EFFECTS OF VARIOUS MRNA-LNP VACCINE DOSES ON NEUROINFLAMMATION IN BALB/C MICE

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It has been proven that mRNA vaccines are highly effective against the COVID-19 outbreak, and low prevalence of side effects has been shown. However, there are still many gaps in our understanding of the biology and biosafety of nucleic acids as components of lipid nanoparticles (LNPs) most often used as a system for intracellular delivery of mRNA-based vaccines. It is known that LNPs cause severe injection site inflammation, have broad biodistribution profiles, and are found in multiple tissues of the body, including the brain, after administration. The role of new medications with such pharmacokinetics in inflammation developing in inaccessible organs is poorly understood. The study was aimed to assess the effects of various doses of mRNA-LNP expressing the reporter protein (0, 5, 10, and 20 μg of mRNA encoding the firefly luciferase) on the expression of neuroinflammation markers (*Tnfα*, *Il1β*, *Gfap*, *Aif1*) in the prefrontal cortex and hypothalamus of laboratory animals 4, 8, and 30 h after the intramuscular injection of LNP nanoemulsion. It was shown that mRNA-LNP vaccines in a dose of 10–20 μg of mRNA could enhance Aif1 expression in the hypothalamus 8 h after vaccination, however, no such differences were observed after 30 h. It was found that the *Gfap*, *Il1β*, *Tnfα* expression levels in the hypothalamus observed at different times in the experimental groups were different. According to the results, mRNA-LNPs administered by the parenteral route can stimulate temporary activation of microglia in certain time intervals in the dose-dependent and site specific manner.

Keywords: mRNA vaccine, neuroinflammation, lipid nanoparticles, *Aif1*, *Gfap*

Funding: the study was supported by the Ministry of Science and Higher Education of the Russian Federation (agreement № 075-10-2021-113, project ID RF----193021X0001).

Author contribution: Kirshina AS — RNA extraction, conducting PCR; Kazakova AA, Kolosova ES, Imasheva EA, Vasileva OO — generating genetic constructs, RNA extraction, manuscript writing; Zaborova OV — RNA formulation in LNP, manuscript writing; Terenin IM — RNA synthesis, manuscript writing; Muslimov AR — animal experiment, manuscript editing; Reshetnikov VV — animal experiment, data analysis, preparing illustrations, manuscript wr

Compliance with ethical standards: the study was appoved by the Ethics Committee of Pavlov First St.Petersburg State Medical University (protocol № 83 of 21 September 2022); it was conducted in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS No. 123, Strasbourg, 1986, with the 2006 Appendix), international convention on the humane treatment of animals (1986), Guide for the Care and Use of Laboratory Animals, 8th ed. (2010); Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes (2010), Principles of Good Laboratory Practice (2016).

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Received: 01.12.2022 **Accepted:** 15.12.2022 **Published online:** 30.12.2022

DOI: 10.24075/brsmu.2022.068

ВЛИЯНИЕ РАЗЛИЧНЫХ ДОЗ МРНК-ЛНЧ-ВАКЦИН НА НЕЙРОВОСПАЛЕНИЕ У BALB/C МЫШЕЙ

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Доказана высокая эффективность мРНК-вакцин в борьбе с эпидемией COVID-19, продемонстрирована низкая частота развития побочных эффектов. Тем не менее существует еще много пробелов в нашем понимании биологии и биобезопасности нуклеиновых кислот в составе липидных наночастиц (ЛНЧ), наиболее часто используемых в качестве системы внутриклеточной доставки вакцин на основе мРНК . Известно, что ЛНЧ приводят к сильному воспалительному ответу в месте введения, имеют широкий профиль биораспределения и обнаруживаются после введения во многих тканях организма, в том числе в головном мозге. Роль новых препаратов с такой фармакокинетикой в воспалительных процессах, развивающихся в забарьерных органах изучена недостаточно. Целью исследования было оценить влияние различных доз мРНК-ЛНЧ, экспрессирующих репортерный белок (0, 5, 10 и 20 мкг мРНК, кодирующей люциферазу светлячка) на экспрессию маркеров нейровоспаления (*Tnfα*, *Il1β*, *Gfap*, *Aif1*) в префронтальной коре и гипоталамусе лабораторных животных через 4, 8 и 30 ч после внутримышечной инъекции наноэмульсии ЛНЧ. Показано, что мРНК-ЛНЧ-вакцины в дозе 10–20 мкг мРНК способны усиливать экспрессию *Aif1* в гипоталамусе через 8 ч после вакцинации, но через 30 ч эти различия не определялись. Обнаружено, что уровень экспрессии *Gfap*, *Il1β*, *Tnfα* в экспериментальных группах различался в различных временных точках в гипоталамусе. Согласно полученным результатам, введенные парентерально мРНК-ЛНЧ могут стимулировать временную активацию микроглии в определенных временных промежутках дозо- и регион-зависимым образом.

Ключевые слова: мРНК-вакцины, нейровоспаление, липидные наночастицы, *Aif1*, *Gfap*

Финансирование: исследование выполнено при поддержке Министерства науки и высшего образования Российской Федерации (соглашение № 075-10-2021-113, уникальный идентификатор проекта РФ----193021X0001).

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Соблюдение этических стандартов: исследование одобрено этическим комитетом ПСПбГМУ им. И. П. Павлова (протокол № 83 от 21 сентября 2022 г.); проведено в соответствии с Европейской конвенцией ETS № 123 о защите позвоночных животных, используемых для экспериментов или в научных целях (Страсбург) (1986 г. с приложением от 2006), Международным соглашением о гуманном обращении с животными (1986 г.), Guide for the care and use of laboratory animals, 8th ed. (Руководством по уходу и использованию лабораторных животных, 2010 г.); Directive 2010/63/EU of the European parliament and of the council on the protection of animals used for scientific purposes, 2010 г.; «Правилами надлежащей лабораторной практики» (2016 г.).

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Статья получена: 01.12.2022 **Статья принята к печати:** 15.12.2022 **Опубликована онлайн:** 30.12.2022

DOI: 10.24075/vrgmu.2022.068

Advances in the development of mRNA (LNP) vaccines have made it possible to obtain two FDA approved vaccines (Pfizer/BioNTech and Moderna) against the SARS-CoV-2 virus in less than a year [1, 2]. The LNP–mRNA-based medications can be used for both treatment of a number of socially significant disorders and as vaccines for prevention of infections caused by many pathogens. The mRNA–LNP platform flexibility is due to the possibility of specific selection of the antigenic sequence comprised in the mRNA molecule, it is also due to different variants of the lipid composition and their ratios in LNPs that can modulate the mRNA vaccine efficiency and immunogenicity [3]. The Pfizer/BioNTech and Moderna lipid particles comprise charged ionized lipids, neutral ionized lipids, poly(ethylene glycol)-containing lipids, cholesterol, and distearoylphosphatidylcholine (DSPC) [4]. LNPs ensure mRNA–LNP internalization into the cell and play an adjuvant role, stimulating a moderate increase in the injection site inflammation. In has been shown that different variants of ionizable lipids recognized by the toll-like receptor 4 (TLR4) play a central role in the induction of inflammation caused by LNPs [5]. Furthermore, the mRNA molecule being a vaccine component can exert pro-inflammatory activity via TLR-3,7,8, RIG-I, MDA5 [6, 7]. Moderate pro-inflammatory activity contributes to effective antigen presentation of the antigen-presenting cells, as well as to the humoral and T-cell immunity formation. However, inflammation may sometimes cause adverse effects. In particular, recent studies have shown that LNPs cause severe injection site inflammation, have a broad biodistribution profile, and are found in multiple tissues of the body, including the brain [4, 8]. Uninhibited crossing the blood-brain barrier together with pro-inflammatory activity can cause adverse effects in the form of immune activation in the central nervous system. The study was aimed to perform the dynamic assessment of neuroinflammatory markers in the prefrontal cortex and hypothalamus of the Balb/c mice after administration of various mRNA–LNP vaccine doses.

METHODS

Experimental design

The conventional experiment involved 75 adult Balb/c males (age 9–10 weeks, body weight 19–22 g) obtained from the Rappolovo Breeding Nursery of the Russian Academy of Medical Sciences (St. Petersburg, Russia) and kept at the Center of Experimental Pharmacology, St. Petersburg State Chemical and Pharmaceutical University, under fixed lightning conditions (12.00 : 12.00 h). The animals had free access to the standard food (granules) and water. The animals were distributed into the study groups by randomization before the study. Intramuscular injections of 30 μL of various doses of mRNA–LNP (three concentrations: 5, 10, and 20 μg of RNA) or control (empty) LNPs in phosphate buffer were performed. The animals inhaled the 2.0% isoflurane (Laboratories Karizoo, S.A.; Spain) mixed with oxygen for 5 min and were subsequently decapitated within 4, 8, and 30 h after administration of the particle suspension (Fig. 1). The samples of the hypothalamis and prefrontal cortex (PFC) were obtained as earlier reported [9]. The same volume (30 μL) of phosphate buffer was administered to the control animals. Five animals per experimental point were used in each group.

Cloning

Amplification of the target gene comprising the 5'-UTR Moderna (gggaaataagagagaaaagaagagtaagaagaaatat aagaccccggcgccgccacc) encoding the firefly (*Photinus pyralis*) luciferase and the 3'-UTR Moderna (gctggagcctcgg tggcctagcttcttgccccttgggcctccccccagcccctcctcccctttcctgc acccgtacccccgtgtctttgaataaagtctgagtgggcggca) sequences was performed via linking together three fragments by the overlapping primer-based PCR. Then the resulting fragment was incubated with the EcoRI and BglII restriction endonucleases, purified from agarose gel and ligated to the pSmart commercial vector (Lucigen; USA) prepared by the same method. The vector comprised a polyA-tail with the size of 110. A NEB-stable *E. coli* strain (New England Biolabs; UK) was used for transformation. Clones were selected from the colonies by PCR, and the sequence of the insert was confirmed by sequencing. To generate the verified plasmid, *E. coli* was grown in the incubator shaker at 30 °C and 180 rpm. Then plasmid DNA was extracted from bacterial cells using the QIAGEN Plasmid Maxi Kit (Qiagen; USA). The resulting plasmid preparation was linearized by the unique SpeI restriction site and subsequently visualized in agarose gel.

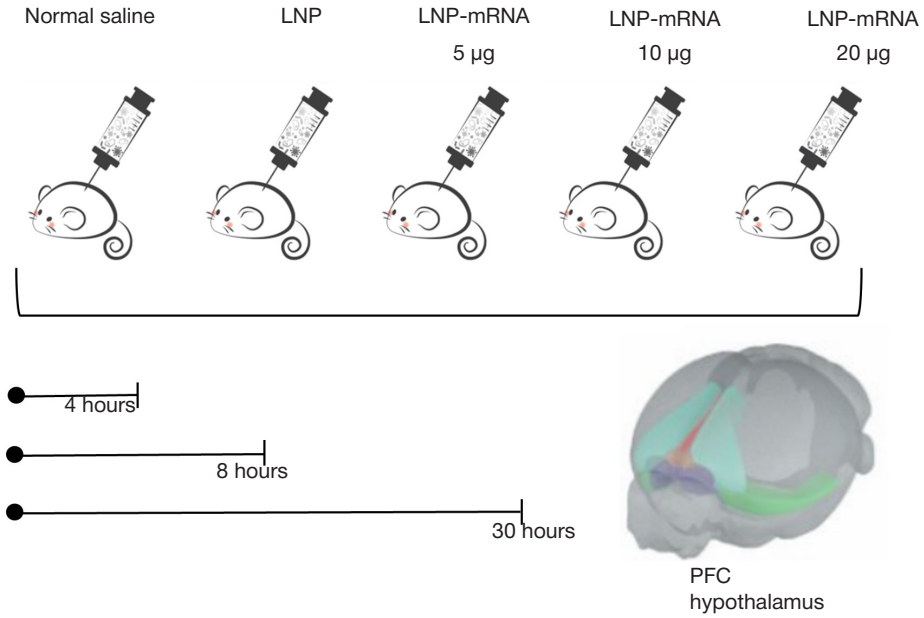

Fig. 1. Experimental design

Table. Nucleotide sequences of primers and probes

Gene	Sequence 5'→3'	
Aif1	Probe	ROX-AGAGAGGCTGGAGGGGATC-BHQ2
	For	GCTTTTGGACTGCTGAAGGC
	Rev	GAAGGCTTCAAGTTTGGACG
Gfap	Probe	ROX-GCAAGAGACAGAGGAGTGG-BHQ-2
	For	CCTGAGAGAGATTCGCACTC
	Rev	GACTCCAGATCGCAGGTCAAG
TNFα	Probe	ROX-CGAGTGACAAGCCTGTAGC-BHQ2
	For	CATCAGTTCTATGGCCCAGACCCT
	Rev	GCTCCTCCACTTGGTGGTTTGCTA
Il1β	Probe	ROX-CTGCTTCCAAACCTTTGACCTGG-BHQ2
	For	CCTGTTCTTTGAAGTTGACGG
	Rev	CTGAAGCTCTTGTTGATGTGC
Gapdh	Probe	CCATCAACGACCCCTTCATTGACCTC
	For	TGCAGTGGCAAAGTGGAGAT
	Rev	TGCCGTGAGTGGAGTCATACT

In vitro mRNA transcription

In vitro transcription was carried out in the buffer solution containing 20 mmol of DTT, 2 mmol of spermidine, 80 mmol of HEPES-KOH (pH 7.4), 24 mmol of $MgCl_2$. The reaction mixture also contained 3 mmol of each ribonucleoside triphosphate (Biosan; Russia), 12 mmol of anti-reverse cap analog (ARCA) (Biolabmix; Russia). Other components per 100-μL reaction volume: 40 units of the RiboCare ribonuclease inhibitor (Evrogen; Russia), 500 units of the T7 RNA polymerase (Biolabmix; Russia), 5 μg of the linearized plazmid, and 1 μL of the enzyme mix from the RiboMAX Large Scale RNA Production System kit (Promega; USA) as the source of inorganic pyrophosphatase. The reaction was carried out for 2 h at a temperature of 37 °C, then another 3 mmol of each ribonucleoside triphosphate were added to the reaction and incubated for 2 h. DNA was hydrolyzed using the RQ1 nuclease (Promega; USA), RNA was precipitated by adding LiCl to a concentration of 0.32 mol and EDTA (pH 8.0) to a concentration of 20 mmol with subsequent incubation on ice for an hour. Then the solution was centrifuged for 15 min (25,000 g, 4 °C). RNA precipitate was washed with 70% ethanol, diluted in the ultrapure water and once more precipitated by alcohol using the standard method. RNA concentration was defined by spectrophotometry based on absorbance at a wavelength of 260 nm.

Formulation of LNPs containing mRNA

Encapulation of mRNA into lipid nanoparticles was performed by mixing the 0.2 mg/mL mRNA aqueous solution (10 mmol citrate buffer, pH 3.0) with the alcohol solution of the lipid mixture in the microfluidic cartridge using the NanoAssemblr Benchtop system (Precision Nanosystems; USA). The lipid mixture contained the following components: ALC-0315 ionizable lipidoid (BroadPharm; USA), distearoylphosphatidylcholine (DSPC) (Avanti Polar Lipids; USA), cholesterol (Sigma-Aldrich; USA), DMG-PEG-2000 (BroadPharm; USA) in a molar ratio (%) of 46.3 : 9.4 : 42.7 : 1.6. The amount of lipids per unit of mRNA was calculated based on the following ratio: N/P = 6 (ALC-0315 ionizable lipidoid/mRNA base). To generate particles of the desired size, the aqueous and alcohol phases were mixed in a ratio of 3 : 1 v/v with the total mixing speed of 10 mL/min. After mixing the phases the resulting water-alcohol particle suspension was dialyzed in 300 volumes of phosphate buffered saline (pH 7.4, 18 h, +15 °C). After dyalisis the particle suspension was concentrated using the Amicon Ultra-4 10,000 Da molecular weight cutoff filter. Then particles were filtered through the filter with the 0.22 μm PES membrane (Merck; USA) and stored at 4 °C. Empty LNPs were obtained by mixing the 10 mmol citrate buffer (pH 3.0) with the lipid mixture alcohol solution in the microfluidic cartridge by the same method that was used to obtain the mRNA-loaded LNPs.

After filtration, the quality of the particles generated was assessed based on two parameters: mRNA load and particle size. The concentration of mRNA loaded into lipid nanoparticles was defined based on the differences in the fluorescence signal levels obtained for the particle suspension stained with the RiboGreen reagent (Thermo Fischer Scientific; USA) before and after the particle disruption. The Triton X-100 detergent (Sigma-Aldrich; USA) was used to disrupt the particles. The LNP size was defined by the dynamic light scattering method in the Zetasizer Nano ZSP system (Malvern Panalitycal; USA).

Estimation of gene expression in the brain

Total RNA was extracted from the PFC and hypothalamus using the kit for column-based RNA isolation (Biolabmix; Russia) in accordance with the manufacturer's protocol. RNA concentration and purity were assessed with the NanoDrop OneC spectrophotometer (Thermo Scientific; USA).

To carry out the reverse transcription reaction, 500 ng of RNA and the OT-M-MuLV-RH reverse transcription kit (Biolabmix; Russia) with random hexanucleotide primers were used. The resulting cDNA was used to assess gene expression. Expression levels of the genes encoding pro-inflammatory cytokines and interleukins (*Il1β*, *Tnfα*), marker genes of microglia (*Aif1*) and astroglia (*Gfap*) activation were assessed as neuroinflammation markers. The study involved the use of quantitative PCR with fluorescent Taq-man probes. The sequences of primers and probes are provided in Table 1.

The expression was assessed relative to mRNA of the housekeeping gene (Gapdh). PCR was carried out using the BioMaster HS-qPCR (2×) kit (Biolabmix; Russia) in the Real-Time CFX96 Touch system (Bio-Rad Laboratories; USA) in

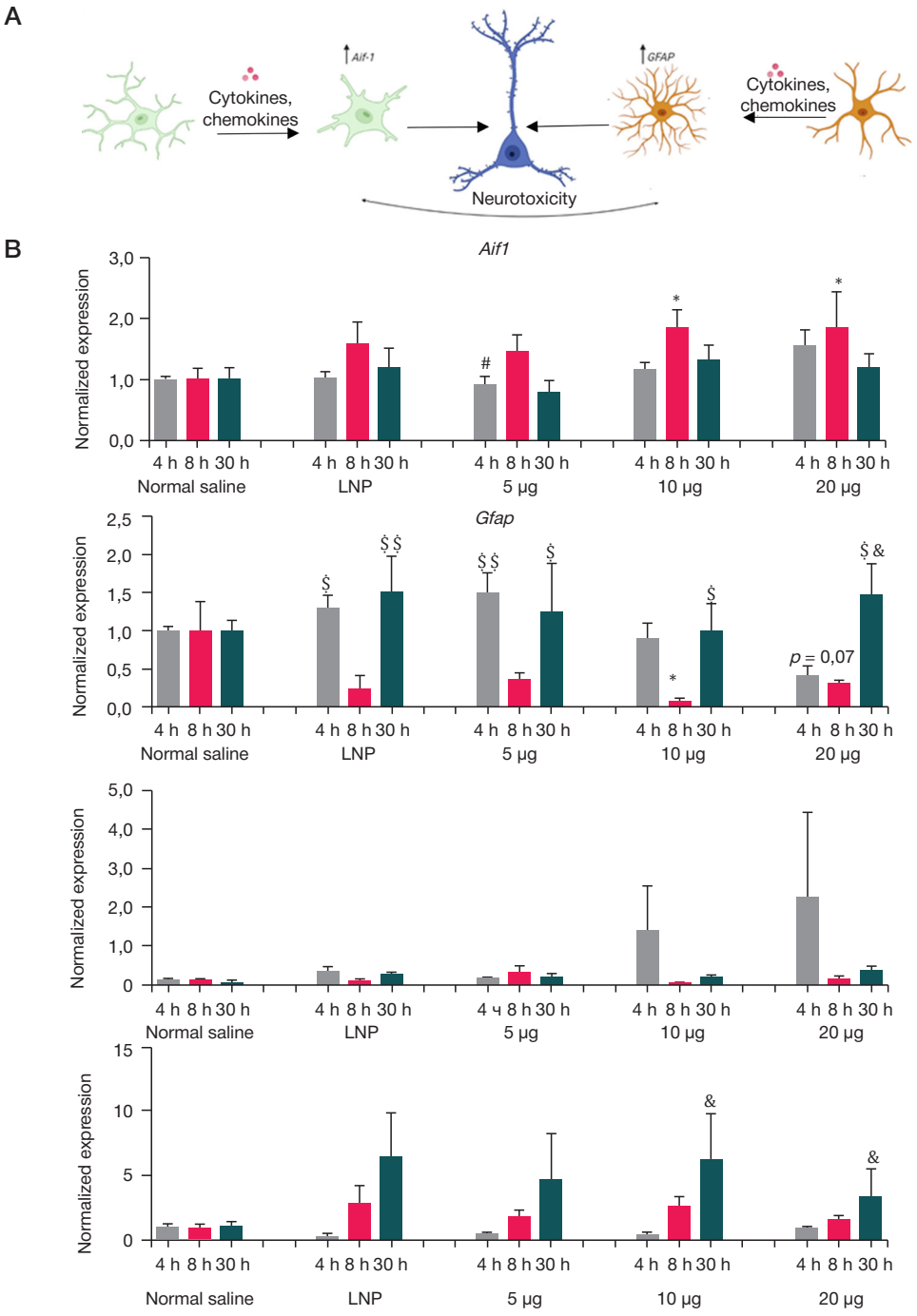

Fig. 2. Expression of neuroinflammatory marker genes in the hypothalamus **A**. Astroglial and microglial response to acute inflammation. **B**. Relative expression of genes encoding pro-inflammatory cytokines (*Il1β*, *Tnfa*) and markers of glial activation (*Aif1*, *Gfap*) at various time points after the mRNA-LNP vaccine administration. The data are presented as mean ± standard error. * — $p < 0.05$, ** — $p < 0.01$, compared to the group that received normal saline at the same time point; # — $p < 0.05$, compared to the group that received 20 μg of mRNA-LNP at the same time point; $ — $p < 0.05$, $$ — $p < 0.01$, compared to the point within 8 hours after administration; & — $p < 0.05$, compared to the point within 4 hours after administration. Post hoc analysis using the Fisher's LSD test

accordance with the following protocol: 95 °C for 15 s, 60 °C for 20 s. Three iterations of all tests per cDNA sample were performed. The expression was quantified by the ΔΔCt method.

Statistical analysis

Statistical processing of the results was performed by ANOVA (the "group" and "time after administration" were used as factors) and Fisher's least significant difference (LSD) test as a post hoc test. The differences between the experimental groups were considered singnificant at $p < 0.05$, while at the level of trends these were considered significant at $p < 0.1$. Data analysis was performed using the Statistica 8.0 software package (Statsoft Inc.; USA).

RESULTS

The findings show that various mRNA–LNP vaccine doses induce activation of *Aif1* in the hypothalamus (Fig. 2), but not in the prefrontal cortex (Fig. 3). The two-way analysis of variance (ANOVA) made it possible to reveal significant effects of the "group" and "time after administration" factors on the *Aif1* expression in the hypothalamus ($F(4.70) = 2.866$ at $p = 0.032$; $F(2.72) = 4.246$ at $p = 0.019$). In the groups of mice that

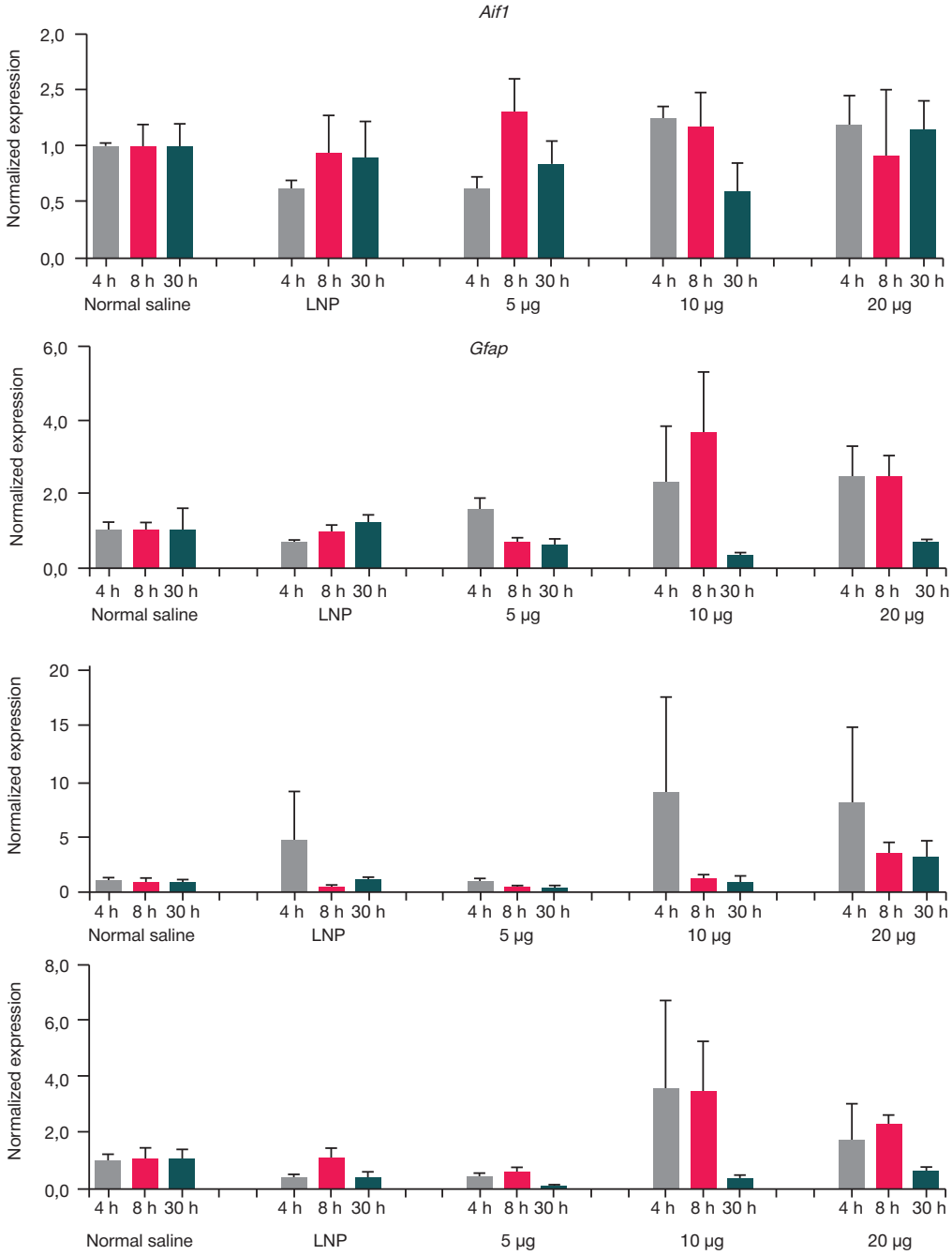

Fig. 3. Relative expression of genes encoding pro-inflammatory cytokines (*Il1β, Tnfa*) and markers of glial activation (*Aif1*, *Gfap*) at various time points after the mRNA-LNP vaccine administration in the prefrontal cortex

received 10 μg of mRNA and 20 μg of RNA as part of the mRNA–LNP vaccine, the expression of *Aif1* mRNA within 7 h after the vaccine administration was about 80% higher than in the control group that received phosphate buffer ($p > 0.05$). It is interesting to note that the groups that received 5 μg of RNA as part of the mRNA–LNP vaccine and empty LNPs (with no mRNA) also showed elevated espression of *Aif1* (by 40–55%) within 8 h, however, these differences were non-significant. No differences in the hypothalamic *Aif1* expression between animals of different groups were observed 30 h after the vaccine administration. No significant effects of the "group" factor or the interaction of the "group" and "time after administration" factors on the expression of other assessed genes in the hypothalamus (*Tnfα*, *Il1β*, *Gfap*) and gene expression in the prefrontal cortex were revealed. Thus, we observed moderate mRNA–LNP effects on the neuroinflammation associated with the elevated expression of the markers of active microglia in the hypothalamus, but not in the prefrontal cortex. Furthermore, these effects were dose-dependent.

Comparison of gene expression at various time points between animals of various groups after administration of the mRNA–LNP vaccine showed that *Il1β* expression was dramatically increased 4 h after vaccination in both hypothalamus and prefrontal cortex of certain animals in the groups that received 10 μg of mRNA and 20 μg of RNA as part of the mRNA–LNP vaccine. However, no such effects were observed in the later measurement points. Despite the profound effects on the *Il1β*, these differences were non-significant, since only a few animals in the groups showed a pronounced response. Such results demonstrate heterogeneity of the response to the mRNA–LNP vaccine associated with individual characteristics of the animals.

The effect of the "time after administration" factor on the *Gfap* and *Tnfα* expression in the hypothalamus was revealed ($F(2.72) = 10.179$ at $p < 0.0001$; $F(2.72) = 5.181$ at $p = 0.008$). The *Gfap* expression decreased within 8 h in all experimental groups, however, it increased in 30 h. It is interesting that the *Tnfα* expression also increased in 30 h after vaccination compared to the levels observed within 4 h in the majority of experimental groups. Such results suggest that mice in the experimental group develop the second wave of pro-inflammatory activation involving astrocytes and interleukin TNFα.

DISCUSSION

The findings show that mRNA–LNP vaccines with the mRNA doses of 10–20 μg are capable of increasing the *Aif1* expression within 8 h in the hypothalamus, but not in the prefrontal cortex. We have found that experimental groups demonstrate the differences in the *Gfap*, *Il1b*, *Tnfα* expression levels measured at various time points in the hypothalamus, which is also an indirect evidence of the fact that the expression levels of these genes may be correlated to the mRNA–LNP vaccine administration.

The mRNA–LNP vaccine can cause both local and systemic inflammation [4, 8]. Inflammation can be caused by various vaccine components: mRNA molecules, lipids forming part of LNPs or protein product encoded by mRNA. The mRNA–LNPs most often transfect cells near the injection site, after that LNPs are rapidly transported to the proximal lymph nodes by passive drainage and are also actively transported by the professional antigen-presenting cells and neutrophils [10, 11]. Then mRNA–LNP can reach any cell of the body via systemic circulation; low amounts of mRNA–LNP are found in the brain, thus suggesting its capability of crossing the blood-brain barrier [12, 13].

It is known that peripheral inflammatory stimuli can also cause immune response in the brain that results in activation of astrocytes, the main immunocompetent cells of the brain [14]. Because of their cytokine-producing and phagocytic activity, these cells affect the development and maturation of the CNS structures [15], participate in the normal formation and development of neural circuits during onthogenesis [16], maintain the pool of neurons, mediate synapse maturation and reduction, thereby regulating the number of synapses and receptor expression [17].

Thus, the signs of microglia activation we have found in certain experimental groups may be both evidence of mRNA–LNP directly crossing the blood-brain barrier and triggering neuroinflammation, and the result of the increasing peripheral inflammation. Since our study does not involve assessment of the peripheral immune activation parameters, we cannot answer this question explicitly.

Significant differences in the *Aif1* expression revealed 8 h after immunization are consistent with the data showing that the peak of microglia activation falls between 6–24 h after induction of inflammation [14, 18–20]. At the same time, the peak of cytokine activation after induction by inflammatory agents, such as bacterial lipopolysaccharide or the synthetic analog of double-stranded RNA (Poly I:C), falls between 1.5–3.0 after administration of inflammatory mimetics. That is why the lack of significant effects on the expression of *Il1β* and *Tnfα* observed across the groups may be due to the fact that peak activation of gene expression is passed. At the same time, a number of studies show that elevated cytokine levels in the brain and periphery may persist up to 24 h after inflammation induction by mimetics.

In our study we assessed the expression of pro-inflammatory genes in two brain structures. The more pronounced effects were observed in the hypothalamus, while prefrontal cortex showed no significant alterations. The hypothalamus is an important brain structure that functions as a metabolic center responsible for regulation of multiple fundamental physiological processes involved in metabolism of the whole body, including food intake, regulation of appetite, energy consumption; thus, the hypothalamus plays a crucial role in systemic homeostatic regulation [22]. Clinical data have shown that various stimuli, such as peripheral inflammation or the increased intake of saturated fatty acids, may cause neuroinfllammation in this brain structure [23–25]. Furthermore, the hypothalamus contains various cell populations of microglia and astroglia [26]. Taken together, these data show that the hypothalamus may be a kind of the peripheral inflammation sensor and respond to pro-inflammatory signals more actively than the prefrontal cortex.

CONCLUSIONS

The mRNA–LNP vaccine can activate the hypothalamic *Aif1* expression 8 h after vaccination in a dose-dependent manner. However, no significant effects of mRNA–LNP vaccines on the gene expression have been found in the prefrontal cortex. Despite the fact that alterations in the *Aif1* expression observed within 30 h after vaccination are non-significant, these findings show that mRNA–LNP vaccine can induce neuroinflammation. Further experiments involving larger groups of animals and focused on assessing the parameters of peripheral inflammation and broader analysis of neuroinflammation involving the use of immunoassays and immunohistochemistry for assessment of pro-inflammatory agents and microglial cell morphology in the hypothalamus and other brain structures are required to understand the mechanisms underlying the mRNA–LNP vaccine capability of inflammation stimulation.

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