key mechanisms governing the development of neuroinflammation and neurodegeneration resulting from long-term alcohol consumption (Lim Y., et al., 2021; Lehmann S.M., et al., 2012; Lippai D., et al., 2013; Coleman L.G., et al., 2017; Ureña-Peralta J.R., et al., 2018, 2020). Several TLR

subtypes are also known to be associated with the development of neuroinflammatory and neurodegenerative events in the brain during long-term alcohol consumption (Lim Y., et al., 2021; Lehmann S.M., et al., 2012; Lippai D., et al., 2013; Coleman L.G., et al., 2017; Ureña-Peralta J.R., et al., 2018, 2020). Several studies have reported a possible functional relationship between miR-let7b, miR-96, miR-182, miR-155 and TLRs in the CNS (Lehmann S.M., 2012; Lippai D., 2013; Coleman L.G., 2017; Ureña-Peralta J.R., 2018, 2020). It is therefore of interest to assess the level of these microRNAs and the expression of TLR genes in Nac under alcoholization conditions in rats, which was the aim of this study.

It is known from the literature that the miRs we investigated may have a functional relationship with toll-like receptor (TLR7 and TLR4) signaling pathways that are involved in the initiation of neuroinflammatory events that develop during chronic alcoholization (Lim Y., et al., 2021; Lehmann S.M., et al., 2012; Lippai D., et al., 2013; Coleman L.G., et al., 2017; Ureña-Peralta J.R., et al., 2018, 2020). It is known that miR-let7b is an endogenous TLR7 agonist (Lehmann S.M., 2012; Airapetov M.I., 2020). Altered expression of miR-let7b may have a functional relationship with the TLR7 signaling response cascade (Lehmann S.M., 2012; Coleman L.G., et al., 2017; Airapetov M.I., et al., 2020). It has been described that upon TLR4 activation there is an increase in miR-155 in brain microglia of mice, whereas this is not observed in Tlr4 gene knockout (TLR4-KO) mice (Lippai D., et al., 2013). TLR4-KO mice do not have altered cortical miR-96 content, whereas long-term alcoholization in wild-type mice decreased in miR-96 levels (Ureña-Peralta J.R. et al., 2018, 2020).

1.6 New potential neuroimmune therapy approaches for alcoholism

Neuroimmune therapies are likely to be of little benefit where severe neurodegenerative changes have already occurred. However, neuroimmune therapies could be helpful in preventing the acute response of innate immune system signaling that can cause neurotoxic brain damage. Currently, there are already pharmacologic agents that have immunomodulatory effects in the brain, such as minocycline (microglial inhibitor), rapamycin (mTORC1 inhibitor), azithromycin (microglial inhibitor), rifampin (TLR4 inhibitor), indomethacin (COX-2 inhibitor), simvastatin (NF-κB inhibitor), pioglitazone (PPARγ agonist), naltrexone (TLR4 inhibitor), etanercept (TNFα antagonist), amlexanox (IKK/TBK1 inhibitor), glycyrisin (HMGB1 inhibitor) (Coleman L. G., et al., 2018).

1.6.1 Glycyrrhizin

Glycyrrhizin (GL) is found in large quantities in the roots of licorice (Glycyrrhiza radix) and consists of glycyrrhizic acid (Figure 6) and two molecules of glucuronic acid. GL has been used for many years in East Asian countries as an anti-inflammatory, anti-allergic, and antiviral agent. In Japan, GL injections have been used for allergic inflammation since 1948. In addition, it has been suggested that GL exerts neuroprotective effects in post-ischemic brain damage in rats (Kim S.W., et al., 2012).

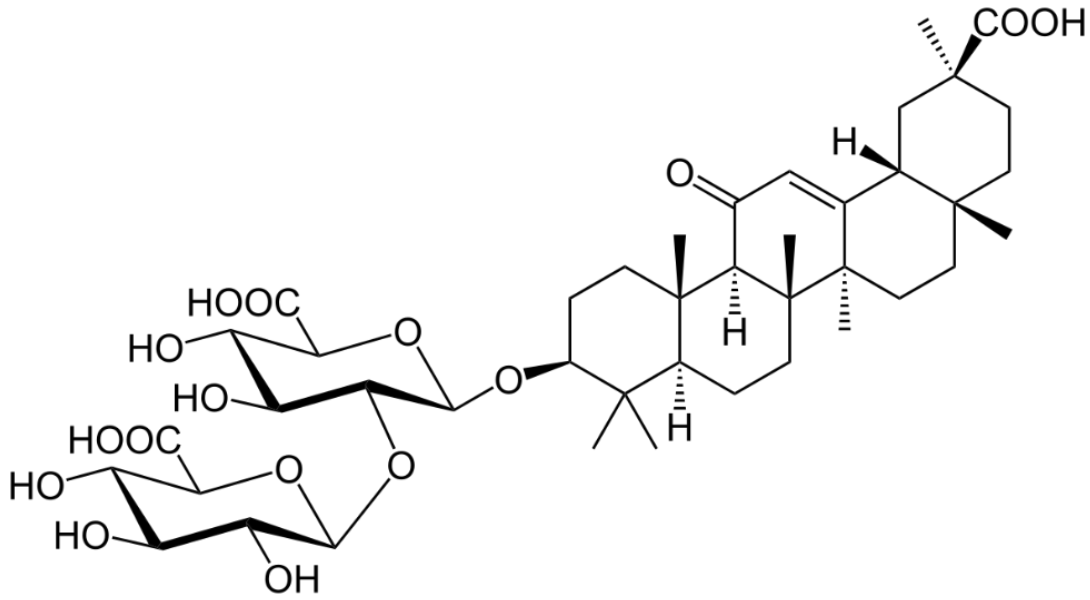

Figure 6 - Structural formula of glycyrrhizic acid

Subsequently, GL was shown to directly bind to HMGB1 and subsequently inhibit HMGB1 translocation from activated or damaged cells (Kim S.W. et al., 2012.). Meanwhile, glycyrisin therapy is considered quite safe due to the long clinical experience with its use (Figure 7). Pseudoaldosteronism is the only major side effect of glycyrrhizin (Okuma Y. Et al., 2014).

These anti-inflammatory effects of glycyrrhizin have been shown to reduce plasma levels of HMGB1, which may contribute to the protection of the brain from injury. The use of glycyrrhizin in brain injury shows that 6 h after administration leads to 90% inhibition of the expression of factors such as TNFα, IL-1β, IL-6 (Coleman L.G., 2018; Okuma Y. Et al., 2014).

Thus, this compound may be promising for the purpose of pharmacocorrection of neuroimmune signaling under conditions of long-term alcohol consumption.

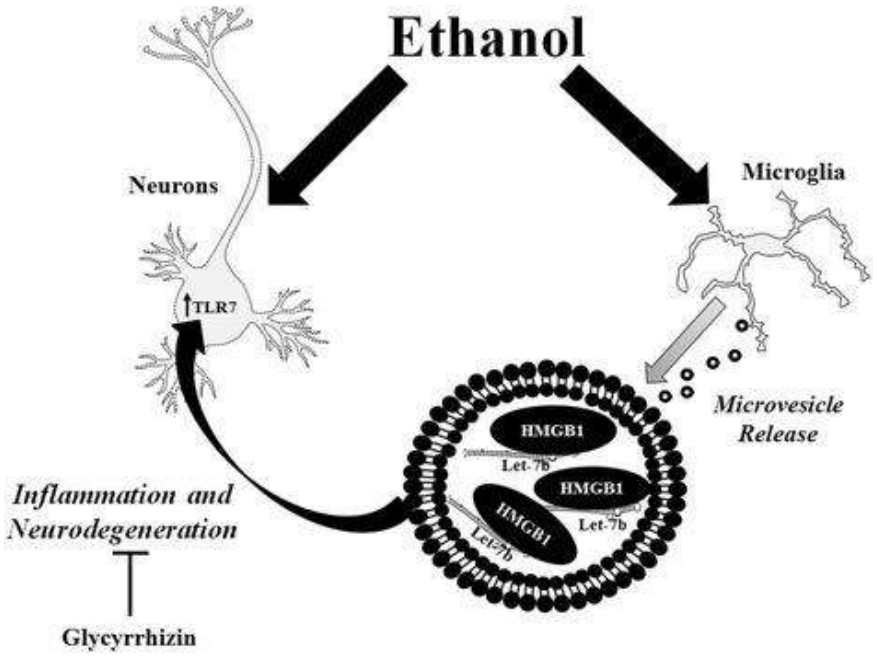

Figure 7 - Putative mechanism of HMGB1 and let-7 secretion by microvesicles (Coleman L.G., 2017).

1.6.2 Rifampicin

Rifampicin (Rif) (Figure 8) is a broad-spectrum antibiotic from the ansamycin group that is used in the treatment of tuberculosis as well as other infections: staphylococcal, meningococcal, and lepra (Ebenezer G.J., et al., 2021; Cao Y., et al., 2022). Rif can be either in a bound form - with blood plasma proteins - or in an unbound form, in which case its passage through the barriers of the body, including the blood-brain barrier, is facilitated (Ebenezer G.J., et al., 2021; Cao Y., et al., 2022). The ability of Rif to penetrate the blood-brain barrier is of interest for its application for the pharmacological correction of pathological processes of the brain.

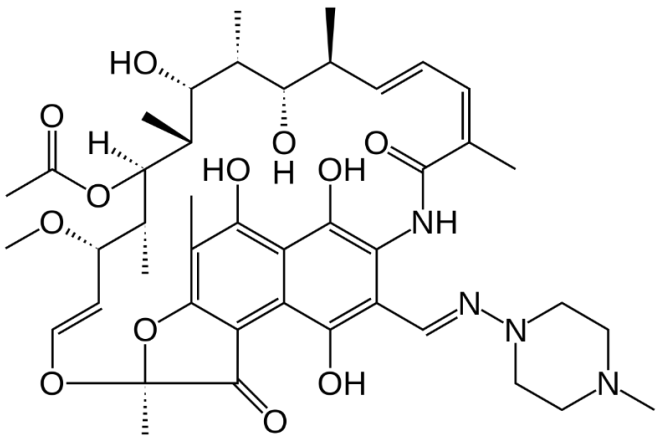

Figure 8 - Structural formula of rifampicin

Epidemiologic studies have shown that a lower incidence of neurodegenerative pathologies was observed in leprosy patients treated with Rif (Bi W., et al., 2021). It has been shown experimentally in various models of neuroinflammation that Rif can reduce the levels of pro-inflammatory cytokines, β-amyloid content in a model of Alzheimer's disease, α-synuclein in a model of Parkinson's disease. There are studies showing improvement of autophagy process in mice in the hippocampus when exposed to Rif (30 mg/kg). In a model of cerebellar demyelination, it was shown that administration of Rif (40 mg/kg) to mice recorded decreased Bax expression and increased Bcl2 expression, as well as decreased caspase 3 and caspase 12 activity (Zahednasab H., et al, 2019), indicating a decrease in apoptotic activity, and rifampicin (20 mg/kg) pre-administration suppressed lithium-pilocarpine induced hippocampal neurodegeneration by reducing cytochrome c levels in the hippocampus (Ali A.E., et al., 2018).

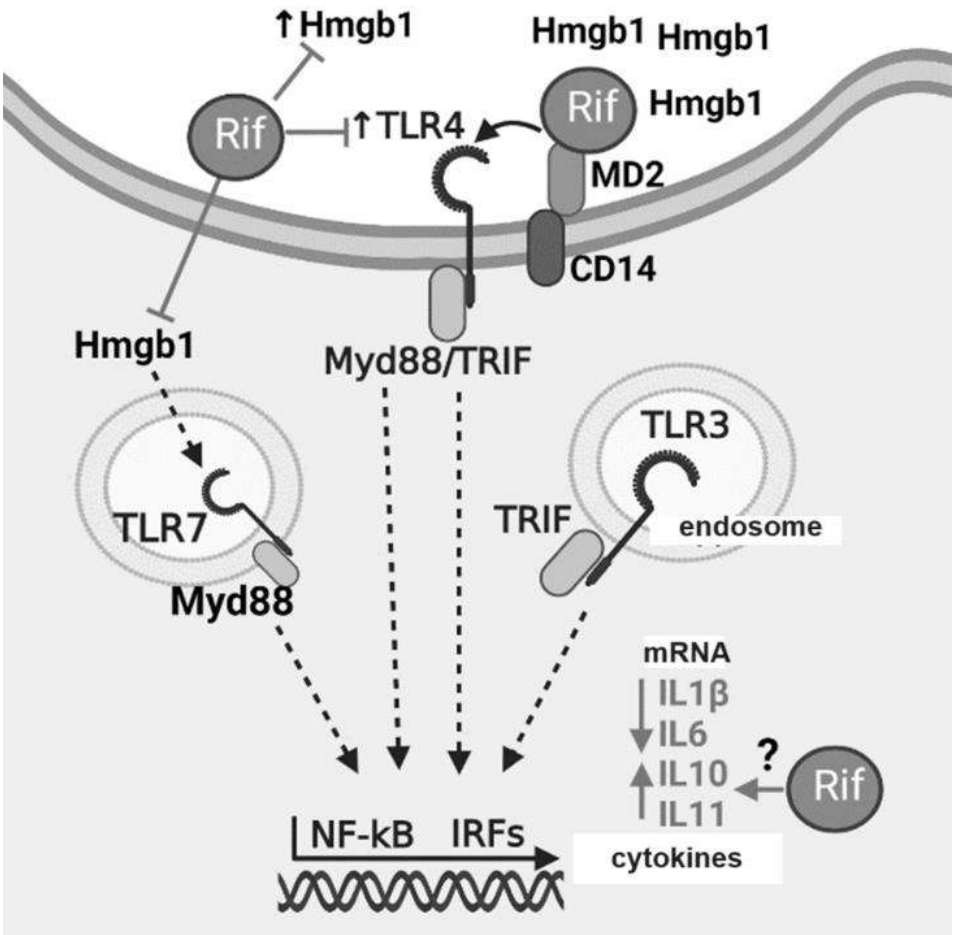

Figure 9 - Anticipated pathways of Rif action (Airapetov M.I. et al., 2021)

These data are sufficient to conclude that Rif has neuroprotective properties, but the exact mechanism of action remains unknown. In one of the studies performed on microglial cell culture,

there is evidence of competition between Rif and lipopolysaccharide for binding to MD2 protein. MD2 protein is required for TLR4 ligand binding and subsequent signaling. Rif, by binding to this protein, contributed to the inhibition of LPS-CD14-MD2-TLR4-NFκB signaling in the experiment, and as a consequence, there was a decrease in NF-κB activation and proinflammatory factors (NO, IL-1β, TNFα). Thus, Rif, competitively binding to MD2, can block TLR4-dependent signaling (Wang X., et al., 2013) (Figure 9).

1.6.3 Ginsenosides

Various species of plants in the genus Panax (Ginseng), including P. Ginseng, P. quinquefolius and P. notoginseng, have been used as medicinal plants for several millennia (He Y., et al., 2018). The plant's widespread use is due to its chemical composition containing a wide variety of pharmacologically active components, including ginsenosides (Figure 10), phytosterol, polyacetylene, polyphenols, polysaccharides, organic acids, amino acids, vitamins and minerals, each of which may play a potential role in the treatment and prevention of several diseases (He Y., et al., 2018; Mahady G.B., et al., 2000; Cui L., et al., 2016; Nguyen N.H., et al., 2019; Tam D.N.H., 2018). Studies on molecular targets that may explain the observed pharmacological effects in different ginseng compounds are actively realized in several countries (He Y., et al., 2018). One of the main biologically active components of plants of the genus Panax are ginsenosides, which, according to the literature, have anti-inflammatory, antioxidant and anticancer effects (Tam D.N.H., 2018).

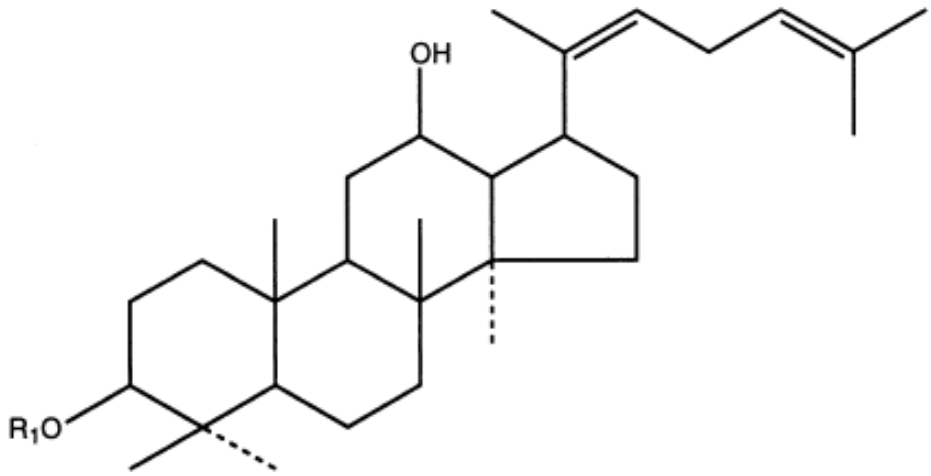

Figure 10 - General structural formula of ginsenosides

It is reported that 152 drugs based on ginseng ginsenosides alone have been registered in the world and 119 are in clinical trials (Fan S., et al., 2020). In recent years, ginsenosides have been actively investigated as potential pharmacological agents to reduce the development of neuroinflammatory process, which is manifested in the first stages during the development of various pathological conditions of the nervous system (neurodegenerative diseases, brain injuries, bacterial and viral infectious diseases, intoxications) (He Y., et al., 2018; González-Burgos E., et al., 2015; Wang X., et al., 2015; Xu X., et al., 2020; Li J., et al., 2021; Cai L., et al., 2021; Sun Y., et al., 2022; Gong L., et al., 2022; Zheng Z., et al., 2022; Xu X., et al., 2022).

Recent research findings support the fact that alcohol consumption serves as a signal to activate neuroinflammatory pathways in various brain structures (Ramos A., et al., 2022; Coleman L.G.J., et al., 2021; Airapetov M., et al., 2021; Becker H.C., et al., 2014; Gano A., et al., 2022; Griffin W.C., et al., 2022), which in turn affects the triggering of those pathogenetic molecular mechanisms involved in the formation of alcoholism, a serious socially significant disease (Becker H.C., et al., 2014; Gano A., et al., 2022; Griffin W.C., et al., 2022). Over the past more than 20 years, research findings have supported the fact that certain Toll-like receptor (TLR) subtypes mediate the development of neuroinflammation in brain structures during long-term ethanol consumption (Ramos A., et al., 2022; Coleman L.G.J., et al., 2021; Airapetov M., et al., 2021). There is evidence that TLR signaling not only contributes to the development of neuroinflammation in the brain, but may also be involved in the mechanisms of regulation of functional activity of neurotransmitter systems (Femenia T., et al., 2018; Tripathi A.S., et al., 2021; Shirayama Y., et al., 2022), which may also contribute to the formation of pathological craving for alcohol.

There are studies that point to the ability of Panax genus plant extract and some individual ginsenosides of ginseng to influence the mechanisms of neuroinflammation by altering the state of TLR signaling system (Pannacci M., et al., 2006; Rhule A., et al., 2008; Beamer C.A., et al., 2012; Kim T.W., et al., 2012; Zhao B.S., et al., 2014). It seems interesting to investigate the effect of ginsenosides on the TLR signaling system in the brain after prior alcohol ingestion. Thus, the aim of our work was to evaluate the effect of the amount of ginsenosides, obtained from the extract of Panax Japonicus cell culture, on the state of the TLR-signaling system at the mRNA level in the nucleus accumbens and hippocampus of the rat brain on the model of prolonged alcoholization during alcohol withdrawal.

CHAPTER 2. MATERIALS AND METHODS

2.1 In vivo experiments on rats

The work was performed on 120 male Wistar rats (initial age 2-3 months, body weight = 280±30 g) obtained from the laboratory animal nursery («Rappolovo», Russia), in compliance with the principles of humaneness (European Community Directive No. 86/609 EC). The animals were divided into 10 per group (12 groups in total), which is necessary to obtain statistically reliable data. Laboratory animals were kept in the vivarium of FGBNU "Institute of Experimental Medicine". Animals were kept in a separate room for rats at room temperature and under natural light regime. Feeding was carried out ad libitum, in the first half of the day with free access to water (except for those experimental groups where there is a need to simulate prolonged exposure to alcohol only by semi-coercive drinking of rats with ethanol solution, where ethanol solution serves as the only source of liquid).

The work was reviewed and approved by the Ethical Committee for Animal Research of SPbSU, conclusion No. 131-03-8 dated April 29, 2024. Throughout the experiment, the animals had unrestricted access to food and water.

2.2 Modeling the long-term effects of ethanol and withdrawal

Modeling of long-term ethanol exposure and withdrawal state in rats was performed in the study using several protocols:

Protocol 1: Modeling of prolonged ethanol exposure and withdrawal state was performed by semi-forcibly administering rats a 20% ethanol solution as their only source of fluid for 1 month followed by withdrawal. Brains were removed on the last day of long-term ethanol exposure, and at different time points of ethanol withdrawal - 1st day, 7th day, and 14th day.

Protocol 2: Modeling of the withdrawal state after prolonged ethanol exposure was performed by semi-forced consumption of 20% ethanol solution as the only source of fluid by rats for 2 months, followed by its withdrawal. The brain was removed on the 7th day of withdrawal of prolonged ethanol exposure.

Protocol 3: Modeling of withdrawal after prolonged ethanol exposure was performed by intragastric administration of a 20% ethanol solution via gastric tube for 2 months (2 g/kg ethanol, daily Monday through Friday, 40 injections total) followed by withdrawal. Control group - water was administered through the probe in equivalent amounts. The brain was withdrawn on the 10th day of withdrawal.

Protocol 4: Long-term ethanol exposure was modeled by intragastric administration of a 20% ethanol solution via gastric tube for 1 month (2 g/kg ethanol, daily Monday through Friday, total of 20 administrations). Control group - water was administered through the probe in equivalent amounts. The brain was withdrawn on the last day of prolonged ethanol exposure.

The protocols we used simulate moderate levels of ethanol exposure over long durations and are based on previously described protocols by different groups of researchers (Burov Y.V. et al., 1985; Lelevich V.V. et al., 2017; Coleman L.G.J. et al., 2018; McMahan R.H. et al., 2021; Tucker A.E. et al., 2022).

2.3 Cultivation of *Panax japonicus* cells

The suspension cell culture of Japanese ginseng (Panax japonicus C. A. Mey. Var. Repens Maxim.) was obtained in 1998 from the root callus of an intact biennial plant (Primorsky Krai, Russia). The strain was registered in the All-Russian Collection of Cell Cultures of Higher Plants under No. 62 (Nosov A.M., 2014). P. japonicus cell culture was grown at the Department of Plant Physiology, Lomonosov Moscow State University (Moscow) (according to the collection passport) on modified nutrient medium with mineral base according to Murashige-Skooga with sucrose (2.5%), vitamins according to White, α-NUC (2 mg/L), and kinetin (1 mg/L). Cultivation was carried out in the dark at 26°C, on a rocker (90 rpm), in 250 mL flasks capped with a double layer of aluminum foil and a layer of offset paper (30-40 mL of suspension per flask).

2.4 Sample preparation for the analysis of ginsenosides

A sample of plant material (25 mg) was extracted 3 times with 1 ml of 70% (by volume) aqueous methyl alcohol for 30 min under ultrasound (UZV-12, "Sapphire", Russia), then centrifuged at 10000 rpm for 10 min (Microcentrifuge MCF, Russia) and the supernatant was collected in a pear-shaped flask. The combined alcoholic extracts were evaporated under vacuum (at 40°C). The obtained extract was suspended in 1 ml of 5% (by volume) acetic acid solution in distilled water and applied to a Supelclean ENVI-18 solid phase extraction cartridge («Supelco», USA). The cartridge was washed with 3 ml of 5% (by volume) acetic acid solution in distilled water, and the analytes were washed with 3 ml of ethanol. The resulting solution was evaporated under vacuum at 40°C. Before analysis, the extracts were dissolved in 1 mL of acetonitrile-water mixture (1:1, by volume) and filtered using a 0.2 μm pore size nylon filter (Acrodisc, "Pall Corporation", USA).

2.5 UPLC-MS

Analysis was performed on a Waters Aquity UPLC chromatograph («Waters», USA) equipped with a XEVO QTOF hybrid quadrupole time-of-flight mass spectrometer («Waters», USA). A 1 μL sample volume was applied to an ACQUITY UPLC BEH Phenyl column (50 × 2.1 mm, 1.7 μm; «Waters», Ireland). The column temperature was 40°C and the volume flow rate of the mobile phase was 0.4 mL/min. A 0.1% (by volume) solution of formic acid in water (solvent A) and a 0.1% (by volume) solution of formic acid in acetonitrile (solvent B) were used as mobile phase. The chromatographic separation of ginseng triterpene glycosides was carried out in the gradient elution mode. During the analysis, the composition of the mobile phase was changed as follows (solvent B, % by volume): 0-1 min - 15%, 1-5 min - 15 → 30%, 5-15 min - 30 → 38%, 15-15.5 min - 48 → 45%, 15.5-23 min - 45%, 23-23.5 min - 45 → 95%. The analysis was performed in the positive ion detection mode (m/z range 100-1200). Ionization source parameters: ionization source temperature - 120°C, desolvation temperature - 250°C, capillary voltage - 3.0 kV, voltage at the sample input cone - 30 V, nitrogen feed rate (desolvation gas) - 600 l/h. The results were processed using MassLynx program (Waters, USA).

2.6 Intraperitoneal administration of of ginsenosides

Modeling of long-term exposure to ethanol and its withdrawal was performed according to Protocol 2 (see above). During 7 days of alcohol withdrawal, one group of animals (n=10) received intraperitoneal injections of sum of ginsenosides (50 mg/kg) and the other group (n=8) received injections of saline in equivalent amounts. The control group of rats (n=9), which were not subjected to alcoholization, received injections of saline solution in equivalent amount.

2.7 Intraperitoneal administration of rifampicin

Modeling of long-term ethanol exposure and withdrawal was performed according to Protocol 3 (see above). During 7 days of alcohol withdrawal, one group of animals (n=8) received intraperitoneal injections of rifampicin (100 mg/kg) and the other group (n=8) received injections of saline in equivalent amounts. A commercial preparation of rifampicin (Rifampicin, lyophilizate d/preg.concentrate d/preg solution for infusion", 150 mg, "Belmedpreparaty", Belarus) was used. The control group of rats (n=9) received injections of saline solution.

2.8 Biomaterial collection

At the end of the experiment rats were decapitated on the last day of ethanol exposure and at different periods of ethanol withdrawal, the necessary brain structures (amygdala, striatum, medial entorhinal cortex, hippocampus, nucleus accumbens) were sampled. The boundaries of the brain structure were defined according to the rat brain atlas (Paxinos G., et al., 2017). Brain samples were immediately frozen and stored at -80°C.

2.9 RNA isolation

Total RNA isolation was performed with ExtractRNA reagent («Eurogen», Russia) in full accordance with the manufacturer's instructions. The purity of the isolation and the concentration of the obtained RNA were measured on an Implen NanoPhotometer P330 spectrophotometer («Implen», Germany); the purity of the isolated product was evaluated by the A260/A280 ratio (normal ≥ 1.8).

2.10 RT-PCR

CDNA synthesis was performed by reverse transcription (RT) in 20 μl using the MMLV RT kit («Eurogen», Russia) in full compliance with the manufacturer's instructions. Prior to microRNA RT, polyadenylation of microRNAs was performed using E.coli poly(A)-polymerase («New England Biolabs Inc.», USA) according to the previously described method. OT for microRNA was performed in 10 μl using the MMLV RT kit reagent kit ("Eurogen", Russia) and a specific PolyT adapter (5'-GCGAGCACAGACAGAATTAATTAATACGACTCACTATAGGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN-3').

The concentration of the resulting cDNA during OT was measured on an Implen NanoPhotometer P330 spectrophotometer («Implen», Germany).

Polymerase chain reaction (PCR) with real-time detection was performed in the amplifier Mx3005P («Stratagene», USA) in 10 μl of the reaction mixture containing SYBR Green («Eurogen», Russia) and a mixture of specific forward and reverse primers (Table 3) («Beagle», Russia). The relative level of mRNA and miR content was calculated using the $2^{\Delta\Delta\Delta Ct}$ method, mRNA content was normalized to the expression level of Gapdh gene, miR level was normalized to the expression level of U6 gene.

Table 3 - Sequence of primers

Gene	Primers	
	Right (5'-3')	Reverse (5'-3')
Tlr3	AACTGGAGAACCTCCAAGA	CACCCTGGAGAAAACTCTTT
Tlr4	ACTCTGATCATGGCATTGTT	GTCTCAATTTCACACCTGGA
Tlr7	TGAAAATGGTATTTCCAATGTG	TAAGGGTAAGGTTGGTGGTA
Myd88	TCATTGAGAAAAGGTGTCGT	AGTGCAGATAGTGATGAAC
Ticam	GCTCAGCTAGATGATGTGAT	TGACAGTGCAGACCTGG
NfkB1	ATACTGCTTTGACTCACTCC	AGGTATGGGCCATCTGTT
Irf3	AATTCCTCCCCTGGCTC	CATGGGATCCTGAACTTTGT
Irf1	CGGAAGTTACCTTCTAGCTC	CGGAAGTTACCTTCTAGCTC
Hmgb1	CTCTGATGCAGCTTATACGA	AAAAGACTAGCTTCCCCTTG
Il1β	TGTCTGACCCATGTGAGCTG	TTTGGGATCCACACTCTCCAG
Ccl2	AAGATGATCCCAATGAGTCG	TGGTGACAAATACTACAGCTT
Il10	CTGCAGGACTTTAAGGGTTA	CCTTTGTCTTGGAGCTTATT
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

2.11 Statistical processing of data

Graph Pad Prizm v.6 program was used for statistical processing of the obtained data. The Mann-Whitney U-criterion for small independent samples was used to compare groups. Differences were considered statistically significant at $p \leq 0.05$.

Correlation analysis was also performed in the program Graph Pad Prizm v.6. The level of relationship between two compared variables was determined by the coefficient of determination (R^2).

CHAPTER 3. RESULTS OF OWN RESEARCH

3.1 The state of expression of TLR genes and proinflammatory cytokines in rat brain structures under conditions of long-term ethanol exposure and upon withdrawal

Modeling of long-term ethanol exposure and withdrawal state was performed according to protocol 1 (see chapter "Materials and Methods").

3.1.1 Expression level of Tlr3 in rat brain under prolonged ethanol exposure and withdrawal conditions

The results of the performed experiment showed that under conditions of long-term ethanol exposure there was an increase in the expression level of Tlr3 in the medial entorhinal cortex of the rat brain, although this increase was statistically insignificant. In the amygdala and striatum, the gene expression level remains unchanged. In the hippocampus, a decreased expression level was observed in the long-term ethanol exposure group compared to the control group (Figure 11).

During ethanol withdrawal, the expression level of Tlr3 in the amygdaloid body of the brain increases relative to the long-term alcoholization group on the 1st day, the level of control values is reached on the 7th day, and on the 14th day the index is lower than the level of values in the control group (Figure 11).

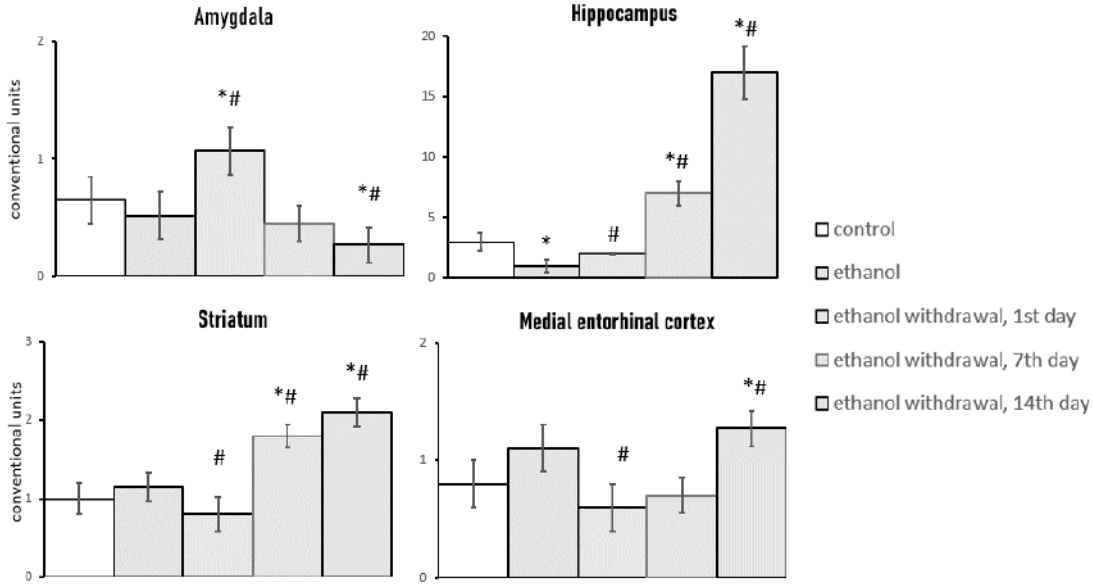

Figure 11 - Expression of Tlr3 gene in different structures of rat brain under chronic alcoholization and under conditions of ethanol withdrawal

(*p<0.05 in relation to the control group, #p<0.05 in relation to the alcoholization group)

In the medial entorhinal cortex, a decrease in expression is found on the 1st day in relation to the alcohol group, on the 7th day and 14th day there is an increase in expression, exceeding the level of values in the control group. In the striatum, there is a decrease on the 1st day relative to the alcohol group and an increase on the 7th and 14th days of ethanol withdrawal, exceeding the levels in the control group. In the hippocampus of the rat brain there is an increase in mRNA levels on all studied terms of withdrawal of prolonged ethanol exposure (Figure 11).

3.1.2 Tlr4 expression level in rat brain under conditions of long-term ethanol exposure and upon withdrawal

The expression level of Tlr4 had no statistically significant changes in any of the studied rat brain structures in the long-term ethanol exposure group (Figure 12).

During ethanol withdrawal, the level of Tlr4 expression was elevated in the amygdaloid body on the 1st day, but on the 7th day there was a decrease to the values of the control group. On the 14th day of ethanol withdrawal, the expression level acquired values lower than those of the control group (Figure 12). In the medial entorhinal cortex on the 1st day of withdrawal the expression level increased, then on the 7th day it decreased to the level of the control group. On the 14th day, the expression index acquired values below the level of the control group (Figure 12).

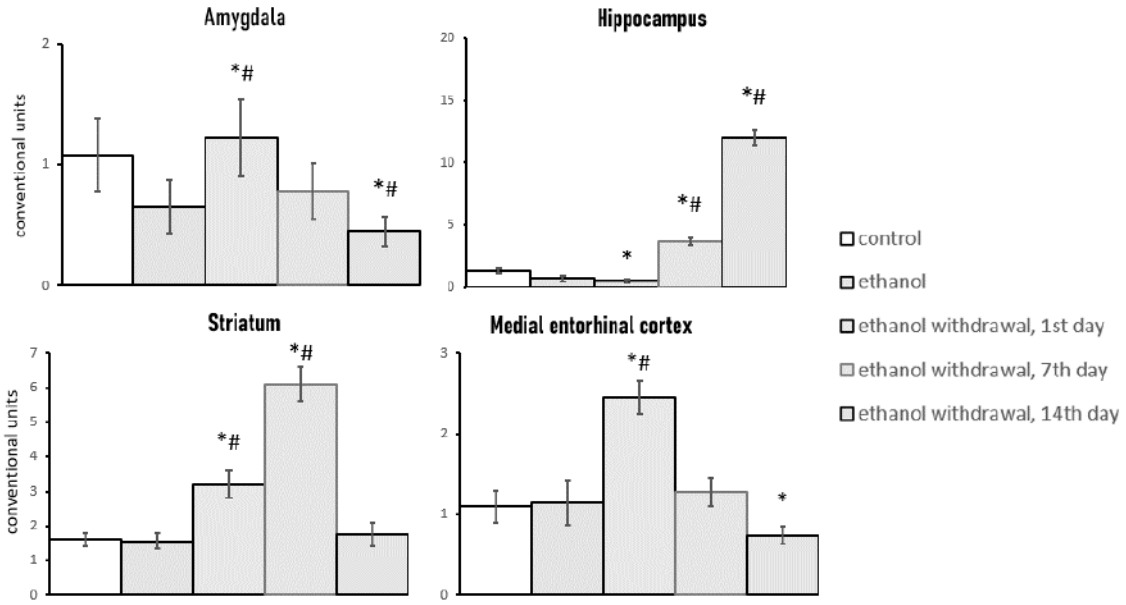

Figure 12 - Expression of Tlr4 gene in different structures of the rat brain under chronic alcoholization and under conditions of ethanol withdrawal

(*p<0.05 in relation to the control group, #p<0.05 in relation to the alcoholization group)

In rat brain striatum, Tlr4 expression was increased on the 1st and 7th days of ethanol withdrawal relative to the values in the control and long-term ethanol exposure groups. On the 14th day of ethanol withdrawal, Tlr4 expression in rat brain striatum decreases to the level of control group values (Figure 12). In the hippocampus, the expression level of Tlr4 increased on the 7th and 14th day of ethanol withdrawal relative to the values in the control and long-term ethanol exposure groups (Figure 12).

3.1.3 Expression level of Tlr7 in rat brain under prolonged ethanol exposure and upon withdrawal

In our experiment, the expression level of Tlr7 had no statistically significant changes among the rat brain structures we studied in the long-term ethanol exposure group (Figure 13).

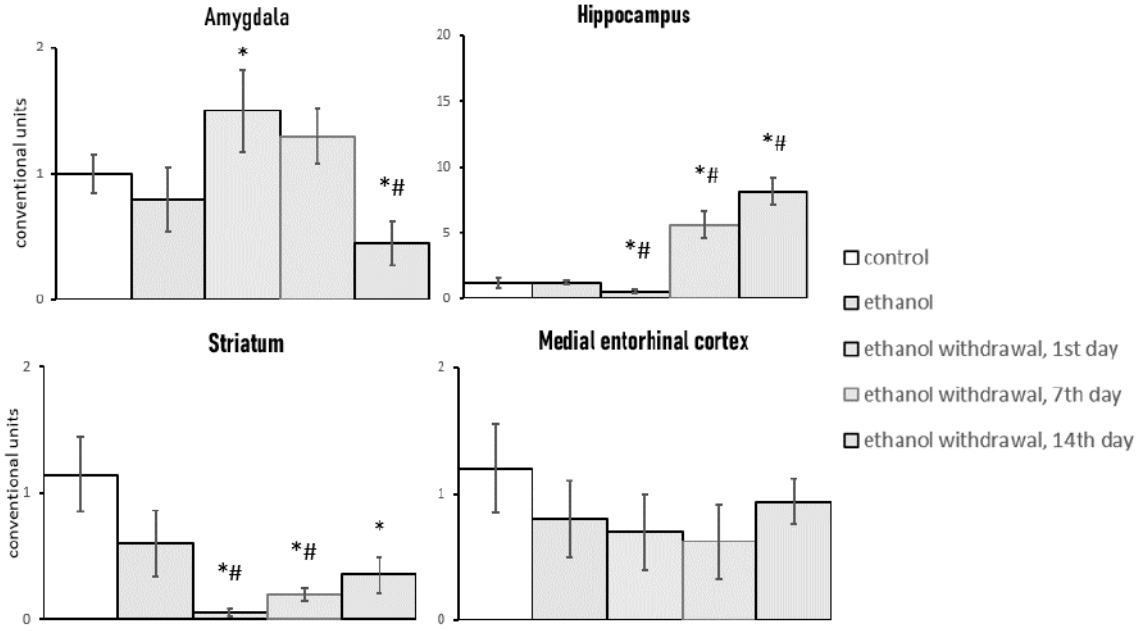

Figure 13 - Expression of Tlr7 gene in different structures of rat brain under chronic alcoholization and under conditions of ethanol withdrawal
(*p<0.05 in relation to the control group, #p<0.05 in relation to the alcoholization group)

At the same time, in the group of animals with ethanol withdrawal, the level of Tlr7 expression was increased in the amygdaloid body on the 1st day of withdrawal, but on the 7th and 14th days of withdrawal it was reduced to levels lower than in the control group (Figure 13). In the medial entorhinal cortex, no changes were detected in any of the studied experimental groups of animals (Figure 13). In the rat brain striatum, the level of Tlr7 expression is reduced in the

ethanol withdrawal group on the 1st day relative to the values in the control and long-term ethanol exposure groups. In addition, there is a persistence of the decreased expression level in the groups on the 7th and 14th day of ethanol withdrawal (Figure 13). In the hippocampus, the level of expressi is reduced on the 1st day of ethanol withdrawal, but an increase is detected on the 7th and 14th day of withdrawal, exceeding the level of values in the control group (Figure 13).

3.1.4 Expression level of proinflammatory cytokines under conditions of long-term ethanol exposure and withdrawal

Modeling of long-term ethanol exposure caused an increase in the level of Il1β gene expression in the amygdaloid body of the rat brain relative to the values in the control group. The increased level of Il1β gene expression was also detected during ethanol withdrawal on the 1st day; however, on the 7th and 14th days of withdrawal Il1β gene expression did not differ from the level of control group values (Figure 14).

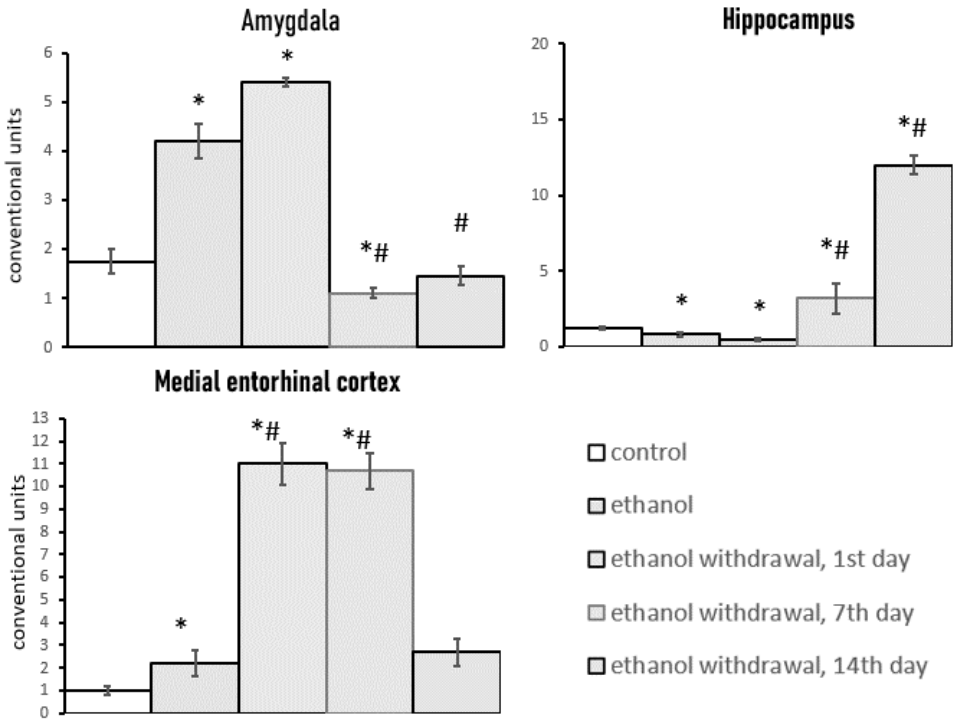

Figure 14 - Il1β gene expression in different structures of rat brain under chronic alcoholization and ethanol withdrawal conditions

(*p<0.05 in relation to the control group, #p<0.05 in relation to the alcoholization group)

In the medial entorhinal cortex, there is an increased level of gene expression in the group of long-term ethanol exposure, while in all studied ethanol withdrawal groups the value does not

have significant deviations from the level of values in the control group (Figure 14). In the hippocampus of rat brain, the expression level of Il1β gene is significantly unchanged in the long-term ethanol exposure group and in the ethanol withdrawal group on the 1st day; however, on the 7th and 14th days of ethanol withdrawal, there is an increase in Il1β gene expression relative to the control and long-term ethanol exposure groups (Figure 14).

The level of Ccl2 gene expression had no significant changes in the amygdala in the group of long-term ethanol exposure, but in the conditions of ethanol withdrawal there was a decrease in Ccl2 gene expression on the 1st day, on the 7th day there was a gradual increase, on the 14th day the indices did not differ from the level of values in the control group (Figure 15).

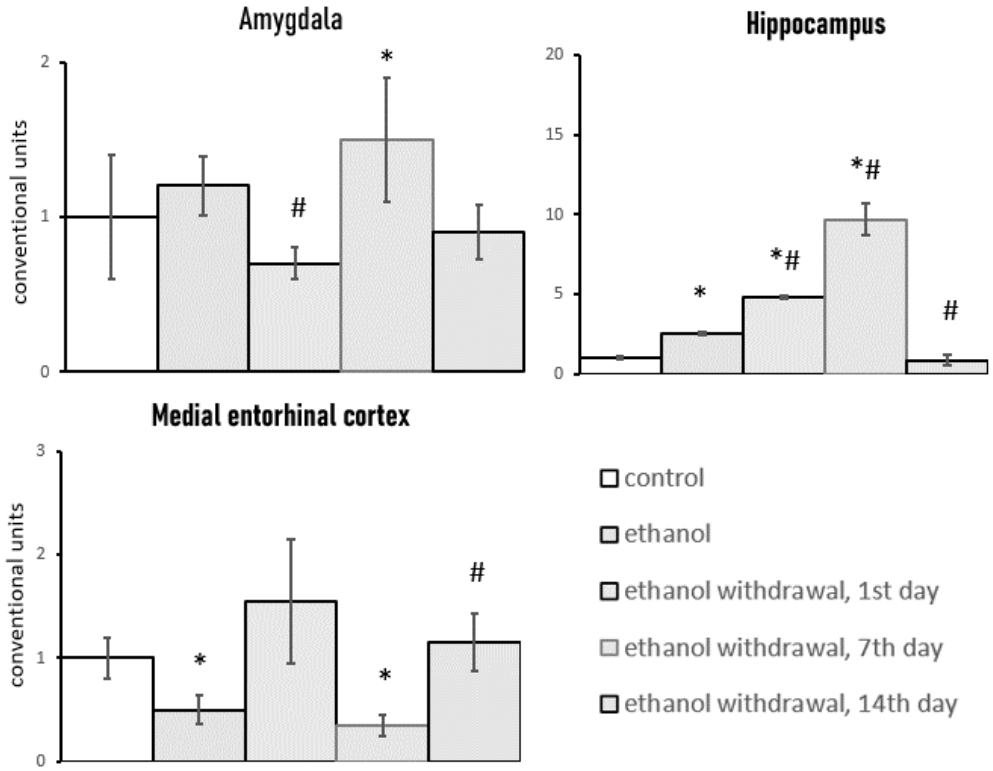

Figure 15 - Expression of Ccl2 gene in different structures of rat brain under chronic alcoholization and under conditions of ethanol withdrawal

(*p<0.05 in relation to the control group, #p<0.05 in relation to the alcoholization group)

The expression level of Ccl2 gene is reduced in the medial entorhinal cortex in the group of long-term ethanol exposure; however, on the 1st day the expression level is increased, and on the 7th day of withdrawal the expression level is lower than the level in the control group. On the 14th day of ethanol withdrawal, the expression level of Ccl2 gene is not significantly different from the level of values in the control group (Figure 15). The expression of Ccl2 gene in the hippocampus

was increased in the alcoholization group as well as on the 1st and 7th days of alcohol withdrawal, on the 14th day of withdrawal the value reaches the level of control (Figure 15).

3.1.5 Correlation analysis

Correlation analysis was performed between changes in the expression levels of TLR genes and pro-inflammatory cytokines genes in the long-term ethanol exposure groups and in the ethanol, withdrawal groups in the dynamics on the 1st, 7th and 14th set days. The results of the performed correlation analysis are clearly presented in the paper, where a reliable correlation between the analyzed indicators of the selected groups was revealed (R2≥0.75) (Figure 16).

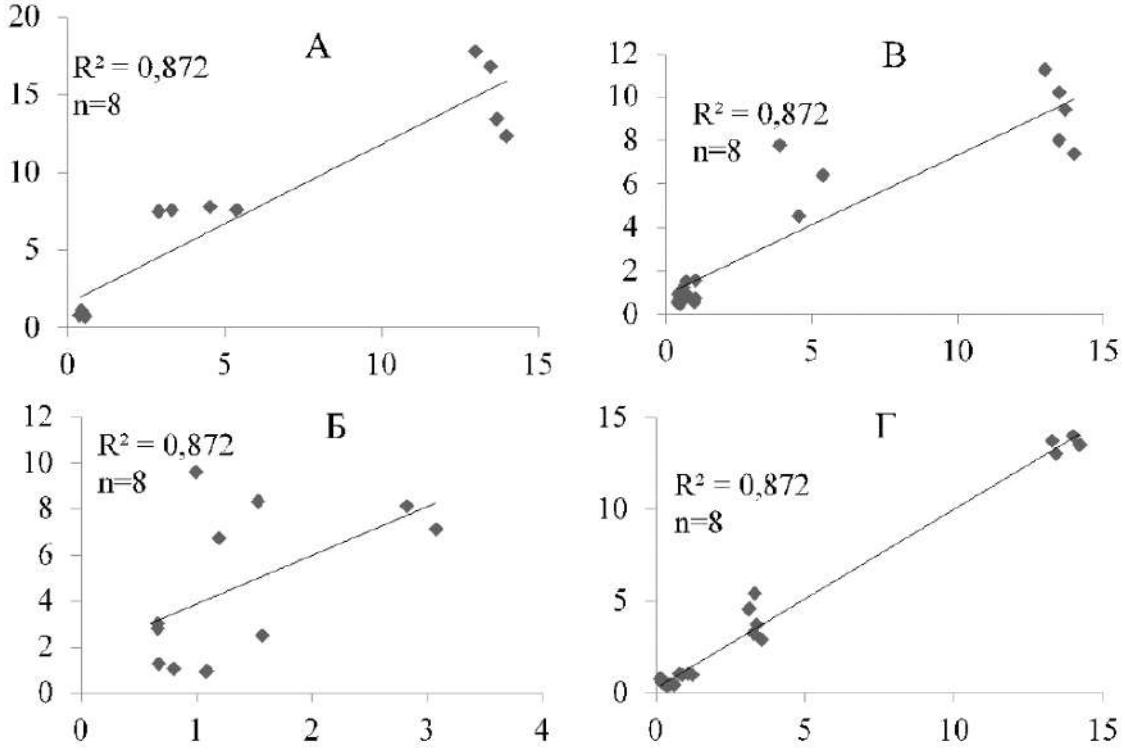

Figure 16 - Results of correlation analysis. (A) TLR3 mRNA level correlates with IL-1β mRNA level in hippocampus; (B) TLR7 mRNA level correlates with IL-1β mRNA level in hippocampus; (C) TLR4 mRNA level correlates with IL-1β mRNA level in mEC and (D) in hippocampus.

3.2 Hmgb1 gene expression level in rat brain structures under prolonged ethanol exposure and withdrawal

The results of the study revealed that the rat brain structures under study undergo multidirectional changes in Hmgb1 gene expression under prolonged ethanol exposure and during ethanol withdrawal (Figure 17).

Prolonged ethanol exposure caused an increase in Hmgb1 gene expression levels in the rat brain amygdala and striatum. No significant changes in the level of Hmgb1 expression were detected in the hippocampus when modeling long-term ethanol exposure in the experiment.

The level of Hmgb1 gene expression was investigated during the abolition of long-term ethanol exposure in dynamics. Thus, an increased level of Hmgb1 gene expression was detected in rat brain striatum on the 1st day of ethanol withdrawal, on the 7th day the gene expression was decreased relative to the values in the control group, on the 14th day of withdrawal the expression level was significantly increased in comparison with the expression level in the control group. In the amygdala, an increased level of gene expression was detected on the 1st day of ethanol withdrawal, but no significant changes were found on the 7th day and 14th day. In the hippocampus of rat brain, a trend towards increased gene expression level on the 1st and 7th day of ethanol withdrawal was observed, however statistically not significant. No changes were detected in the hippocampus of rat brain on the 14th day of withdrawal (Figure 17).

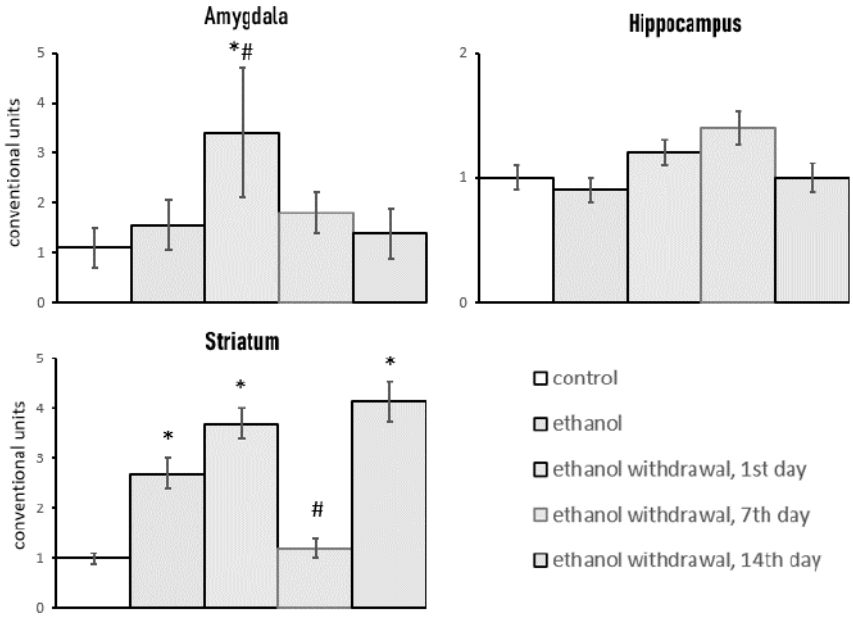

Figure 17 - Expression of Hmgb1 gene in different structures of rat brain under chronic alcoholization and under conditions of ethanol withdrawal

(*p<0.05 in relation to the control group, #p<0.05 in relation to the alcoholization group)

3.3 Gene expression status of TLR system in the nucleus accumbens of rat brain during withdrawal of long-term ethanol exposure and during rifampicin use

Modeling of long-term ethanol exposure and withdrawal state was performed according to protocol 3 (see Materials and Methods chapter).

3.3.1 Effects of rifampicin on toll-like receptor gene expression

The results of the performed PCR analysis showed no significant changes in the expression of Tlr3 and Tlr7 genes at the mRNA level on the 10th day of ethanol withdrawal in rat brain Nac (Figure 18).

It is likely that the gene expression level normalizes in Nac, i.e., the gene expression changes here are of a less prolonged nature. However, there was an increase in the level of Tlr4 gene expression on the 10th day of ethanol withdrawal in Nac. Rifampicin injections decreased the increased level of Tlr4 gene expression to the level of values in the control group (Figure 18).

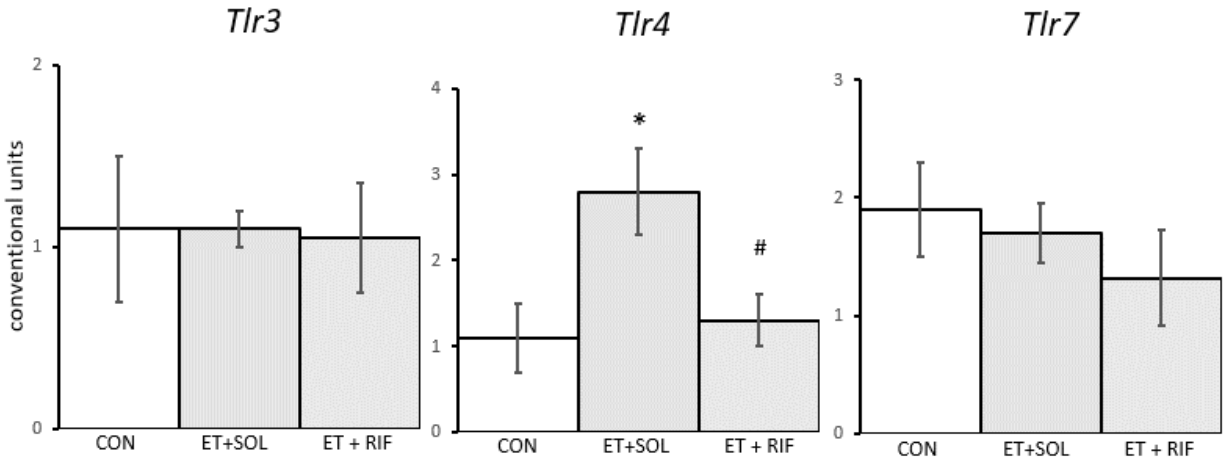

Figure 18 - TLRs mRNA levels in Nac when corrected with rifampicin
(*p<0.05 in relation to the control group, #p<0.05 in relation to the alcoholization group)

3.3.2 Effect of rifampicin on gene expression of adaptor proteins and transcription factors

Next, the gene expression level of TLR adaptor proteins, which are involved in the realization of TLR-dependent intracellular signal transduction, was investigated.

Myd88 and Trif proteins signal from TLRs to a cascade of intracellular kinases, activating the operation of transcription factors responsible for regulating the expression of pro- and anti-

inflammatory genes (Airapetov M.I., 2020, Crews F.T., Zou J., Qin L. (2011), Coleman L.G. Jr. (2020). However, we did not detect significant changes in Myd88 and Trif gene expression (Figure 19).

In addition, we did not detect significant changes in gene expression of the TLR-dependent intracellular signaling transcription factors NfκB (aka NfκB1), IRF3, and IRF1 (Figure 20).

The expression level of adaptor proteins has previously been studied by other researchers in the Nac of rat brain at the time of long-term alcoholization and during alcohol withdrawal on the 1st day, the researchers also found no changes in the mRNA content of Myd88 and Trif (McCarthy G.M., Warden A.S., Bridges C.R., Blednov Y.A., Harris R.A. (2017).

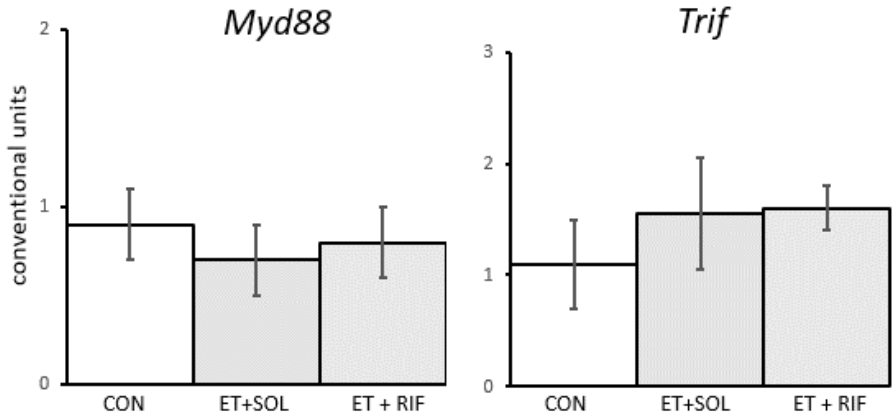

Figure 19 - Adaptor protein mRNA levels in Nac when corrected with rifampicin

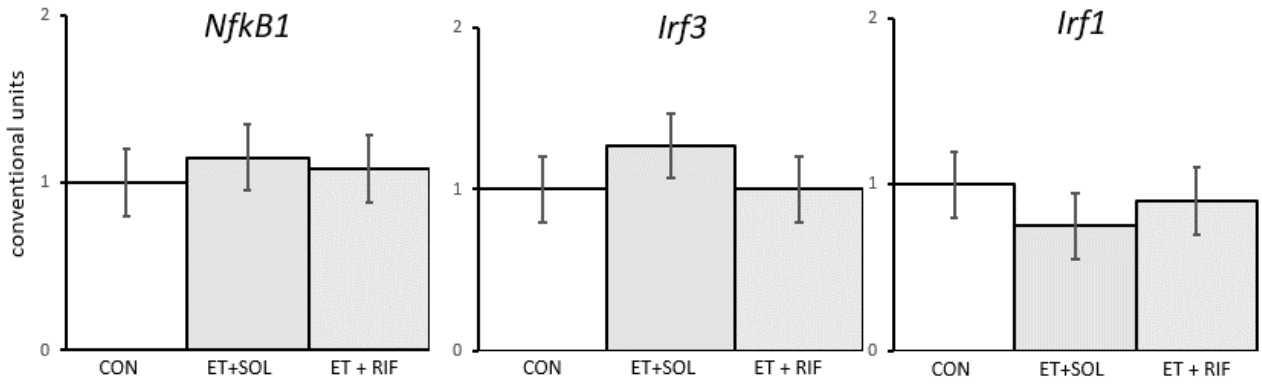

Figure 20 - mRNA levels of transcription factors in Nac during rifampicin correction

3.3.3 Effect of rifampicin on cytokine gene expression in the nucleus accumbens of rat brain during withdrawal of long-term ethanol exposure

The results of our experiment showed that in addition to an increase in the expression level of Tlr4 gene, there was also an increase in the expression level of Ccl2 gene, and a slight increase in the expression of Il1β Nac gene of rat brain on the 10th day of ethanol withdrawal (Figure 21).

In addition, we found that concomitantly with the increase in gene expression of pro-inflammatory cytokines, there was a decreased expression of the anti-inflammatory cytokine genes Il10 and Il11 (Figure 21).

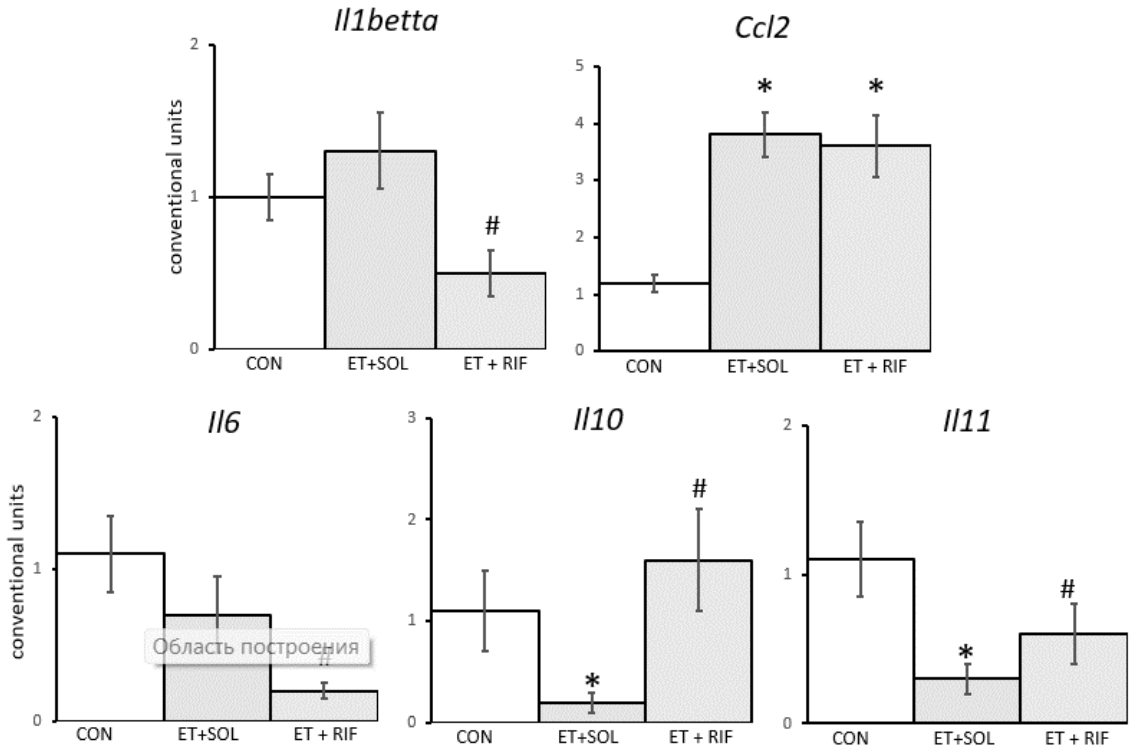

Figure 21 - Cytokine mRNA levels in Nac during rifampicin correction
(*p<0.05 in relation to the control group, #p<0.05 in relation to the alcoholization group)

Thus, the data obtained could possibly indicate the development of a persistent proinflammatory state in rat brain Nac induced by prolonged ethanol ingestion. It is possible that TLR4-dependent signaling pathways may be involved in the development of these effects.

At the same time, rifampicin injections decreased the increased level of Il1β gene expression and had no significant effect on Ccl2 gene expression. Importantly, rifampicin injections increased the decreased expression level of the anti-inflammatory cytokine genes Il10 and Il11 (Figure 21). We examined the expression level of one pro-inflammatory cytokine gene in Nac on the 10th day of withdrawal of long-term ethanol exposure, however, we did not find significant changes in the ethanol withdrawal group, but rifampicin injections decreased the expression level of this pro-inflammatory gene as well to levels below those observed in the control group.

3.3.4 Effect of rifampicin on Hmgb1 gene expression in the adjacent nucleus of the rat brain during ethanol withdrawal

The experiment performed revealed an increase in the expression level of Hmgb1 gene in Nac (Figure 22).

Hmgb1 protein is known to be an endogenous agonist of TLR4 receptor. It has also been found that the protein content is increased in the brain under various pathological conditions, including alcoholism. There is evidence that increased Hmgb1 content may initiate TLR4-dependent signaling, leading to increased gene expression of proinflammatory cytokines in nervous tissue, which serves as a reason for the development of neuroinflammatory process. Rifampicin injections decreased the increased level of Hmgb1 gene expression to the level of values in the control group (Figure 22).

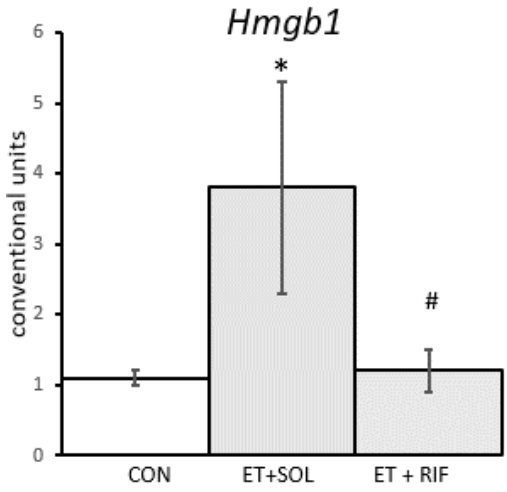

Figure 22 - Hmgb1 mRNA levels in Nac

(*p<0.05 in relation to the control group, #p<0.05 in relation to the alcoholization group)

3.4 Effect of purified sum of ginsenosides on toll-like receptor system in the nucleus accumbens and hippocampus of rat brain during withdrawal from long-term ethanol exposure

Modeling of long-term ethanol exposure and withdrawal state was performed according to protocol 2 (see Materials and Methods chapter).

3.4.1 Analysis of the sum of ginsenosides obtained from suspension culture of *Japanese ginseng* cells

Preliminary chromatographic analysis showed that the purified extract obtained from the suspension culture of Japanese ginseng cells contained 83% of ginsenosides. Chromatographic analysis of the obtained sum of ginsenosides was also performed. The sum of ginsenosides used for further study contained in its composition: triterpene glycosides of oleanolic acid (main component - ginsenoside R0) and triterpene glycosides of protopanaxadiol (main ginsenosides - Rb1, Rc, Rb2/Rb3, Rd). Approximate content of major ginsenosides (% of total mass): 1. R0 - 33 %; 2. Rb1 - 12 %; 3. Rc+Rb2+Rb3+Rd -13 %; 4. Other triterpene glycosides (in total) - 25 %.

3.4.2 Effect of the sum of ginsenosides on toll-like receptor gene expression

In our study, long-term alcoholization of rats resulted in a significant decrease in Tlr3 gene expression level on the 7th day of ethanol withdrawal in Nac (Figure 23). Tlr4 gene expression level was significantly increased in Nac on the 7th day of ethanol withdrawal. The level of Tlr7 gene expression in Nac was not altered in any of the studied experimental groups of animals. At the same time, application of the sum of ginsenosides caused a significant increase in the level of Tlr3 gene expression to levels exceeding the values in the control group of animals. In addition, gisenosides caused a decrease in Tlr4 gene expression in Nac almost to the level of values in the control group (Figure 23).

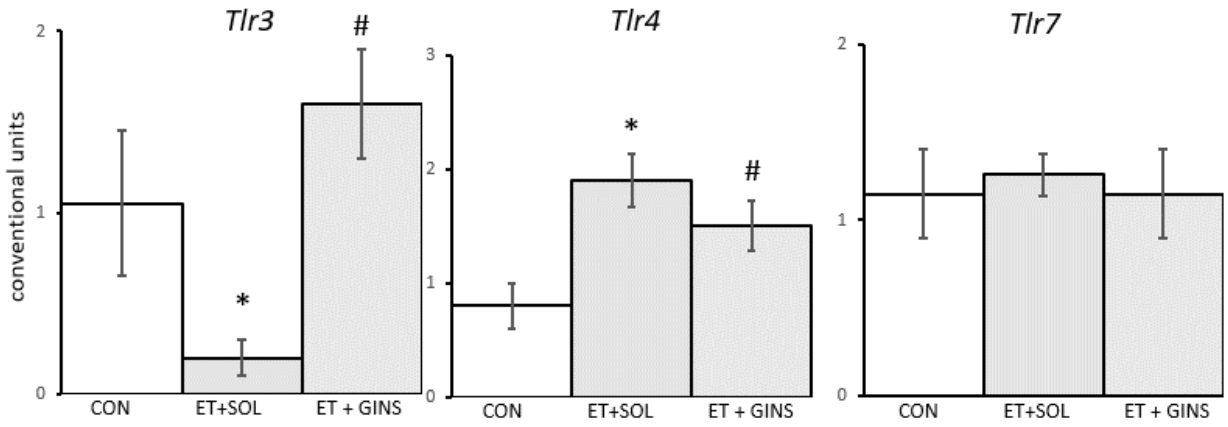

Figure 23 - TLRs mRNA level in Nac

(*p<0.05 in relation to the control group, #p<0.05 in relation to the alcoholization group)

In the hippocampus (HIP) on the 7th day of withdrawal of long-term ethanol exposure, an increased level of gene expression of all TLR subtypes studied by us (Tlr3, Tlr4, Tlr7) was observed, with the most significant increase in expression level being characteristic of Tlr4.

Administration of the sum of ginsenosides had no significant effect on the expression of Tlr3 and Tlr3 genes in the hippocampus, but decreased the expression level of Tlr7 gene to the level of values in the control group (Figure 24).

Thus, the purified sum of ginsenosides used in our experiment had an effect on the pathologically altered TLR gene expression level associated with long-term ethanol consumption in rodents.

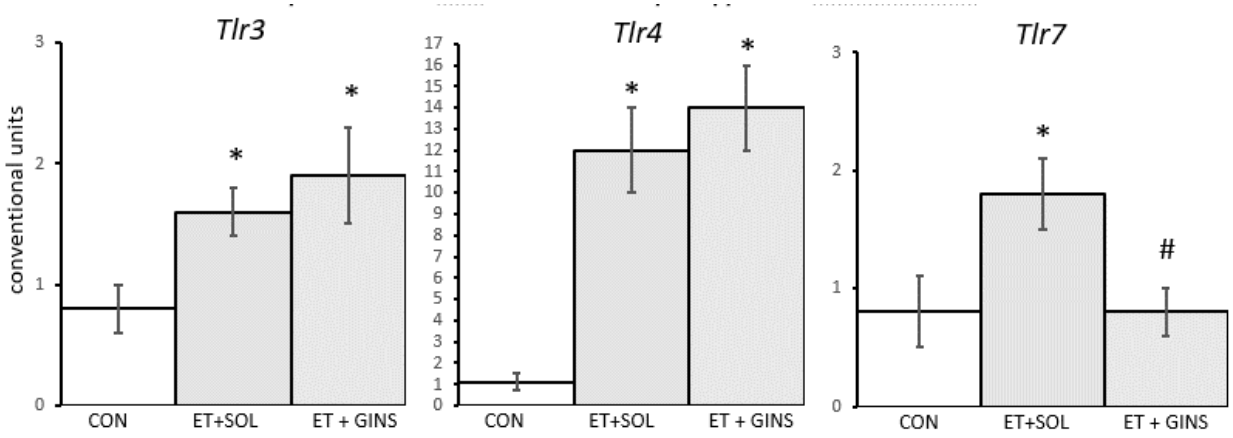

Figure 24 - TLRs mRNA levels in HIP

(*p<0.05 in relation to the control group, #p<0.05 in relation to the alcoholization group)

3.4.3 Effect of the sum of ginsenosides on transcription factor gene expression

We evaluated the gene expression levels of several transcription factors that are key components of TLR-dependent signaling. The expression level of the NfκB (aka NfκB1) gene had no statistically significant differences in any of the animal groups we studied in Nac (Figure 25).

The level of Irf3 gene expression was elevated in Nac on the 7th day of withdrawal of long-term ethanol exposure, and administration of the sum of ginsenosides restored the level of Irf3 gene expression to the level of values in the control group (Figure 25).

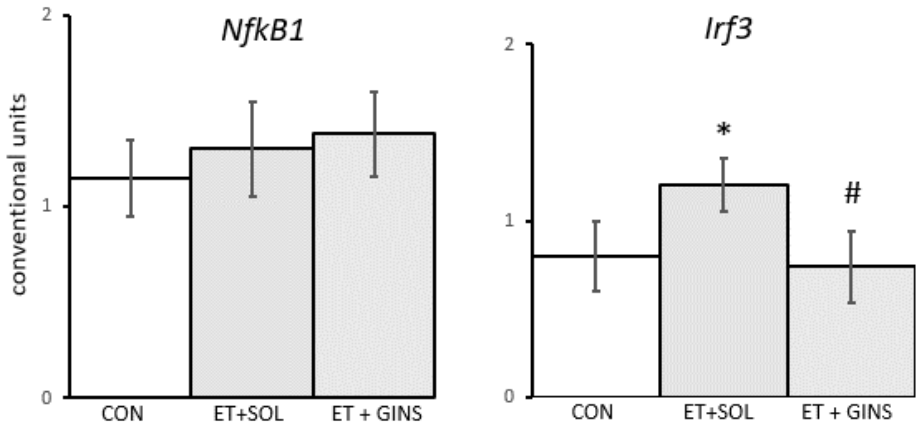

Figure 25 - Transcription factor mRNA levels in Nac
(*p<0.05 in relation to the control group, #p<0.05 in relation to the alcoholization group)

No changes in Irf3 gene expression were detected in the hippocampus of rat brain. However, an increase in the level of Irf1 gene expression was detected in the hippocampus on the 7th day of ethanol withdrawal, while application of ginsenosides had no significant effect on the level of Irf1 gene expression (Figure 26). In Nac, we did not examine Irf1 gene expression.

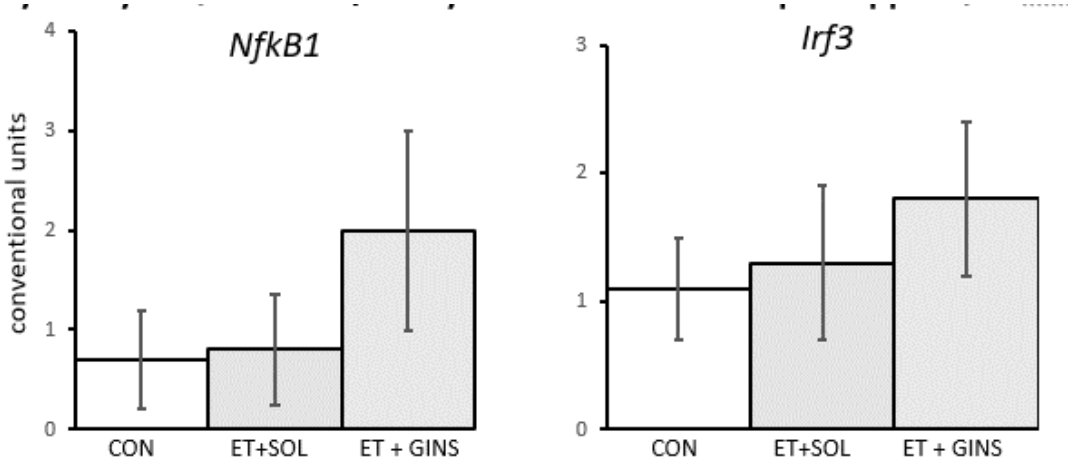

Figure 26 - Transcription factor mRNA levels in HIP

3.4.4 Effect of sum of ginsenosides on the expression level of cytokine genes in the nucleus accumbens and hippocampus of rat brain during ethanol withdrawal

Changes in the expression of a number of pro- and anti-inflammatory cytokine genes serve as a response to changes in the functional activity of molecular intracellular pathways of TLR-dependent signaling. In this experiment, we focused our attention on the expression level of key neuroinflammatory genes - Il1β and Ccl2.

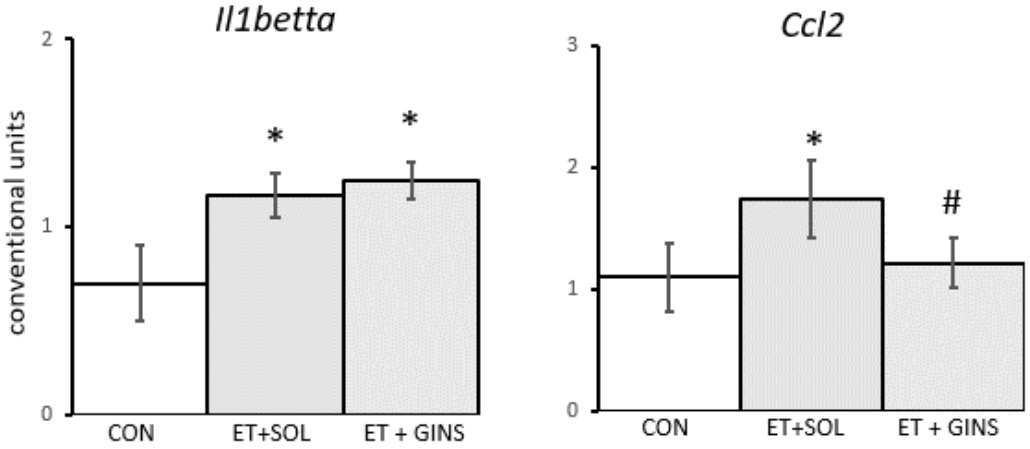

Figure 27 - Cytokine mRNA levels in Nac

(*p<0.05 in relation to the control group, #p<0.05 in relation to the alcoholization group)

The expression level of Il1β gene in Nac was upregulated in both ethanol withdrawal control group, animal group, and group with injected sum of ginsenosides. The gene expression state of another proinflammatory cytokine Ccl2 was elevated only in the ethanol withdrawal control group, whereas injections of the sum of ginsenosides decreased this index to the values of the control group (Figure 27).

In HIP, the expression level of Il1β gene did not change in any of the experimental groups of animals, while the gene expression level of another pro-inflammatory cytokine here was increased both in the control group of ethanol withdrawal and in the group of animals with performed sum of ginsenosides (Figure 28).

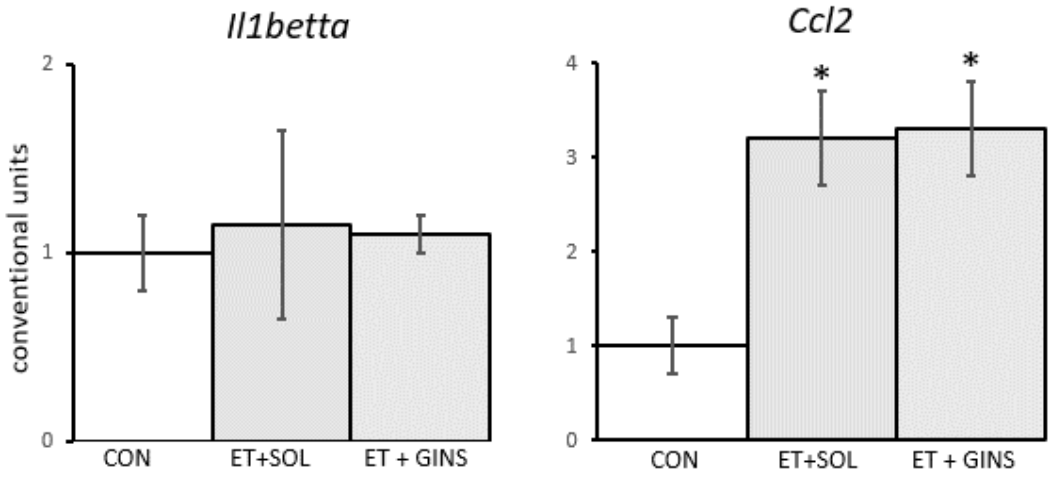

Figure 28 - Cytokine mRNA levels in HIP

(*p<0.05 in relation to the control group, #p<0.05 in relation to the alcoholization group)

3.4.5 Effect of the sum of ginsenosides on the level of Hmgb1 gene expression in the nucleus accumbens and hippocampus of rat brain during ethanol withdrawal

Experimental results showed an increased level of Hmgb1 gene expression in the Nac (Figure 29) and HIP (Figure 30) of the rat brain in the withdrawal group of long-term ethanol exposure on the 7th day. both in the Nac (Figure 29) and HIP of the rat brain (Figure 30).

In the group of animals with performed injections of the sum of ginsenosides, the level of Hmgb1 gene expression normalized to the level of values in the control group.

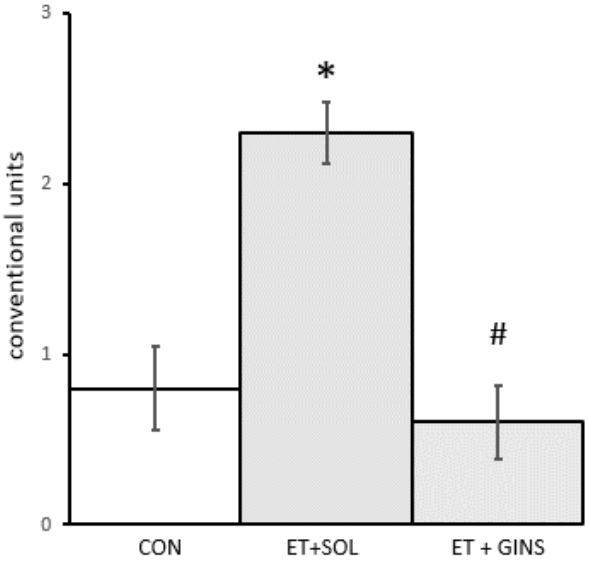

Figure 29 - Hmgb1 mRNA level in Nac (*p<0.05 vs. control group, #p<0.05 vs. alcoholization group)

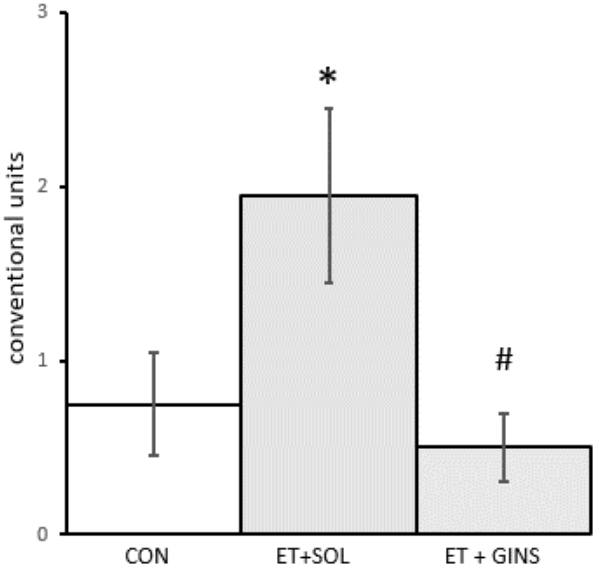

Figure 30 - Hmgb1 mRNA level in HIP (*p<0.05 vs. control group, #p<0.05 vs. alcoholization group)

3.5 Micro-RNA content in the nucleus accumbens of rat brain during prolonged ethanol exposure

Modeling of prolonged ethanol exposure and withdrawal state was performed according to protocol 4 (see chapter "Materials and Methods").

Prolonged ethanol exposure for 1 month resulted in a 1.71-fold decrease in miR-let7b levels in rat brain Nac, miR-96 levels decreased 3.87-fold, and miR-182 levels decreased 2.29-fold. In contrast, miR-155 content increased 1.41-fold (Figure 31).

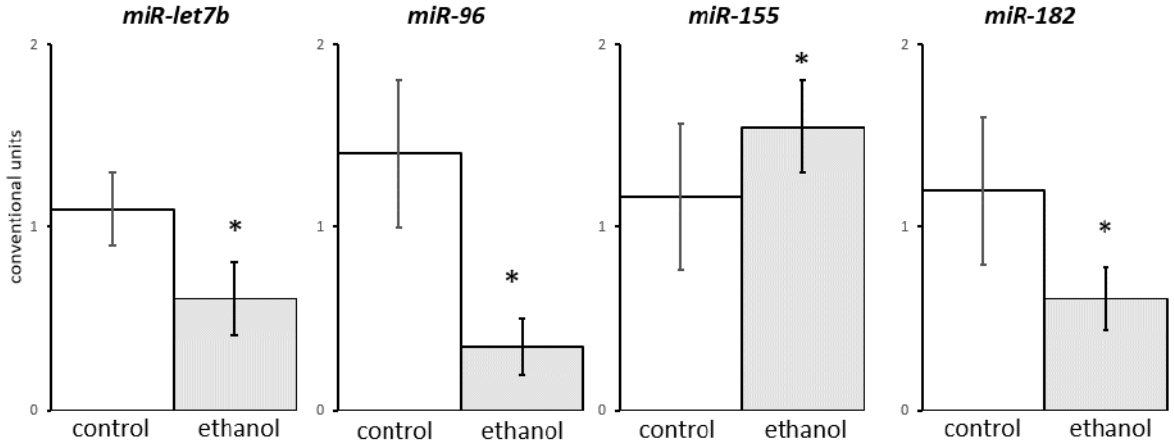

Figure 31 - miR levels in Nac (*p≤0.05 vs. control)

The results of our experiment showed that TLR7 mRNA level was also downregulated in Nac as a result of long-term alcoholization (Figure 32), as was miR-let7b. B. We obtained evidence of increased TLR4 mRNA content (Figure 32), and the level of miR-155 was also elevated.

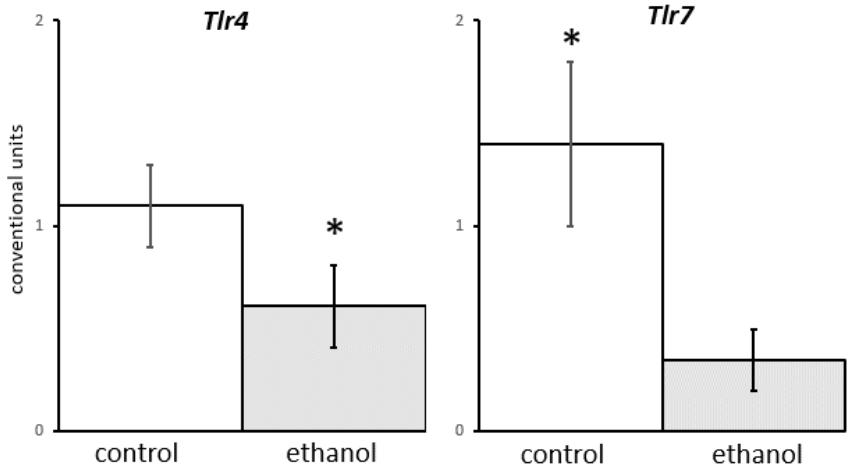

Figure 32 - TLR mRNA levels in Nac (*p≤0.05 vs. control)

The mechanism of interaction of the microRNA cluster miR-183C (which includes miR-96 and miR-182) with TLR4 is not fully understood, but there are works that report possible such interactions using miR-182-5p as an example (Ureña-Peralta J.R., 2018, 2020). In our experiment, we observe reduced expression levels of miR-96 and miR-182 when TLR4 mRNA is upregulated in Nac.

In addition, there are observations that ethanol induces the formation of HMGB1-miR-let-7 complexes in microvesicles, which cause the development of neurotoxic effects through the activation of TLR7 in neurons (Coleman L.G., et al., 2017). The results of our experiment revealed elevated levels of Hmgb1 mRNA in Nac (Figure 33), whereas miR-let7b and TLR7 mRNA were downregulated. Such

data seem to indicate that there is no functionally significant relationship between Hmgb1 and the miR-let7b-TLR7 system in the Nac, as divergent responses were obtained with respect to Hmgb1 and miR-let7b expression levels in this brain structure.

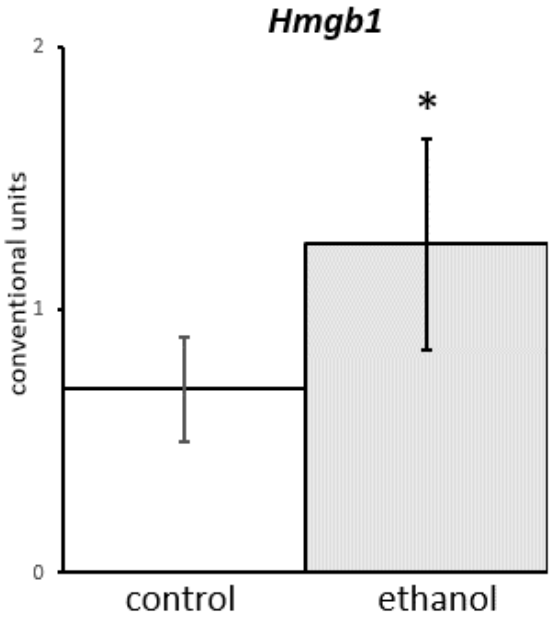

Figure 33 - Hmgb1 mRNA levels in Nac (*p≤0.05 vs. control)

CHAPTER 4. DISCUSSION OF THE RESULTS OBTAINED

4.1 Expression of TLR genes and proinflammatory cytokines under conditions of long-term ethanol exposure and during ethanol withdrawal in rat brain

TLR3 mRNA levels in rat brain under conditions of long-term alcohol exposure and during alcohol withdrawal. In mouse experiments, TLR3 signaling has been shown to be involved in the development of a pro-inflammatory response during alcohol consumption, as well as mediating mechanisms of pathological attraction to alcohol and, consequently, increased alcohol consumption (Warden A.S., et al., 2018). However, there is very little data at all to draw full conclusions about the contribution of TLR3 to the pathogenesis of alcoholism. There are no studies that show what happens to TLR3 gene expression in limbic brain structures such as the hippocampus, amygdala, and striatum. There are no data on the changes in TLR3 gene expression in the brain during alcohol withdrawal on different terms after long-term alcoholization. Therefore, the aim of this experiment was to evaluate the level of TLR3 gene expression in different structures of the rat brain during long-term alcoholization (1 month), as well as at different terms of alcohol withdrawal (1st, 7th, 14th days).

We obtained that the TLR3 mRNA level under alcoholization conditions changes only in the hippocampus of the rat brain (Airapetov M.I. et al., 2020); however, during alcohol withdrawal, changes in TLR3 gene expression at the mRNA level were observed in all brain structures we studied. By the 14th day of withdrawal, the mRNA level is lower than the control level in the amygdala (4.6 times), higher than the control level in the hippocampus (15.5 times) and striatum (2.1 times), less significant changes in the medial entorhinal cortex (1.9 times higher than the control level).

It is known from the literature that TLR3-dependent signaling can influence the level of voluntary alcohol consumption in rodent experiment (Warden A.S., et al., 2018). For example, a single intraperitoneal injection of a TLR3 agonist (poly (I:C)) into mice results in increased levels of voluntary alcohol consumption in a two-tailed test (Warden A.S., et al., 2018). By investigating the effects of poly (I: C) on gene expression in the nucleus accumbens of the rat brain, it was shown that TLR3 activation leads to increased mRNA levels of TLR3, COX2 (cyclooxygenase 2, cyclooxygenase 2), and glutamatergic system genes (mGluR2 - metaβotropic glutamate receptor 2; mGluR3; GLT1 - glutamate transporter 1, glutamate transporter 1) and the BDNF (βrain-derived neurotrophic factor, neurotrophic brain factor) gene in the contiguous brain nucleus (Nacc, nucleus accumβens). In addition, increases in mRNA of each of these genes correlated with increases in TLR3 mRNA levels. These results suggest that the operation of the glutamatergic system undergoes alterations upon TLR3 activation (Randall P.A., et al., 2018).

Based on the obtained data, we assume that the obtained TLR3 changes at the mRNA level in limbic structures of the brain during alcohol withdrawal may be associated not only with the involvement of TLR3 in the activation of proinflammatory gene expression, but also in the development of mechanisms aimed at increasing the level of pathological craving for alcohol during alcohol withdrawal. So it is quite possible that in the structures studied by us the role of TLR3 may be related to their mediated involvement in the dysregulation of neurotransmitter systems, which may lead, for example, to the motivation to use alcohol during alcohol withdrawal. In order to confirm the obtained assumptions it is necessary to conduct additional studies in the future.

TLR4 mRNA level in the rat brain under conditions of long-term alcoholization and during alcohol withdrawal. The largest number of works was aimed at studying the involvement of TLR4 in the mechanisms of activation of proinflammatory signaling as a result of ethanol consumption, but most of such works were performed on cell cultures and on the cerebral cortex of mice (Blednov Y.A., et al., 2017, 2012; Warden A.S., et al., 2018). In contrast, we focused our attention on studying the expression level of TLR4 gene at the mRNA level in such rat brain structures as the amygdala body, medial entorhinal cortex (mEC), striatum and hippocampus.

The results of our experiment showed no significant changes in the structures of the long-term alcoholized brain after 1 month of alcoholization (Airapetov M.I. et al., 2020). However, during the period of alcohol withdrawal, the mRNA level changes in all the rat brain structures we studied. Thus, by the 14th day of withdrawal the TLR4 mRNA level decreases in the amygdala (2.4 times) and medial entorhinal cortex (1.6 times) below the level of control values, in the striatum it returns to the level of control values, in the hippocampus the value of TLR4 mRNA level exceeds 13.5 times the level of control values.

A large number of studies performed in rats and mice using genetic and pharmacological manipulations (TLR4 knockout and antagonist application), have shown that TLR4 activity does not regulate the level of ethanol intake, but nevertheless, changes in TLR4-mediated signaling after alcohol consumption are observed (Blednov Y.A., et al., 2017, 2012; Warden A.S., et al., 2018). However, despite the fact that TLR4 is not directly involved in the formation of pathological craving for alcohol, it is involved in the processes of activation of pro-inflammatory genes, resulting in the development of neuroinflammatory process in the CNS. There are also suggestions that TLR4-MyD88-dependent signaling can alter the activity of GABAergic transmission in the CNS (Blednov Y.A., et al., 2017), which indicates other physiological roles of TLR4, different from the activation of genes of the innate immune system. There is also evidence that TLR4 forms heterodimers with corticoliberin receptor type 2 (CRFR2) in the CNS (Lebedev K.A., Ponyakina I.D., 2017). Intraperitoneal administration of LPS (lipopolysaccharide, lipopolysaccharide), an exogenous TLR4 ligand, accelerates the development of anxious behavior in animals subsequently exposed to ethanol. Mice deprived of TLR4 or MyD88

became less sensitive to the sedative and intoxicating effects of ethanol, whereas mice deprived of TLR2 did not differ from control mice in these tests (Blednov Y.A., et al., 2017). All this indicates that TLR4 may mediate changes in the activity of neurotransmitter systems, which may also contribute to the pathogenesis of alcoholism.

TLR7 mRNA levels in rat brain under conditions of long-term alcoholization and during alcohol withdrawal. In addition to the already described TLR3 and TLR4, a small number of studies have focused on the contribution of TLR7 to the pathogenesis of alcoholism (Coleman L.G., et al., 2017). Meanwhile, the level of TLR7 gene expression in different brain structures has not been previously studied.

In our experiment, we obtained that the level of TLR7 mRNA in the long-term alcoholized brain does not change in any of the studied brain structures (Airapetov M.I. et al., 2020). During alcohol withdrawal by the 14th day of withdrawal, TLR7 mRNA level was decreased in the amygdala (3.2-fold) and striatum (3.5-fold), increased in the hippocampus (8.5-fold), and no changes in mRNA level were detected in the medial entorhinal cortex.

It is difficult to draw any significant conclusions regarding the obtained changes at the mRNA level, as there are not many works paying attention to TLR7-dependent signaling in the brain.

The mRNA levels of proinflammatory cytokines under conditions of alcoholization and alcohol withdrawal. In our experiment, using a model of semicompulsory alcoholization of rats for 1 mo. 20% ethanol, we obtained data on IL-1β and CCL2 mRNA levels to detect the presence of increased expression of proinflammatory genes of the innate immune response under conditions of alcoholization as well as under conditions of ethanol withdrawal. High mRNA levels of proinflammatory cytokines may indicate the presence of neuroinflammatory process in the rat brain (Coleman L.G., et al., 2018, 2017, 2017).

In our experiment, IL-1β mRNA levels were elevated in the amygdala (2.5-fold) and in the mES (2.2-fold), with a slight decrease in the hippocampus (1.6-fold). During alcohol withdrawal, by day 14 of withdrawal, IL-1β mRNA levels return to normal, reaching the level of control values, in the amygdala and in the mES; however, in the hippocampus, IL-1β mRNA levels by day 14 of withdrawal exceed the control level by 13.6-fold.

CCL2 mRNA level changes insignificantly under conditions of alcoholization, by the 14th day of ethanol withdrawal in all studied brain structures the mRNA level is at the level of control values. However, it is worth noting that in the hippocampus on the 7th day of withdrawal the CCL2 mRNA level exceeds the control level by 9.1 times. It is well known that cytokine CCL2 is the most important chemokine, which is responsible for the migration of monocytes to the focus of inflammation (Lebedev K.A., Ponyakina I.D. 2017; Crews F.T., et al., 2016). Consequently, such increased expression of CCL2 gene on the 7th day of alcohol withdrawal indicates the presence of neuroinflammatory process in the

hippocampus, while the mRNA level of proinflammatory IL-1β gene in the hippocampus is also increased 4.3-fold on the 7th day of withdrawal and 13.6-fold by the 14th day of withdrawal. All these data suggest the presence of a long-term neuroinflammatory process in the hippocampus of rat brain during alcohol withdrawal.

Correlation analysis. It is well known that activation of TLR-dependent signaling involving different TLR subtypes leads to activation of various pro-inflammatory genes, including IL-1β and CCL2 (Coleman L.G., et al., 2017, 2018; Crews F.T., et al., 2017). Based on our data we assumed that activation of proinflammatory genes can be and in our case, in conditions of prolonged semi-coercive alcoholization of rats with ethanol and in conditions of ethanol withdrawal, caused by activation of TLR-dependent signaling, which is realized with the participation of toll-like receptors. That is why our further task was to try to find out the contribution of different TLR subtypes to the activation of our selected genes of the innate immune system (IL-1β and CCL2) in different brain structures. For this purpose, we performed a correlation analysis, which showed the relationship between the dynamics of variability of mRNA levels of proinflammatory cytokines and TLRs in the rat brain structures we studied under conditions of prolonged alcoholization and alcohol withdrawal.

Thus, the data were obtained that the level of TLR3 mRNA correlates with the level of IL-1β mRNA in the hippocampus of rat brain. The obtained correlation may indicate the involvement of TLR3-dependent signaling in the increase of gene expression of the proinflammatory cytokine IL-1β in the hippocampus of the rat brain. In other brain structures (striatum, amygdala, mEC) TLR3 mRNA levels do not correlate with IL-1β mRNA levels, which may indicate that activation of TLR3-dependent signaling in other brain structures may lead to other events at the level of intracellular signaling. For example, it is known that TLR3-dependent signaling along with TLR4-dependent signaling can lead to activation of interferon transcription factors (IRF3), which further leads to increased expression of interferon genes (Lebedev K.A., Ponyakina I.D., 2017).

Further, correlation analysis showed us that TLR4 mRNA level correlates with the mRNA level of pro-inflammatory cytokine IL-1β in hippocampus and in mEC. Based on this correlation, we can also conclude that TLR4-dependent signaling is probably also involved in the activation of proinflammatory IL-1β gene expression in mEC and hippocampus. Previously, researchers have shown that TLR4 activation leads to increased activity levels of not only IL-1β but also CCL2 (Crews F.T., et al., 2014, 2016, 2017). However, in our experiment, TLR4 mRNA levels do not correlate in any of the brain structures with CCL2 cytokine levels. The obtained result may be explained by the different alcoholization technique we chose or by the selected experimental groups for the mRNA level study. It is possible that we could have observed different results at other alcoholization periods and alcohol withdrawal periods. It is also possible that TLR4 activation does not lead to activation of the pro-inflammatory cytokine CCL2 gene in the brain structures we studied, but does in others, as has been

shown previously, for example, in the prefrontal cortex in a mouse experiment (Crews F.T., et al., 2014, 2016, 2017). The observed changes in TLR4 gene activity at the mRNA level in other brain structures where mRNA levels do not correlate with IL-1β may be due to the fact that TLR4-avoidance signaling in the amygdala and striatum possibly leads to the activation of other pro-inflammatory genes. It is known that TLR4, as well as TLR3 is able to activate interferon transcription factors (IRF3), which leads to increased expression of interferon genes (Lebedev K.A., Ponyakina I.D., 2017).

Further, we obtained data that TLR7 mRNA level correlates with IL-1β mRNA level in the hippocampus of rat brain, which may also indicate the involvement of TLR7-dependent signaling in the activation of pro-inflammatory genes in this brain structure. In contrast, TLR7 mRNA levels in other brain structures did not show such a correlation, indicating that TLR7-dependent signaling in other brain structures may play a different role.

4.2 Hmgb1 gene expression under long-term ethanol exposure and ethanol withdrawal in rat brain

In our study, the expression level of Hmgb1 gene under conditions of long-term alcoholization is increased in the striatum of rat brain, whereas it remains unchanged in the amygdala and hippocampus. Alcohol withdrawal leads to changes in Hmgb1 mRNA content in the striatum and amygdala of the rat brain, whereas no such changes are observed in the hippocampus. In the striatum, increased mRNA content was observed on days 1 and 14 of alcohol withdrawal, while on day 7 the value of mRNA content was the same in the control group. In the amygdala, the mRNA level was elevated on the 1st day of alcohol withdrawal, while on the 7th and 14th days the index did not differ from the control values.

There is no correlation between the mRNA content of proinflammatory cytokine IL-1β and mRNA in the brain structures we studied. In the long-term alcoholization group, the IL-1β mRNA level was increased in the striatum of the rat brain, whereas this was not observed in the amygdala and hippocampus. During alcohol withdrawal, IL-1β mRNA levels are elevated in the striatum on day 1 of alcohol withdrawal, whereas there is no change in the amygdala and hippocampus on day 1 of alcohol withdrawal. In the amygdala, there is an increase in IL-1β mRNA levels only on day 14 of withdrawal. In the hippocampus there are no changes in mRNA content in comparison with the control group during alcohol withdrawal on the terms we studied.

In the experiments of Vetreno R.P. and Crews F.T. (Vetreno R.P., et al., 2012) on the model of adolescent alcoholization (adolescent binge drinking) of rodents it was shown that ethanol increases the content of mRNA and protein HMGB1 in the cerebral cortex of adolescent rats, which is preserved later in adult rats. It was hypothesized that adolescent alcoholization leads to a persistent increase in the activity of the innate immune system, which persists at elevated levels in the rat brain, which correlated in the study and with neurocognitive dysfunction in adult rats (Vetreno R.P., et al., 2012). It was later

hypothesized that HMGB1 protein may be involved in the increased activation of the innate immune system due to the fact that it is an endogenous agonist of many receptors of the innate immune system.

Whitman B.A.'s 2013 paper (Whitman B.A., et al., 2013), using an antagonist to the HMGB1 protein, supports the hypothesis that increased amounts of HMGB1 protein leads to increased mRNA levels of pro-inflammatory cytokines in the prefrontal cortex of the brain in long-term alcoholized mice, but allows for different results to be obtained in other brain structures.

The cerebellum of long-term alcoholized mice showed a slight increase in HMGB1 mRNA content relative to the control group. In addition, increased mRNA levels of receptors to HMGB1 (TLR2, TLR4, TLR9 and RAGE) and mRNA levels of pro-inflammatory cytokines (pro-IL-1β, TNFα, MCP-1) were observed in the cerebellum (Lippai D., et al., 2013). Ethanol exposure in adolescent rats increases TLR4 receptor and HMGB1 protein expression in the prefrontal cortex, which persists at elevated levels in the adult rat brain (Vetreno R.P., et al., 2012). In another study with long-term alcoholization of rats, it was shown that HMGB1 protein levels in the frontal cortex of the rat brain were unchanged in the long-term alcoholization and day 1 alcohol withdrawal groups, but decreased in the day 28 ethanol withdrawal group; in addition, the content of none of the pro- and anti-inflammatory cytokines studied was altered by day 28 of alcohol withdrawal in the frontal cortex of the rat brain (Sanchez-Alavez M., et al., 2019).

4.3 Gene expression analysis of TLR signaling system during rifampicin use

Ethanol exposure promotes an increase in the mRNA and protein levels of some TLR subtypes (TLR3, TLR4, TL7) in the cerebral cortex and hippocampus with a subsequent increase in the levels of a number of pro-inflammatory signaling molecules, while the use of antagonists to TLR signaling response cascades or knockout on these receptor genes leads to a decrease in the levels of pro-inflammatory factors in the brain (Airapetov M.I., et al., 2020, 2024; Lewohl J.M., et al., 2000; Crews F.T., et al., 2011; Coleman L.G. Jr., et al., 2020). Elevated TLR3 mRNA levels have also been reported in postmortem samples of the orbifrontal cortex of alcoholic humans (Crews F.T., et al., 2011). Alcoholization of mice for 10 days leads to an increase in TLR3 mRNA levels in the brains of mice, as well as an increase in TLR3 protein levels in the orbifrontal and entorhinal cortex (Crews F.T., et al., 2011). In cell culture work, ethanol supplementation was shown to induce TLR3 activation and this promoted the release of IFNβ and IFNγ by neurons and astrocytes, while blocking the TRAIL gene, an adaptor protein to TLR3, resulted in decreased levels of IFNβ and IFNγ in both astrocytes and neurons (Lawrimore C.J., et al., 2019). In the same work, the combined effects of ethanol and a TLR3 agonist showed increased mRNA levels of TNF-α, IL-1β, and IL-6, as well as increased levels of p38 and IRF3 proteins in microglia and neuronal cell cultures (Lawrimore C.J., et al., 2019). In addition, there is evidence to suggest that alterations in TLR3 signaling function influences voluntary ethanol

consumption in rodent experiments (Warden A.S., et al., 2019), suggesting that intracellular pathways from TLR3 are linked to neurotransmitter pathways. There is an observation that when TLR3 is activated by a specific agonist, the expression of glutamatergic system genes: mGluR2 (metabotropic glutamate receptor 2), mGluR3, Glt1 (glutamate transporter 1) is increased. The increase in mRNA of each of these genes correlated with an increase in TLR3 mRNA (Randall P.A., et al., 2019). There is only one study that examined TLR3 mRNA content in Nac in rats and showed a small increase in mRNA at day 1 of ethanol withdrawal, whereas mRNA content was not examined at later time points (McCarthy G.M., et al., 2017).

Previously, researchers showed that ethanol consumption in mice for 2 weeks. Led to activation of TLR4-dependent pro-inflammatory signaling cascades in the cerebral cortex, which was expressed by activation of MAP-kinases and NF-κB with subsequent release of COX-2 (English Cyclooxygenase 2), iNOS (English Inducible nitric oxide synthase), HMGB1 (English High-mobility group protein B1). The development of neuroinflammatory process against the background of increased activity of these proinflammatory factors was observed, which served as a cause of axon demyelination and led to structural synaptic changes as a result of myelin and synaptic proteins damage. These events subsequently affected the deterioration of cognitive parameters in tests of object recognition, passive avoidance and olfactory behavior in mice (Montesinos J., et al., 2015). The use of genetic and pharmacological manipulations (TLR4 knockout and antagonist administration) has shown that TLR4 activity does not regulate ethanol consumption (Harris R.A., et al., 2017), but TLR4 gene knockdown inhibits the production of pro-inflammatory mediators by blocking the activation of MAP kinases and NF-κB pathways in astrocytes (Alfonso-Loeches S., et al., 2010). In TLR4 gene knockout mice, it was shown that such mice are protected from ethanol-induced 5-months. increase in the level of cytokine and chemokine concentrations in the brain, whereas in a group of wild-type animals (in the presence of the TLR4 gene), ethanol consumption of TLR4 leads to an increase in the concentration of cytokines (IL-1β, IL-17, TNF-α) and chemokines (CCL2, MIP-1α, CX3CL1) in the blood and in the striatum of the animals' brains (Airapetov M. I., et al., 2020; Pascual M., et al., 2015).

The results of our experiment showed that in addition to the increased TLR4 mRNA level, there was also an increase in the level of cytokine CCL2, as well as a slight increase in cytokine IL1β. In addition, an interesting observation in our experiment for us was that at the same time we found a decreased mRNA content of the anti-inflammatory cytokines IL10 and IL11. Thus, the data obtained may indicate the development of a long-term pro-inflammatory state in Nac induced by long-term ethanol consumption, and it is possible that TLR4-dependent intracellular signaling pathways may play a key role here.

In addition, we detected an increase in Hmgb1 mRNA levels in Nac. The Hmgb1 protein was found to serve as an endogenous TLR4 agonist. The content of the protein is increased in the brain in

various pathologic conditions, including alcoholism. It is suggested that an increase in its content may initiate TLR4-dependent signaling, triggering the neuroinflammatory process. In addition, there are observations that ethanol induces the formation of Hmgb1-miR-let-7 complexes in microvesicles, which cause the development of neurotoxic effects through TLR7 activation (Coleman L.G., et al., 2017). In our experiment, TLR7 mRNA level by day 10 of withdrawal had no significant changes relative to the control group. On this basis, we can assume that increased Hmgb1 mRNA content in Nac may mediate a prolonged increase in pro-inflammatory TLR4-signaling cascades of reactions, but has no significant effect on TLR7 signaling due to the absence of a pronounced increase in Tlr7 gene expression during alcohol withdrawal. It should be noted that the TLR7 mRNA content in Nac was assessed for the first time during the withdrawal of long-term alcoholization, and it is not possible to compare our data with similar works of researchers earlier.

We have previously shown that long-term alcoholization (1 month) also alters the Hmgb1 mRNA content in the striatum and amygdala of the rat brain, which apparently indicates the presence of mechanisms regulating the local expression of the Hmgb1 gene in various brain structures during long-term alcoholization and ethanol withdrawal (Airapetov M.I., et al., 2021).

To understand how adaptor proteins Myd88 and Trif (aka Ticam) contribute to the conductance of intracellular TLR4-dependent signaling, we assessed the relative content of their mRNA levels. Myd88 and Trif are known to signal from TLR4 to a cascade of intracellular kinases, triggering activation of transcription factors responsible for regulation of pro-inflammatory genes (Airapetov M.I., et al., 2020; Crews F.T., et al., 2011; Coleman L.G. Jr., et al., 2020). We found no significant changes in Myd88 and Trif mRNA content. In addition, we found no significant changes in the mRNA content of the transcription factors NfκB, IRF3, and IRF1.

The mRNA content of adaptor proteins was studied previously in rat brain Nac at the time of prolonged alcoholization and upon withdrawal at day 1, researchers also found no changes in the mRNA content of Myd88 and Trif (McCarthy G.M., et al., 2017). Thus, the expression of these genes is not altered in this brain structure; however, this does not rule out the possibility that the rate of signaling to pro-inflammatory genes may be enhanced through the existing constitutive protein content of transcription factors. Perhaps only increased expression of TLR4, which initiates a signaling cascade of reactions upon ethanol ingestion, is sufficient for this. In addition, there is a need to search for possible unknown new TLR signaling pathways. For example, there are suggestions that TLR4 may mediate modulation of neurotransmitter and neuropeptide systems by regulating ethanol intake as well as other higher nervous activity processes (Liu J., et al., 2011; June H.L., et al., 2015; Garcia Bueno B., et al., 2016; Figueroa-Hall L.K., et al., 2020; Aurelian L., et al., 2019).

Rifampicin (Rif) is a broad-spectrum antibiotic from the group of ansamycins, which is used in the treatment of tuberculosis, as well as other infections: staphylococcal, meningococcal, leprosy

(Ebenezer G.J., et al., 2021; Cao Y., et al., 2022). Rif can be either in a bound form - with blood plasma proteins - or in an unbound form, in which case its passage through the barriers of the body, including the blood-brain barrier, is facilitated (Ebenezer G.J., et al., 2021; Cao Y., et al., 2022). The ability of Rif to penetrate through the GEB is of interest for its application for the pharmacologic correction of brain pathological processes. Epidemiologic studies have shown that a lower incidence of neurodegenerative pathologies was observed in leprosy patients treated with Rif (Bi W., et al., 2021). It has been shown experimentally in various models of neuroinflammation that Rif can reduce the levels of pro-inflammatory cytokines, β-amyloid content in a model of Alzheimer's disease, α-synuclein in a model of Parkinson's disease. There are studies showing improvement of autophagy process in mice in the hippocampus when exposed to Rif (30mg/kg). In a model of cerebellar demyelination, it was shown that administration of Rif (40mg/kg) to mice recorded decreased Bax expression and increased Bcl2 expression, as well as decreased caspase 3 and caspase 12 activity (Zahednasab H., et al, 2019), indicating a decrease in apoptotic activity, and prior administration of rifampicin (20 mg/kg) suppressed lithium-pilocarpine induced hippocampal neurodegeneration by reducing cytochrome c levels in the hippocampus (Ali A.E., et al., 2018). These findings are sufficient to conclude that Rif has neuroprotective properties, but the exact mechanism of action remains unknown. In one study performed on microglial cell culture, there is evidence of competition between Rif and lipopolysaccharide for binding to the MD2 protein. MD2 protein is required for TLR4 ligand binding and subsequent signaling. Rif, by binding to this protein, contributed to the inhibition of LPS-CD14-MD2-TLR4-NFκB signaling in the experiment, and as a consequence, there was a decrease in NF-κB activation and proinflammatory factors (NO, IL-1β, TNFα). Thus, Rif, by competitively binding to MD2, may block TLR4-dependent signaling (Wang X., et al., 2013).

In our study, Rif (100 mg/kg) had no effect on TLR3 and TLR7 mRNA content, whereas TLR4 content was reduced to control levels on day 10 of alcohol withdrawal (Figure 1). The level of Hmgb1 mRNA returned to the baseline level under the influence of the drug (Fig. 2). Having evaluated the mRNA content of proinflammatory cytokines, we found a decrease of mRNA of proinflammatory cytokines IL1β and IL6 in rat brain Nac, and an interesting observation for us was the increase of mRNA content of anti-inflammatory cytokines IL-10 and IL-11 under the effect of Rif injections (100 mg/kg). The data obtained by us testify to the possible correction of neuroinflammatory conditions by this drug, but the mechanism of its action may have a more complex character associated not only with the competition of Rif with TLR4 ligands for binding to MD2 protein.

4.4 Gene expression analysis of the TLR signaling system using the sum of ginsenosides

Research findings support the fact that long-term alcohol consumption leads to activation of neuroinflammatory pathways with subsequent neurodegeneration in a number of brain structures responsible for emotional and cognitive control (Ramos A., et al., 2022; Coleman L.G.J., et al., 2021; Airapetov M., et al., 2021; Becker H.C., et al., 2014; Gano A., et al., 2022; Griffin W.C., et al., 2022). These structures include, in particular, the hippocampus (Eng. hippocampus, HIP) and the nucleus accumbens (Eng. nucleus accumbens, Nac), which were selected for our study (Becker H.C., et al., 2014).

NAc is the ventral part of the brain striatum, which is an important part of the mesolimbic pathway involved in the internal reinforcement system that mediates the formation of various forms of addiction, particularly alcohol addiction (Koob G.F., 2014; Sobstyl M., et al., 2021; Airapetov M.I., et al., 2022). Neuroimaging data suggest the development of a pronounced neurodegenerative process in the NAc during long-term alcohol consumption (Edith V.S., et al., 2005), but the key mechanisms mediating this process remain unknown. HIP is a brain region that is primarily responsible for memory formation, but being part of the limbic system, is also associated with the regulation of emotional responses. Chronic alcohol consumption leads to activation of neuroinflammatory pathways and neurodegeneration in the hippocampus, including reduction of white matter, loss of oligodendrocytes and other glial cells, and suppresses the process of neurogenesis (Becker H.C., et al., 2014; Griffin W.C., et al., 2022; McClintick J.N., et al., 2013; Shabani Z., et al., 2020).

The effect of ginseng extract containing various compounds as well as the use of selected ginsenosides on the ability to reduce the development of neuroinflammatory process has been observed (He Y., et al., 2018; González-Burgos E., et al., 2015; Wang X., et al., 2015; Xu X., et al., 2020; Li J., et al., 2021; Cai L., et al., 2021; Sun Y., et al., 2022; Gong L., et al, 2022; Zheng Z., et al., 2022; Xu X., et al., 2022; Pannacci M., et al., 2006; Rhule A., et al., 2008; Beamer C.A., et al., 2012; Kim T.W., et al., 2012; Zhao B.S., et al., 2014). Research results show the effects of ginsenosides on the TLR system in a number of pathological conditions of the nervous system (Pannacci M., et al., 2006; Rhule A., et al., 2008;, Beamer C.A et al., 2012; Kim T.W., et al., 2012; Zhao B.S., et al., 2014), however, in a model of long-term alcoholization, the effects of ginsenosides on the TLR system among different brain structures have not been previously investigated. In 2008, a paper by Rhule A. et al. reported that Panax notoginseng extract inhibits the secretion of pro-inflammatory molecules and activation of the innate immune system induced by TLR4 activation (Rhule A., et al., 2008). Beamer C.A. et al. reported that further studies on Panax notoginseng extract may have therapeutic benefits for the correction of neurodegenerative diseases. Application of the extract in experiment caused suppression of microglia activation as measured by decreased expression of CD40 and CD86 molecules, decreased synthesis of

inflammatory mediators IL-6 and TNFα and nitric oxide (NO) levels (Beamer C.A., et al., 2012). By investigating the effect of individual ginsenosides, it was shown that the ginsenoside Rg5 inhibited the expression of iNOS (English Inducible nitric oxide synthase) and COX (English cyclooxygenases) as well as the level of active forms of NF-κB (English nuclear factor kappa-light-chain-enhancer of activated B cells) in LPS (lipopolysaccharide)-stimulated lung inflammation in mice. The inhibitory effect of Rg5 (10 mg/kg) was comparable to that of dexamethasone (5 mg/kg). The authors suggested that the effect is mediated through inhibition of LPS binding to TLR4 on macrophages (Kim T.W., et al., 2012). The ginsenoside Rg1 has activity against inhibition of signaling pathways from TLR3 and TLR4, reducing the synthesis of neuroinflammatory factors induced by Aβ25-35 (a peptide to model Alzheimer's disease) in NG108-15 cells. Rg1 decreased protein and mRNA content of TLR3, TLR4, NF-κB and TRAF6 (TNF Receptor Associated Factor 6) and lowered protein levels of TNF-α, IFN-β and iNOS in cell supernatant lysates (Zhao B.S., et al., 2014). In another work, Rg1 ginsenoside reduced alcohol-induced inflammation by inhibiting the TLR4/NF-κB pathway in the liver of mice, thereby exerting a hepatoprotective effect (Xia T., et al., 2022). Rg4 ginsenoside reduced inflammation and exhibited a protective effect in experimental sepsis. Administration of Rg4 decreased the levels of cytokines including TNF-α and IL-1β as well as NO levels in mouse kidney tissues. Addition of Rg4 to HMGB1-activated human umbilical vein endothelial cell culture decreased the expression of TLR4 and TNF-α (Kim G.O., et al., 2022). The effects of Rg2 ginsenoside were evaluated in a rat model of brain injury with intracerebral hemorrhage. Rg2 administration decreased the levels of IL-1β and TNF-α in the affected hemisphere, treatment with Rg2 ginsenoside inhibited the increased expression of TLR4, MyD88, p-IκBα and p-NFκB, and reduced the decreased expression of occludin and claudin-5 in the affected hemisphere (Cai L., et al., 2021). Rb1 ginsenoside directly reduced the level of reactive oxygen species, inhibited mitochondrial apoptotic pathway to protect neonatal rat cardiomyocytes from ischemic injury, reduced cytochrome c release and the level of neural tissue damage induced by mitochondrial stress in a cerebral ischemia model (Zhou P., et al., 2019).

There are isolated studies that have examined TLR3 mRNA content in Nac after ethanol exposure. One study noted a small increase in TLR3 mRNA at 1 day of withdrawal of prolonged alcoholization in Nac in rats; at later time points, mRNA content has not previously been examined (McCarthy G.M., et al., 2017). In another study, activation of TLR3 by a poly(I:C) agonist resulted in increased mRNA levels of neuroimmune genes (TLR3, COX2), glutamatergic genes (mGluR2, mGluR3, GLT1), and the neurotrophic factor BDNF in Nac, and increases in mRNA content of each of these genes correlated with increases in TLR3 mRNA. Thus, TLR3 activation induces long-lasting changes in gene expression and protein content that may be associated with addictive seeking and drinking behaviors. Furthermore, in vitro studies show that both ethanol and poly(I:C) increase TLR3 mRNA in neuronal cells (Randall P.A., et al., 2019). In Tlr4 knockout mice, TLR4 has been shown to play a significant role in Nac function

(Kashima D.T., et al., 2017). Accordingly, changes in TLR4 will also be reflected in Nac functioning. However, there is a study in which knockdown of Tlr4 in Nac in mice did not reduce voluntary alcohol consumption in continuous or intermittent alcohol access tests (Harris R.A., et al., 2017). There have been no studies regarding the contribution of TLR7 to Nac in the development or modeling of any CNS pathologies. Only in our earlier work it was shown that the level of TLR7 mRNA content did not change on the 7th day of withdrawal from prolonged alcoholization (Airapetov M.I., et al., 2022), which is consistent with the results we obtained in this study, although on a different model of alcoholization.

The increased activity of the TLR4 system under conditions of prolonged alcoholization in the hippocampus and its role in the activation of neuroinflammatory pathways has been shown repeatedly. A large amount of data has been obtained in rats and mice using genetic and pharmacological manipulations (TLR4 knockout and application of antagonists), which showed that TLR4 activity does not regulate ethanol consumption, but changes in TLR4-mediated signaling subsequently consumed alcohol are observed (Harris R.A., et al., 2017; Blednov Y.A., 2017). A study on mice showed that ethanol consumption by mice for 2 weeks resulted in activation of TLR4-dependent pro-inflammatory processes characterized by activation of MAP-kinases and NF-κB with subsequent release of COX-2 (Cyclooxygenase 2), iNOS (Inducible nitric oxide synthase), HMGB1 (high-mobility group protein B1). The development of inflammatory process on the background of increased activity of these pro-inflammatory signaling molecules led in the experiment to demyelination of axons and to structural synaptic changes as a result of damage to myelin and synaptic proteins, which subsequently affected in mice the deterioration of cognitive parameters in tests of object recognition, passive avoidance and olfactory behavior (Montesinos J., et al., 2015). Knockdown of TLR4 gene was accompanied by inhibition of proinflammatory mediators production, blockade of activation of MAP-kinases and NF-κB pathways in astrocytes (Alfonso-Loeches S., et al., 2010). Thus, it is possible that the observed neurodegenerative changes under conditions of long-term alcohol consumption in the hippocampus may also be realized through increased activity of TLR4 signaling. Data on changes in the expression level of TLR3 and TLR7 in the hippocampus under conditions of long-term alcoholization are also available, although the physiological meaning of these changes is still not fully defined. The effect of ethanol on TLR7 and microRNA let-7b was investigated on rat hippocampal-entorhinal cortex tissue culture (Coleman L.G., et al., 2017): alcoholized hippocampal tissue was characterized by increased TLR7 content (Coleman L.G., et al., 2017). In addition, ethanol was found to induce the formation of HMGB1-miR-let-7 complexes in microvesicles, which induce the development of neurotoxic effects through signaling via TLR7 (Coleman L.G., et al., 2017). Ethanol causes an increase in TLR7 protein content and release of let-7b and HMGB1 from microglia. Inhibition of HMGB1 by glycyrrhizin prevented the development of neurotoxicity (Coleman L.G., et al., 2017).

The increased mRNA levels of all TLRs we studied in the hippocampus once again indicate that changes in the TLR system occur here under conditions of prolonged alcoholization. What was interesting in this study was that the sum of hinzenosides we used (50 mg/kg) decreased TLR4 mRNA levels in Nac and also normalized TLR7 levels in the hippocampus to control values, indicating the ability of the sum of hinzenosides to modify pathological changes in the TLR system, although it is of interest to further evaluate the contribution of individual hinzenosides as well as their effects on other brain structures.

The Hmgb1 protein is an endogenous agonist of TLR4 and TLR7 and increased levels of gene expression usually correlate with increased activity of TLR4- and TLR7-dependent intracellular signaling pathways. There is a notion that the Hmgb1 protein may be involved in increased activation of the innate immune system under conditions of prolonged alcoholization. In the work of Whitman. et al. (Whitman B.A., et al., 2013) an increase in the amount of HMGB1 protein, correlated with increased mRNA levels of pro-inflammatory cytokines in the prefrontal cortex of the brain in long-term alcoholized mice. In another study with long-term alcoholized rats, however, it was shown that HMGB1 protein levels in the frontal cortex of the rat brain were unchanged in the long-term alcoholization and day 1 alcohol withdrawal groups, but decreased in the day 28 ethanol withdrawal group; furthermore, the content of none of the pro- and anti-inflammatory cytokines studied was altered on day 28 of alcohol withdrawal in the frontal cortex of the rat brain (Sanchez-Alavez M., et al., 2019). A slight increase in Hmgb1 mRNA content was observed in the cerebellum of long-term alcoholized mice. In addition, increased mRNA levels of TLR2, TLR4, TLR9, and RAGE and mRNA levels of pro-inflammatory cytokines pro-IL-1β, TNF-α, and CCL2 were observed in the cerebellum (Lippai D., et al., 2013). The results of our earlier study also demonstrated that the striatum, amygdala, and hippocampus of the brain of long-term alcoholized rats undergo multidirectional changes in Hmgb1 and Il-1β gene expression at different time points during alcohol withdrawal (Airapetov M. I., et al., 2021). In the current work, an important result was that our injections of the sum of ginsenosides (50 mg/kg) restored the pathologically increased level of Hmgb1 mRNA to the level of control values both in the hippocampus and in the Nac of the brain of long-term alcoholized rats.

Very little is known about the effects of gynznosides on IRF3 and IRF1. However, we note that a study using the ginsenoside Rb3 also showed a decrease in the amount of phosphorylated forms of IRF3 in tissues of injured skin, and this change was most evident in microvascular endothelial cells (Li Y., et al., 2022). Meanwhile, another Rg3 ginsenoside already induced IRF3 phosphorylation and its translocation to the nucleus in a different experiment (Choi Y.J., et al., 2014). Ginsenoside Rg1 promoted angiogenesis during diabetic wound healing via signaling from miR-23a and reducing IRF1 levels (Cai H.A., et al., 2019). Thus, the effect of ginsenosides on neuroinflammation pathways through affecting transcription factors is also possible. The results of our experiment also showed that the elevated IRF3

mRNA level in Nac was restored to control values in the ginsenoside-injected group. In the hippocampus, the observed elevated level of IRF1 at day 7 of alcohol withdrawal was maintained in the group with injection of the sum of ginsenosides.

Much has already been said about the effect of ginsenosides on the level of proinflammatory cytokines. The absence of significant changes in our experiment in the IL1β mRNA content in the hippocampus can probably be explained by the fact that by day 7 of withdrawal of long-term alcoholization the level of IL1β gene expression could normalize in this brain structure, probably due to the activation of compensatory anti-inflammatory and regenerative pathways of intracellular signaling. At the same time, mRNA of the proinflammatory cytokine CCL2 remained at an elevated level in both brain structures we studied by day 7 of alcohol withdrawal. We found similar results in our earlier works (Airapetov M.I., et al., 2022; Airapetov M.I., et al., 2021). Injections of ginsenosides corrected this condition only in the adjacent brain nucleus, which once again confirms the multidirectional nature of the action of the sum of ginsenosides with respect to different brain structures.

4.5 MicroRNA content under conditions of long-term ethanol exposure in the rat brain

It is known from the literature that the miRs we studied may have a functional relationship with toll-like receptor (TLR7 and TLR4) signaling pathways, which are involved in the initiation of neuroinflammatory events that develop during chronic alcoholization (Lim Y., et al., 2021; Lehmann S.M., et al., 2012; Lippai D., et al., 2013; Coleman L.G., et al., 2017; Ureña-Peralta J.R., et al., 2018, 2020). It is known that miR-let7b is an endogenous TLR7 agonist (Lehmann S.M., et al., 2012; Airapetov M.I., et al., 2020). Altered expression of miR-let7b may have a functional relationship with the TLR7-signaling response cascade (Lehmann S.M., et al., 2012; Airapetov M.I., et al., 2020; Coleman L.G., et al., 2017). TLR4 activation has been described to increase miR-155 in brain microglia of mice, whereas this is not observed in Tlr4 gene knockout (TLR4-KO) mice (Lippai D., et al., 2013). TLR4-KO mice do not have altered cortical miR-96 content, whereas long-term alcoholization in wild-type mice decreased in miR-96 levels (Ureña-Peralta J.R., 2018, 2020).

In our experiment, long-term alcoholization of rats for 1 month resulted in a 1.71-fold decrease in miR-let7b levels in brain Nac, miR-96 levels decreased 3.87-fold, and miR-182 levels decreased 2.29-fold. In contrast, miR-155 content increased 1.41-fold.

The mechanism of interaction of the microRNA cluster miR-183C (which includes miR-96 and miR-182) with TLR4 is not fully understood, but there are works that report possible such interactions using miR-182-5p as an example (Ureña-Peralta J.R., 2018, 2020). In our experiment, we observe reduced expression levels of miR-96 and miR-182 when TLR4 mRNA is upregulated in Nac.

In addition, there are observations that ethanol induces the formation of HMGB1-miR-let-7 complexes in microvesicles, which cause the development of neurotoxic effects through the activation of TLR7 in neurons (Coleman L.G., et al., 2017). The results of our experiment revealed increased levels of Hmgb1 mRNA in Nac, whereas miR-let7b and TLR7 mRNA were downregulated. Such data seem to indicate that there is no functionally significant relationship between Hmgb1 and the miR-let7b-TLR7 system in Nac, as divergent responses were obtained with respect to the expression levels of Hmgb1 and miR-let7b in this brain structure.

Conclusion

The results of the performed dissertation work provided information about the state of gene expression of toll-like receptor system in different brain structures in long-term alcoholized rats, as well as at different withdrawal periods of long-term ethanol exposure. The results of the study showed that the level of Tlr3 gene expression was not affected by changes in most of the brain structures studied (striatum, nucleus accumbens, amygdala, entorhinal cortex), except for a slight decrease in the hippocampus, Tlr4 and Tlr7 gene expression was not altered in any of the brain structures studied. However, new data were obtained on the presence of pronounced multidirectional changes in the expression of Tlr3, Tlr4 and Tlr7 genes under conditions of abolition of long-term ethanol exposure at different time periods in dynamics. It should be noted that the nature of these changes depended not only on the term of ethanol withdrawal, but also on the brain region, as well as on the modeling of ethanol exposure, i.e., on the duration and, apparently, on the method of administered ethanol. Thus, different ways of modeling prolonged alcoholization are very likely to have different effects on the severity of the neuroinflammatory response. On this basis, it is necessary to note the fact that when analyzing the literature devoted to this issue, it is extremely important to pay attention to the peculiarities of modeling long-term ethanol exposure (duration, concentration, volume, method of ethanol administration).

The results of the work revealed that not only the level of Tlr gene expression is subject to pronounced changes when long-term ethanol exposure is abolished, but the whole system of toll-like receptor genes (genes of adaptor proteins, transcription factors, pro- and anti-inflammatory cytokines) is changed to a greater or lesser extent in the analyzed brain structures in experimental animals. The obtained data are new both for the domestic and world literature, as the analysis of expression of a series of genes from the toll-like receptor system in the studied brain structures has not been previously performed during the abolition of long-term ethanol exposure.

The correction of altered molecular mechanisms by long-term ethanol exposure in such brain structures as hippocampus and nucleus accumbens using potential anti-neuroinflammatory compounds rifampicin and ginsenoids allowed us to obtain original results. The use of these compounds in feces allowed to correct a number of molecular mechanisms in the hippocampus and adjacent nucleus of the rat brain, which were altered by prolonged exposure to ethanol followed by its withdrawal. The selected compounds in this work have proven themselves, according to the literature, as potential neuroprotectors, which are able to eliminate signs of neuroinflammatory response in various pathological conditions of the central nervous system, as well as modulate the level of expression of the studied genes and their translation products. The brain structures selected for gene expression analysis are the most susceptible to various changes during prolonged ethanol exposure. There are assumptions that changes in these structures may be key in the formation of alcohol dependence. Thus, the obtained data on the restoration of a number of altered molecular mechanisms using selected pharmacological compounds are very interesting and open the prospect of a new direction in this field of research for further work.

List of abbreviations

TLR - toll-like receptor

PRR - pattern-recognizing receptors

mEC - medial entorhinal cortex

mPFC- (medial prefrontal cortex

STR - Striatum

HIP - Hippocampus

AMY - amygdala

MCP1/CCL2 - Monocyte Chemoattractant Protein 1/C-C motif ligand 2

NF-κB - nuclear factor kappa-light-chain-enhancer of activated B cells

MAPK - mitogen-activated protein kinase

AP-1 - activator protein 1

IRF3 - Interferon regulatory factor 3

IL - Interleukin

IFN - Interferon

TNFα - Tumor necrosis factor alpha

TRAF6 - TNF receptor-associated factor 6

NOX2 - NADPH oxidase 2

COX2 - Cyclooxygenase 2

S100ß - S100 calcium-binding protein B

iNOS - NOS2, Nitric oxide synthase

TACE/ADAM17 - Tumor necrosis factor-alpha-converting enzyme

MyD88 - Myeloid differentiation primary response 88

TRIF - TIR-domain-containing adapter-inducing interferon-β

HMGB1 - High-mobility group protein B1

NLRP/NALP - Nucleotide-binding oligomerization domain, Leucine rich Repeat and Pyrin domain containing

JNK - c-Jun N-terminal kinases

TAK1 - Transforming growth factor beta-activated kinase 1

TAB - TAK1-Binding Protein

LPS - Lipopolysaccharide

miR – MicroRNA

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