Study of gene expression of the Toll-like receptor system in the forebrain cortex of rat pups with prenatal alcohol exposure and pharmacologic correction with rifampicin

Marat Airapetov[1,2], Sergei Eresko[1], Polina Ignatova[1], Daria Ganshina[1], Daria Sukhanova[1], Alexandra Mikhailova[1], Andrei Lebedev[1], Evgenii Bychkov[1], and Petr Shabanov[1]

[1]Institute of Experimental Medicine, ul. Akademika Pavlova, 12, Saint Petersburg, 197376, Russian Federation
[2]S. M. Kirov Military Medical Academy, ul. Akademika Lebedeva, 6, Saint Petersburg, 194044, Russian Federation

Address correspondence and requests for materials to Marat Airapetov, interleukin1b@gmail.com

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Authors' information: Marat Airapetov, PhD, Associate Professor, Leading Researcher, orcid.org/0000-0002-8318-9069; Sergei Eresko, Research Fellow, orcid.org/0000-0002-0269-6078; Polina Ignatova, Student, orcid.org/0000-0003-0930-8151; Daria Ganshina, Student, orcid.org/0009-0002-7328-1985; Daria Sukhanova, Student, orcid.org/0009-0007-0303-9719; Alexandra Mikhailova, Student, orcid.org/0009-0001-4472-5826; Andrei Lebedev, Dr. of Sci. in Biology, Professor, Head of Laboratory, orcid.org/0000-0003-0297-0425; Evgenii Bychkov, Dr. of Sci. in Medicine, Head of Laboratory, orcid.org/0000-0002-8911-6805; Petr Shabanov, Dr of Sci. in Medicine, Professor, Head of Department, orcid.org/0000-0003-1464-1127

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Abstract

Ethanol causes changes in the toll-like receptor (TLR) system in the brain promoting activation of neuroinflammatory pathways. Alcohol consumption during pregnancy induces neuroinflammatory processes in the fetus, which can lead to the development of symptoms of fetal alcohol spectrum disorder (FASD). Modeling prenatal alcohol exposure in our experiment resulted in changes in the expression of TLR system genes *(Tlr3, Tlr4, Hmgb1, Trif*, cytokine genes) in the forebrain cortex of baby rats. The administration of rifampicin (from the first to the seventh day of neonatal development) normalized the altered expression level of the studied genes. This suggests that rifampicin may prevent the development of persistent neuroinflammatory phenomena in the forebrain cortex of baby rats.

Keywords: brain, PAE, fetal alcohol spectrum disorder, toll-like receptors, cytokine, rifampicin.

Introduction

Prenatal damage of the central nervous system (CNS) results in the development of neurological and cognitive disorders in children (Couch et al., 2021; Woods et al., 2021; Nolvi, Merz, Kataja, and Parsons, 2023; Usui, Kobayashi, and Shimada, 2023). Prenatal alcohol exposure (PAE) causes fetal alcohol spectrum disorders (FASD) (Mattson, Bernes, and Doyle, 2019), in which CNS disorders appear to be the most crucial ones because the fetal CNS is one of the most sensitive systems to maternal alcohol (Ajrapetyancz, 1989; Shabanov and Kalishevich, 1998; Riley, Infante, and Warren, 2011; Mattson, Bernes, and Doyle, 2019). The results of recent studies indicate that inflammatory mediators, including cytokines, are involved in the mechanisms of regulation of higher brain functions (Siegel and Zalcman, 2008). However, there are still not so many publications in the world literature devoted to the study of the state of neuroimmune interactions in the developing brain during PAE, and there is basically no information on the effect of PAE on the innate immune system in the CNS. The aim of our work was to investigate the effect of PAE on the level of gene expression of the innate immunity system in rats in the forebrain cortex in the neonatal period of development and pharmacological correction of neuroimmune mechanisms in the brain of rat pups with PAE by

means of Rifampicin (RIF). Previously, RIF showed anti-inflammatory and neuroprotective properties in various models of pathological conditions of the nervous system (Wang et al., 2013; Bi et al., 2014; 2021; Zahednasab et al., 2019).

Materials and methods

Animals

Adult male (250–300 g, n = 6) and female (200–250 g, n = 6) Wistar rats ("Rappolovo", Russia) were used in the experiment. The animals were kept in separate plastic cages with unlimited access to water and food. Each male was paired with two females. The first day of pregnancy was determined by detection of spermatozoa in females in a vaginal smear. After detection of spermatozoa, female rats were marked, their body weight was determined and they were placed in separate cages, and the countdown of the gestation period was started.

Modeling of prenatal alcohol exposure (PAE)

Pregnant rats (from day 1 of pregnancy) were divided into two groups: a group of females exposed to semi-forced alcoholization with 15 % ethanol solution as the only source of liquid (n = 6) and a control group receiving water (n = 3). There were 8–10 pups in each litter; to continue the experiment, 2 pups per litter were left (12 pups with PAE, 6 pups without PAE). Body weight measurements did not reveal any significant differences between the groups of animals studied (pregnant rats and offspring).

Administration of rifampicin (RIF)

Two animals were randomly selected from each litter. From the first to the seventh day of neonatal development, rat pups with PAE (PAE + RIF group, n = 6) received intraperitoneal (ip) injection of 50 mg/kg Rifampicin ("Belmedpreparaty", Belarus). RIF was dissolved in saline. Rat pups of the second (PAE + saline; n = 6) and the third (control; n=6) groups received ip injections of an equivalent volume of saline during the first 7 days of neonatal development. On day 8 of postnatal development, the forebrain cortex was sampled and immediately frozen and stored at –78 °C.

Real-time polymerase chain reaction

Total RNA was isolated using the ExtractRNA reagent ("Evrogen", Russia) in full accordance with the manufacturer's instructions. Samples were treated with DNase ("Promega", USA). Concentrations of the resultant obtained RNA were measured using an Implen NanoPhotometer P330 spectrophotometer ("Implen", Germany). The purity of the isolated product was evaluated by the ratio A260/A280 (normal ≥ 1.8). cDNA synthesis was performed by reverse transcription (RT) using the MMLV RT kit ("Evrogen", Russia) in full accordance with the manufacturer's instructions in the volume of 20 μl. Polymerase chain reaction (PCR) with real-time detection (RT-PCR) was carried out in an M × 3005P amplifier ("Stratagene", USA) in 10 μl of the reaction mixture containing SYBR Green MIX ("Biolabmix", Russia), a mixture of specific forward and reverse primers ("BioBeagle", Russia), selected using the Primer-BLAST software (Table 1). The data obtained were normalized to the expression level of the *Gapdh* gene and calculated in relative units versus the mRNA content of the studied reference gene (Table 1) using the $2^{\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Data analysis

Statistical processing of the results was performed using the program Graph Pad Prizm v. 6. Groups were compared using the Mann — Whitney U-test for independent small datasets. The normality of distribution was checked using the D'Agostino — Pearson test. Differences were considered as statistically significant at $p \le 0.05$.

Results

The effect of PAE on the expression level of TLR genes

On day 8 after birth the levels of TLR3 and TLR4 mRNAs demonstrated the 1.46- and 1.75-fold increase in the forebrain cortex of animals of the group PAE+saline. The level of TLR7 mRNA did not change significantly (Fig. 1). Analysis of the mRNA content of adapter proteins (Myd88, Trif) (Fig. 2) and transcription factors (NF-κB, IRF1, IRF3, IRF7) (Fig. 3), involved in TLR-mediated signaling, revealed a 1.73-fold increase in the Trif mRNA content (Fig. 2). Analysis of the mRNA content of pro-inflammatory (IL-1β, TNFα, IL-6, IFNγ, CCL2) (Fig. 4) and anti-inflammatory cytokines (TGFβ, IL-13, IL-10, IL-11, IL-4) (Fig. 5) showed that on day 8 the level of the following mRNAs increased in the forebrain cortex of animals: IL-1β (by 1.38 times), TNFα (by 1.54 times), IFNγ (1.94 times), CCL2 (1.29 times), IL-13 (1.88 times), IL-10 (3.64 times), IL-11 (2.24 times), IL-4 (1.71 times). An increased level of Hmgb1 mRNA was also noted (by 1.54 times) (Fig. 6). This protein is known to mediate activation of the TLR4 signaling, which leads to the expression and secretion of pro-inflammatory cytokines.

Table 1. Sequence of primers

Gene	Primers			
	Forward (5′-3′)	Reverse (5′-3′)	Tm	Ampicon length
Gapdh	GCCAGCCTCGTCTCATA	GTGGGTAGAGTCATACTGGA	55	193
Tlr3	AACTGGAGAACCTCCAAGA	CACCCTGGAGAAAACTCTTT	55	167
Tlr4	ACTCTGATCATGGCATTGTT	GTCTCAATTTCACACCTGGA	58	223
Tlr7	TGAAAATGGTATTTCCAATGTG	TAAGGGTAAGGTTGGTGGTA	55	183
Myd88	TCATTGAGAAAAGGTGTCGT	AGTGCAGATAGTGATGAAC	58	140
Trif	GCTCAGCTAGATGATGTGAT	TGACAGTGCAGACCTGG	58	115
NfκB1	ATACTGCTTTGACTCACTCC	AGGTATGGGCCATCTGTT	58	142
Irf1	CGGAAGTTACCTTCTAGCTC	CGGAAGTTACCTTCTAGCTC	58	165
Irf3	AATTCCTCCCCTGGCTC	CATGGGATCCTGAACTTTGT	58	205
Irf7	TTGGTTACACTATCTGTGGC	CTACTGACCTCACCCAAGA	58	175
Hmgb1	CTCTGATGCAGCTTATACGA	AAAAGACTAGCTTCCCCTTG	60	212
Il1β	TGTCTGACCCATGTGAGCTG	TTTGGGATCCACACTCTCCAG	58	114
Ccl2	AAGATGATCCCAATGAGTCG	TGGTGACAAATACTACAGCTT	60	158
Tnfα	CACGTCGTAGCAAACCAC	TATGAAATGGCAAATCGGCT	56	223
Il6	ACTTCACAAGTCGGAGGCTT	AATTGCCATTGCACAACTCTTTTC	58	141
Ifnγ	AGCCTAGAAAGTCTGAAGAAC	ATTTTCGTGTTACCGTCCTTT	58	197
Il10	CTGCAGGACTTTAAGGGTTA	CCTTTGTCTTGGAGCTTATT	58	149
Tgfβ	GGACTACTACGCCAAAGAAG	GGTTTTGTCATAGATTGCGTT	60	144
Il13	TGTAACCAAAAGGCCTCGGA	TGGCCATAGCGGAAAAGTTG	59	185
Il4	CGGTGAACTGAGGAAACT	TCAGTGTTGTGAGCGTGG	59	176
Il11	GGGACATGAACTGTGTTTGT	GGTAGGTAGGGAGTCCAGAT	57	162

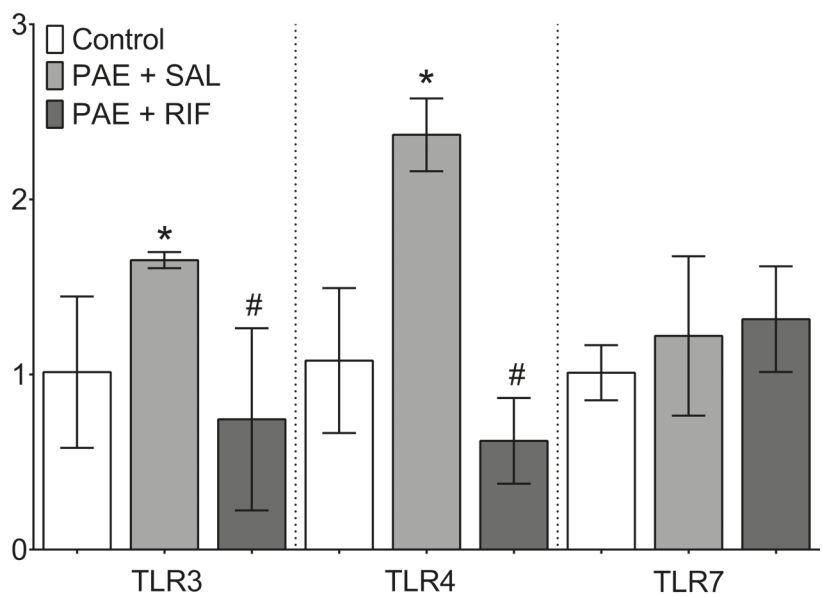

Fig. 1. The content of TLR mRNA in the forebrain cortex of rats on day 8 of their postnatal development (arbitrary units, mean values ± SD; * — $p \le 0.05$ versus control, # — $p \le 0.05$ versus the PAE group).

The influence of RIF administration on the expression level of genes of the TLR-signaling pathways of cytokines in the forebrain cortex of animals subjected to PAE

Administration of RIF (50 mg/kg ip) to animals with PAE day 1 to day 7 of neonatal development caused a decrease in the mRNA level of TLR3 (by 2.23 times) and TLR4 (by 3.02 times) to the control level (Fig. 1). No statistically significant differences were found between the group PAE + RIF and control. In the group PAE + RIF, no statistically significant changes in the mRNA of the studied adapter proteins (Fig. 2) and transcription factors (Fig. 3) were detected versus control. However, administration of RIF normalized the mRNA level of the proinflammatory cytokines IL-1β ($p \le 0.05$) and TNFα ($p \le 0.05$) (Fig. 4), as well as the content of Hmgb1 mRNA ($p \le 0.05$) to the control level (Fig. 6).

Discussion

Recently, of particular interest are studies aimed at investigating the mechanisms of neuroimmune interactions in the developing brain in normal and various pathological conditions (Schepanski, Buss, Hanganu-Opatz, and Arck, 2018; Bergdolt and Dunaevsky, 2019; Couch et al., 2021; Woods et al., 2021; Cattane et al., 2022; Nolvi, Merz, Kataja, and Parsons, 2022; Usui, Kobayashi, and Shimada, 2023). PAE can cause FASN in the fetus, namely morphological and functional disorders of the child's systems, with a special place among them occupied by a spectrum of disorders of the CNS, which is most sensitive to maternal alcohol intake [5–10]. Alcohol consumption by a pregnant mother causes the development of neuroinflammatory process and myelin damage in the brain of the offspring. There are suggestions that these effects may underline persistent cognitive and behaviour disorders observed in PAE (Cantacorps et al., 2017).

Alcohol intake causes various changes in the TLR signaling system in the brain structures (Coleman, Zou, and Crews, 2017; Lawrimore, Coleman, and Crews, 2019; Airapetov et al., 2020; 2021a; 2021b; 2022a; 2022b; Gano, Lebonville, and Becker, 2022). Using TLR agonists and antagonists, as well as genetic manipulations, several groups of researchers confirmed the fact that changes in the activity of the TLR signaling system mediated the development of neuroinflammation in the nervous tissue (Alfonso-Loeches et al., 2010; Ferguson, McKay, Harris, and Homanics, 2013; Chen et al., 2016; Coleman, Zou, and Crews, 2017; Shukla, Meena, Rao, and Rao, 2018; Rizzo et al., 2019; Wang et al., 2019; Qin et al., 2021; Gano, Lebonville, and Becker, 2022). Good evidence exists that all TLR subtypes are expressed in the brain, starting from the earliest stages of ontogenesis (Kaul et al., 2012; Airapetov et al., 2022a; 2022b). Changes in the TLR-signaling system of the brain were detected in offspring with some prenatal pathologies (MacDowell et al., 2017; O'Loughlin, Pakan, Yilmazer-Hanke, and McDermott, 2017; Ali, Mahdy, Elsherbiny, and Azab, 2018; Chin et al., 2019). We have hypothesized that PAE may cause changes in the TLR signaling system in the developing brain.

In an experiment on adolescent and adult rats it was shown that ethanol exposure in adolescence leads to a long-term increase in the level of protein and mRNA expression of TLR4 and TLR3 in the prefrontal cortex

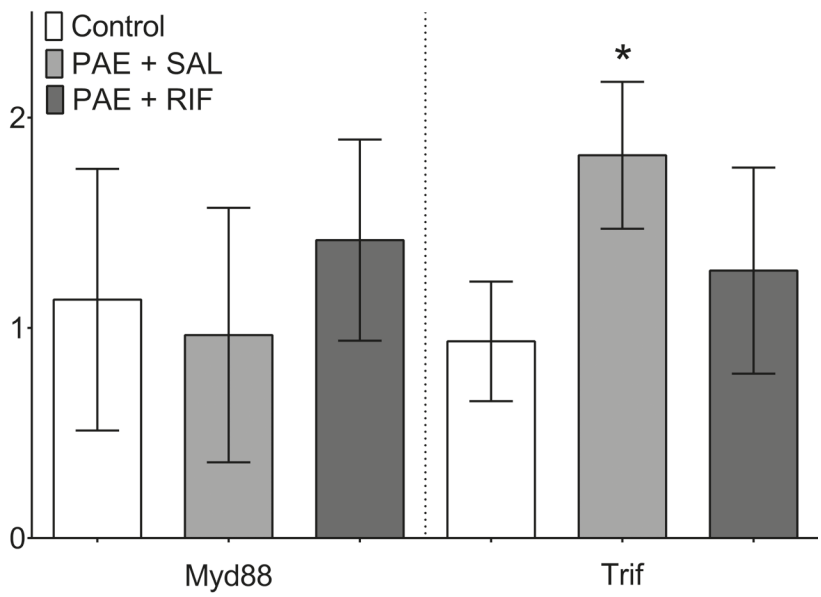

Fig. 2. The content of mRNA of adapter proteins in the forebrain cortex of rats on day 8 of their postnatal development (arbitrary units, mean values ± SD; * — $p \le 0.05$ versus control).

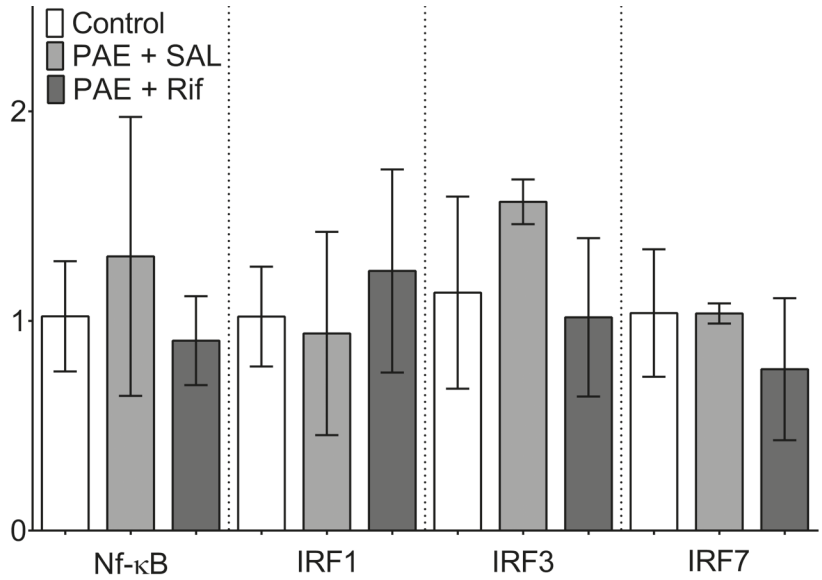

Fig. 3. The content of mRNA of transcription factors in the forebrain cortex of rats on day 8 of their postnatal development (arbitrary units, mean values ± SD; * — $p \le 0.05$ versus control, # — $p \le 0.05$ versus the PAE group).

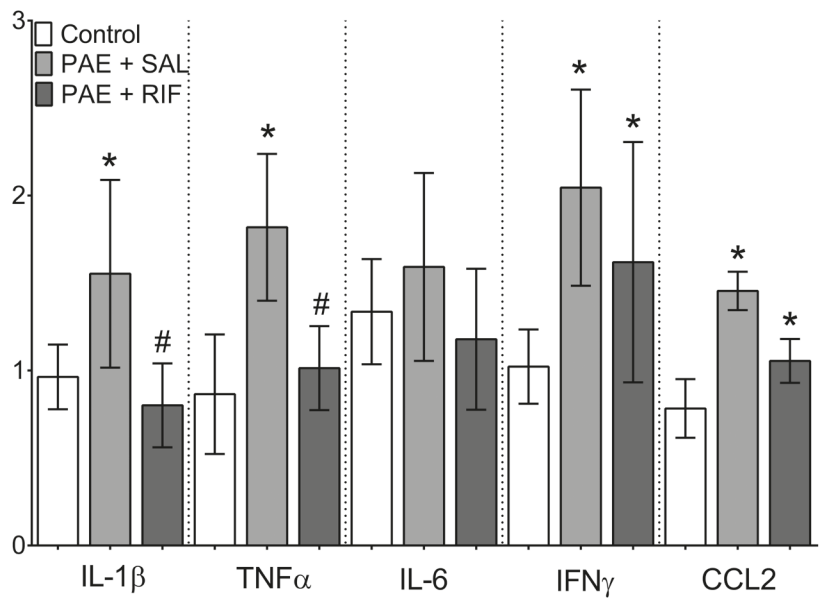

Fig. 4. The content of mRNA of pro-inflammatory cytokines in the forebrain cortex of rats on day 8 of their postnatal development (arbitrary units, mean values ± SD; * — $p \le 0.05$ versus control, # — $p \le 0.05$ versus the PAE group).

of the rats and persists in the adult brain. The increased level of TLR4 and TLR3 expression in the prefrontal cortex of animals correlated with the development of cognitive dysfunction in the study of learning and memory processes using the Barnes test (Vetreno and Crews, 2012).

The PAE modeling performed in this study revealed increased levels of forebrain cortex TLR3 and TLR4 mRNAs on day 8 of postnatal development of rats subjected to PAE. We did not detect any changes in the content of TLR7 mRNA in the forebrain of the animals subjected to PAE. It is possible that the TLR7 system is more resistant to PAE in the cortex, or changes in the expression of the Tlr7 gene are less long-lasting.

Administration of RIF in our experiment reduced the level of TLR3 and TLR4 mRNAs in the group of animals exposed to PAE to the control level. We have chosen this compound based on the known information about RIF effectiveness in different models of neuroinflammation, including the ability of RIF to reduce the level of pro-inflammatory cytokines, the content of β-amyloid in the model of Alzheimer's disease, and the

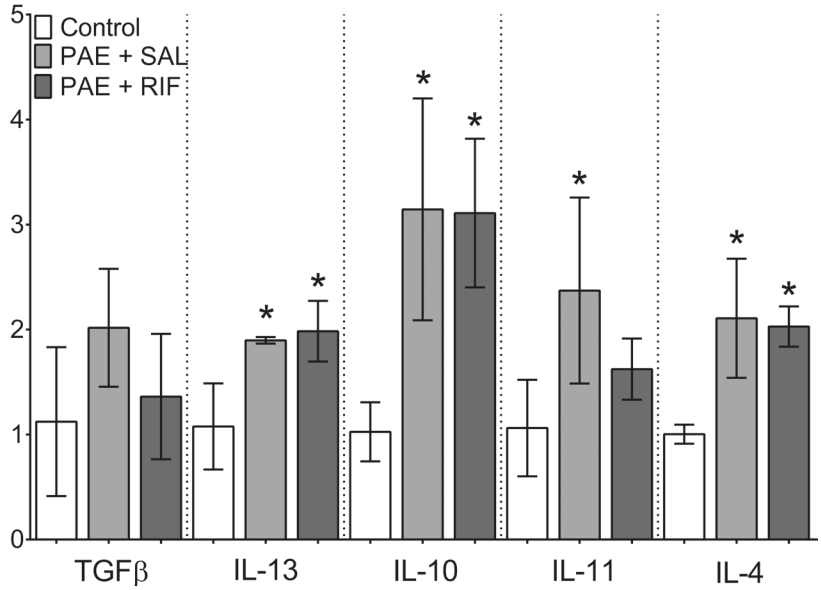

Fig. 5. The content of mRNA of anti-inflammatory cytokines in the forebrain cortex of rats on day 8 of their postnatal development (arbitrary units, mean values ± SD; * — $p \le 0.05$ versus control).

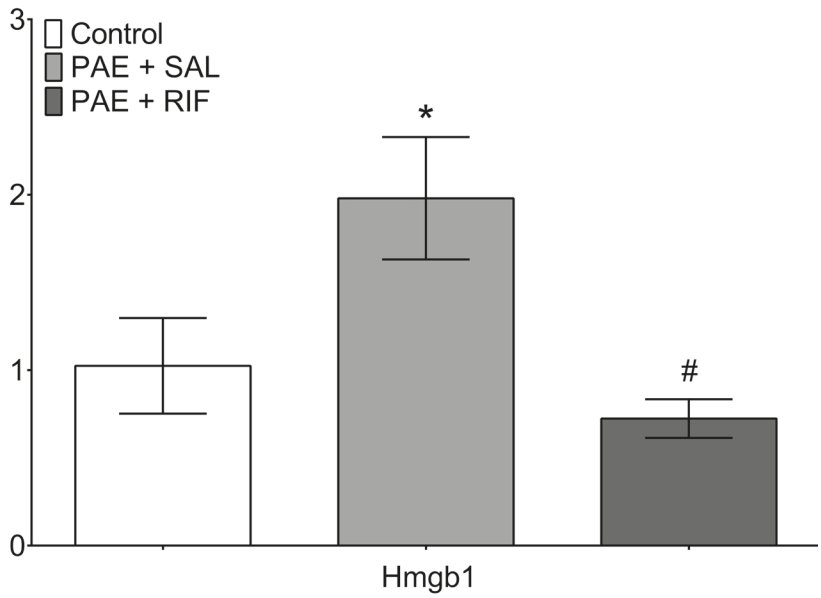

Fig. 6. The content of HMGB1 mRNA in the forebrain cortex of rats on day 8 of their postnatal development (arbitrary units, mean values ± SD; * — $p \le 0.05$ versus control, # — $p \le 0.05$ versus the PAE group).

level of α-synuclein in the model of Parkinson's disease (Qosa, Abuznait, Hill, and Kaddoumi, 2012; Ali, Mahdy, Elsherbiny, and Azab, 2018; Acuña, Hamadat, and Corbalán, 2019; Acuña, Corbalán, and Raisman-Vozari, 2020).

Our study has shown that in the group of animals with PAE, the level of Hmgb1 mRNA increased in the forebrain cortex on day 8 of postnatal development. In the extracellular space, the Hmgb1 protein is able to interact with TLR2, TLR4, TLR5, and TLR7, thus causing activation of intracellular signaling pathways mediated effects of these receptors (Blednov et al., 2012; Airapetov et al., 2021a; 2021b). Administration of RIF decreased the level of HMGB1 mRNA to control values.

Determination of the mRNA level of TLR-signaling components showed an increased content of Trif and IRF3 mRNAs in the forebrain cortex on day 8 of postnatal development of animals subjected to PAE. At the same time, in the group of animals PAE+RIF, the level of IRF3 mRNA corresponded to the control values.

In the group of animals PAE+saline the mRNA levels of IL-1β, TNFα, IFNγ, and CCL2 increased. This suggests long-term changes in gene expression of pro-inflammatory cytokines in the developing brain of animals subjected to PAE. However, the molecular causes that initiate these events, as well as the consequences of such changes (including changes in the level of proteins encoded by these genes) are still to be investigated. Further studies are clearly needed to investigate whether changes in the mRNA level of anti-inflammatory cytokine are accompanied by changes in the corresponding protein products. Thus, on day 8 of postnatal development of animals subjected to PAE the increase in the activity of neuroinflammation pathways probably initiates activation of genes involved in the anti-inflammatory/neuroprotective pathways. It is likely that both the increased expression level of proinflammatory genes and the increased expression level of genes related to the functioning of the anti-inflammatory/neuroprotective mechanisms may have adverse effects on the normal course of neurogenesis in the offspring, which may cause formation of cognitive and other disorders associated with the FASD formation in the future.

For example, it is known that cytokine CCL2 plays an important role in the mechanisms of neurogenesis, participating in the pathways of neuronal differentiation (Turbic, Leong, and Turnley, 2011), in the migration and proliferation of microglia cells (Hinojosa, Garcia-Bueno, Leza, and Madrigal, 2011). There is evidence that alcohol exposure increases CCL2 content in both mature and developing CNS. In an experiment on mice, the effect of alcohol on spinal cord development was studied among wild–type mice and with knockout by CCL2 (CCL2–/–) and CCR2 (CCR2–/–) (Ren et al., 2017). It turned out that both CCR2 deletion and CCL2 deletion were effective in protecting against alcohol-induced spinal cord injury (Cantacorps et al., 2017). One study reported an increase in TRIF, TNFα and IL1β proteins in rat hippocampus after PAE (Rizzo et al., 2019). The mRNA expression of cytokine TNFα and several pro-inflammatory chemokines, including CCL2, was also upregulated in the brains of human embryos with PAE (12.2–21.4 weeks of gestation) (Darbinian et al., 2021). There are results showing an increase in the level of a number of cytokines, including IL10, in children with delayed nervous development after PAE (Bodnar et al., 2018). Thus, there is evidence that cytokine IL10 is expressed in fetal brain tissue already in the first trimester (Mousa, Seiger, Kjaeldgaard, and Bakhiet, 1999) and is elevated in normal, uncomplicated pregnancy (Holmes et al., 2003), which suggests an important participation of this cytokine in brain development. The antigen-induced growth rate of proinflammatory cytokines TNFa, IL1ß, IL6 in fetal brain decreased in genetically modified macrophages overexpressing IL10 (Meyer et al., 2008).

In addition, prenatal exposure to poly(I:C) antigen, an exogenous TLR3 agonist, resulted in behavioral impairment in offspring, but these effects were prevented in offspring that overexpressed IL10 (Meyer et al., 2008; Sowell et al., 2018). In the brain, microglia express low levels of IL4 and IL13 basally, but induction of cytokines can occur in a number of conditions (Shin et al., 2004; Zhou, Spittau, and Krieglstein, 2012). There is evidence that the receptor for IL13 is located on dopaminergic neurons, and hyperactivation of this receptor has been associated with the loss of dopaminergic cells (Mori et al., 2017), prompting studies on the effects of alcohol exposure on the IL13 system because of the importance of dopamine in the development of pathologic changes in the brain following alcohol consumption. In addition, IL4 and IL13 also play a role in learning and memory (Gadani, Cronk, Norris, and Kipnis, 2012; Brombacher et al., 2017). The intracellular molecular pathways of these cytokines lead to the activation of the transcription factor STAT6, which is associated with the expression of the cytokines IL10 and TGFβ, which themselves play key roles in neuroprotection, learning and memory (Vivien and Ali, 2006; Donzis and Tronson, 2014; Caraci et al., 2015; Lobo-Silva et al., 2016). We found no studies in the literature that evaluated the expression status of IL11 in prenatal pathologies. There are a number of studies that report the presence of neuroprotective properties in IL11 (Airapetov et al., 2022a; Airapetov et al., 2022b). It is possible that our observed increase in IL11 expression together with the increase in a number of other neuroprotective genes such as IL10 and TGFβ indicate increased activity of anti-inflammatory/neuroprotective pathways in the group of rats with PAE.

Thus, PAE-induced increased activity of neuroinflammatory pathways likely initiates further activation of genes of anti-inflammatory/neuroprotective pathways, which we apparently observe at day 8 of postnatal development, but there is interest in understanding how increased expression of pro- and anti-inflammatory cytokines may contribute to the course of neurogenesis. It is likely that both increased expression of pro-inflammatory and subsequent increased expression of anti-inflammatory/neuroprotective mechanisms may have adverse effects on the normal course of neurogenesis in the offspring, which may further contribute to the development of cognitive and other impairments associated with the formation of FASN disorders.

Administration of RIF led to a significant decrease in the mRNA level of the key pro-inflammatory cytokines TNFα and IL-1β but had insignificant effects on the mRNA levels of other cytokines analyzed in this study. This may be due to the fact that the dosage of RIF was insufficient to eliminate all the changes in the expression of pro-inflammatory cytokine genes. In addition, it can be assumed that other molecular intracellular

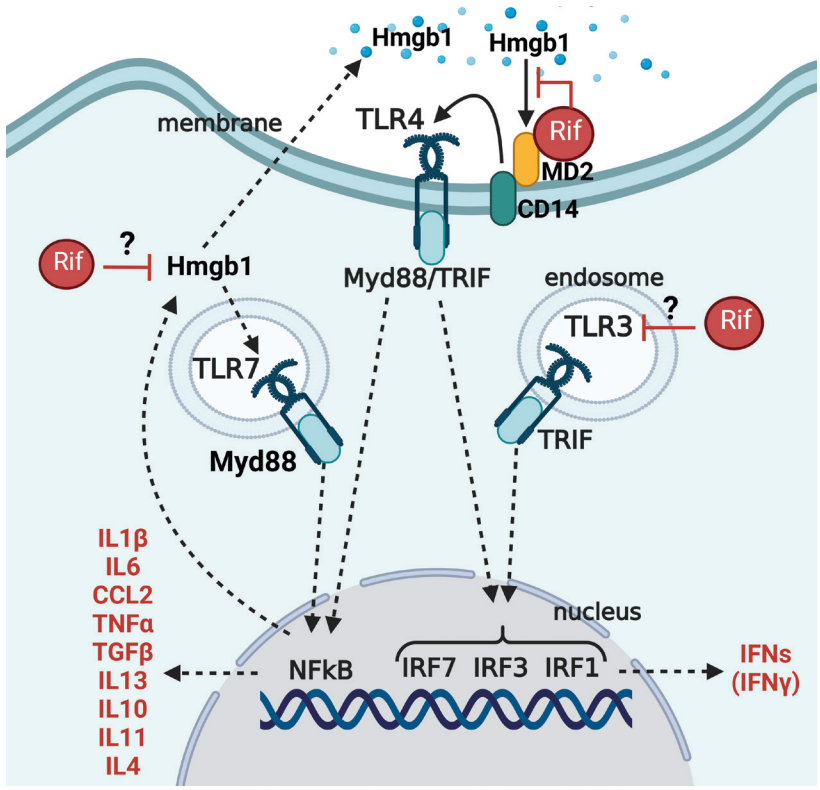

Fig. 7. The scheme of TLR signaling pathways and possible targets of rifampicin action.

mechanisms that have not been investigated yet contribute to the dysregulation of the expression level of cytokine genes associated with PAE.

In one of the studies performed on microglial cell culture there is information about the competition between RIF and lipopolysaccharide for binding to MD2 protein (Myeloid Differentiation factor 2) (Wang et al., 2013). The MD2 protein is necessary for binding the TLR4 ligand with subsequent signal conduction. By binding to this protein RIF contributed to the inhibition of LPS-CD14-MD2-TLR4-NFκB signaling in the experiment, and as a consequence, there was a decrease in NFκB activation and content of pro-inflammatory factors (NO, IL1β, TNFα) (Wang et al., 2013). Several researchers on different models of neuroinflammation have also shown that RIF is able to reduce the level of pro-inflammatory cytokines, β-amyloid content in the Alzheimer's disease model, α-synuclein in the Parkinson's disease model. There are studies showing improvement of autophagy process in mice in the hippocampus when exposed to RIF (Airapetov et al., 2022a; 2022b). Using a model of corpus callosum demyelination, it was shown that when RIF was administered to mice, a decrease in the expression of markers of apoptotic events was recorded, as well as a decrease in the activity of caspases 3 and 12 (Zahednasab et al., 2019), and preliminary administration of RIF suppressed lithium-pilocarpine-induced hippocampal neurodegeneration (Ali, Mahdy, Elsherbiny, and Azab, 2018).

Conclusions

Our study provided new data on the expression state of genes of the TLR signaling system on day 8 of postnatal development in the forebrain cortex of animals subjected to PAE. The results of the study showed the ability of RIF to correct the observed long-term pathological changes in the TLR system (Fig. 7). A decrease in elevated levels of TLR3, TLR4, Hmgb1, IL-1β, and TNFα mRNA in the forebrain cortex of animals subjected to PAE was found.

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