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Comparative Investigation of Expression of Glutamatergic and GABAergic Genes in the Rat Hippocampus after Focal Brain Ischemia and Central LPS Administration

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Abstract—Among the responses in the early stages of stroke, activation of neurodegenerative and proinflammatory processes in the hippocampus is of key importance for the development of negative post-ischemic functional consequences. However, it remains unclear, what genes are involved in these processes. The aim of this work was a comparative study of the expression of genes encoding glutamate and GABA transporters and receptors, as well as inflammation markers in the hippocampus one day after two types of middle cerebral artery occlusion (according to Koizumi et al. method, MCAO-MK, and Longa et al. method, MCAO-ML), and direct pro-inflammatory activation by central administration of bacterial lipopolysaccharide (LPS). Differences and similarities in the effects of these challenges on gene expression were observed. Expression of a larger number of genes associated with activation of apoptosis and neuroinflammation, glutamate reception, and markers of the GABAergic system changed after the MCAO-ML and LPS administration than after the MCAO-MK. Compared with the MCAO-ML, the MCAO-MK and LPS challenges caused changes in the expression of more genes involved in glutamate transport. The most pronounced difference between the responses to different challenges was the changes in expression of calmodulin and calmodulin-dependent kinases genes observed after MCAO, especially MCAO-ML, but not after LPS. The revealed specific features of the hippocampal gene responses to the two types of ischemia and a pro-inflammatory stimulus could contribute to further understanding of the molecular mechanisms underlying diversity of the post-stroke consequences both in the model studies and in the clinic.

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Keywords: hippocampus, middle cerebral artery occlusion, lipopolysaccharide, glutamate, GABA, gene expression

INTRODUCTION

Ischemia in the middle cerebral artery territory can lead to the acute disruption of blood supply in the brain regions, mainly in the neocortex, resulting in cell death and formation of an infarction zone within several hours. Shortage of nutrients and oxygen are considered as main causes of these events. However, during ischemia, cells also are damaged in the "remote" brain structures, such as the hippocampus, a brain region, which does not receive blood supply through the damaged vessels directly [1]. The hippocampus plays a key role in learning and new memory formation [2]. Development of psychopathologies, including post ischemic dementia, is associated with degeneration of hippocampal neurons [3]. Therefore, elucidation of the mechanisms of the damaging effect of ischemia on the hippocampus is important for finding the means to reduce negative consequences of an ischemic event. Since majority of all ischemic brain pathologies of the brain are related to the impaired blood

Abbreviations: LPS, lipopolysaccharide; MCAO, middle cerebral artery occlusion; MCAO-MK, MCAO according to the method of Koizumi; MCAO-ML, MCAO according to the method of Longa.

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flow in the middle cerebral artery [4], occlusion of the middle cerebral artery (MCAO) is widely used for experimental modeling of ischemic stroke in rodents.

Among the proposed approaches for triggering acute cell death after ischemia, considerable attention is paid to excitotoxic effect of glutamate [5, 6]. However, glutamatergic system is involved in numerous vital functions [7] including positive regulation of neuronal differentiation by excitatory stimuli [8]. Ambiguous effect of glutamate on various processes that determine cell viability makes it difficult to find methods to influence this system for the treatment of damage caused by ischemia, as well as timing of administration of such therapeutic agents as, for example, ligands of glutamatergic receptors. There is evidence that increase in the expression of selected types of glutamate receptors during the acute phase of a stroke could aggravate post-ischemic recovery, while their increase at a later phase, on the contrary, facilitates recovery [9]. Obviously, due to incomplete understanding of the role of specific components in the glutamatergic system in the ischemic brain damage, numerous attempts to directly affect this neurochemical system in order to prevent or slow down cell death failed to produce the desired effects [10, 11].

Acute cerebral ischemia is accompanied by the neuroinflammation manifested by the activation of glial cells, production and secretion of cytokines in the hippocampus [12]. In the studies of pathophysiological mechanisms of neurodegenerative diseases, including those induced by ischemia, administration of lipopolysaccharide (LPS) has become widely used as an effective model approach to assess the role of various inflammatory process players in these diseases [13]. The genome-wide analysis of hippocampal transcriptomes one day after experimental ischemia [14-16] or central administration of LPS [16, 17] revealed changes in the expression of the genes associated with apoptosis, inflammation, and neurotransmitter systems. A valuable new information could be obtained by comparing the data received using different models. For example, it turned out that the two most commonly used MCAO models (the "gold standard" for modeling ischemic stroke in rodents [18]) differ significantly in the number of functional parameters, including those associated with glutamate excitotoxicity and inflammatory processes [19, 20]. Taking into account these results, the aim of the present study was to compare expression patterns of the genes encoding glutamate and GABA transporters and receptors as well as markers of inflammation in the hippocampus induced by two types of ischemic exposure, according to the methods of Koizumi et al. (MCAO-MK) [21] and Longa et al. (MCAO-ML) [22], as well as by direct pro-inflammatory activation via central administration of LPS. It was recently found that the Koizumi and Longa ischemia models differ in accumulation of glucocorticoid hormones and pro-inflammatory cytokines in the hippocampus in the acute period after ischemia [20], and these differences persisted for months [23]. The study of specific features of pro-inflammatory activation associated with the responses of glutamatergic and other neurotransmitter systems, primarily the inhibitory GABAergic system, is of fundamental importance for further elucidation of the mechanisms of acute ischemia-induced damage in the hippocampus.

MATERIALS AND METHODS

Animals. Male Wistar rats (2.5 months of age) were used in the experiments. Animals were kept under standard conditions in the vivarium of the Institute of Higher Nervous Activity and Neurophysiology of the Russian Academy of Sciences (models of MCAO) or Institute of Cytology and Genetics, Siberian Branch of the Russian Academy of Sciences (LPS administration) with free access to food and water.

Middle cerebral artery occlusion. *Koizumi et al. MCAO model (MCAO-KM).* Rats were anesthetized with isoflurane. An incision was made in the neck area and, pushing the muscle tissue on the left side, penetration to the common carotid artery was performed and ligatures applied to it, as well as to the external and internal carotid arteries. A nylon filament (3-0) with a rounded end was inserted through the hole at the bifurcation site into the external and internal branches and advanced along the internal carotid artery to the middle cerebral artery. Then the ligature on the internal carotid artery was tightened to fix the filament. The occlusion lasted for 60 min; common, external, and internal carotid arteries remained ligated, and the body temperature of the animal was maintained at 37 ± 0.5°C. Then, the filament was removed and the ligature on the internal carotid artery was tightened. In the control (SHAM-KM) group, all these manipulations were performed, except for the introduction of the filament.

Longa et al. MCAO model (MCAO-LM). Surgery was performed under isoflurane anesthesia. Through an incision in the neck of the animal, the common carotid and external carotid arteries were assessed and ligated. After electrocoagulation and dissection of a fragment of the left external carotid artery near bifurcation site, a filament was inserted through the remaining part of the artery and pushed through the internal carotid artery to the intersection with the middle cerebral artery. The occlusion lasted for 60 min, while the body temperature of the rat was maintained in the range of 37 ± 0.5°C. Then, the filament was extracted, and blood flow restored in the ipsilateral common carotid artery. In the control (SHAM-LM) group, all these manipulations were performed, except for the introduction of the filament.

Evaluation of neurological deficits. Prior to transcriptome analysis, an assessment of neurological deficits

that accompanied formation of an ischemic area was performed to confirm formation of an infarct volume in the animals [20]. Motor and behavioral changes were assessed using a 0-5-point grading scale following MCAO occlusion. This standard test commonly used to assess the efficiency of focal brain ischemia in rodents is based on the 5-point behavioral scale and allows to evaluate functional state of the contralateral foreleg of the rat, presence of turns and circulation in the contralateral side, as well as mobility of the animal: 0) no deficit; 1) failure to extend right forepaw fully; 2) decreased grip of right forelimb while tail pulled; 3) spontaneous circling or walking to contralateral side; 4) walks only when stimulated with depressed level of consciousness; 5) unresponsive to stimulation. In addition, a tongue protrusion test was used that indicates the ability of the rat to lick peanut butter out of a narrow glass cylinder left in the cage overnight. Thickness of the consumed butter layer, from the cylinder opening to the level of the remaining butter, shows ability of the animal to protrude its tongue, which is impaired during the acute phase of MCAO.

All the animals exposed to MCAO exhibited severe neurological deficits, indicating formation of an infarct volume, while the sham-operated animals did not demonstrate such deficits. On a 5-point scale, all the sham-operated animals had a score of 0, while the animals after MCAO in the Koizumi or Longa models showed a severe deficit of 4 points at the time of surgery and 3 points one day after surgery before slaughter. One day after operation, the animals subjected to MCAO, regardless of the model, demonstrated inability to stick out their tongue, while in the sham-operated rats this ability was retained by 92-100% of the animals. It should be noted that the deficit values in this work one day after ischemia are similar to those reported in the previous experiments [20].

LPS administration. LPS (30 μg in 4 μl of sterile saline) or saline (SAL) were administered stereotactically for 5 min into the right striatum under isoflurane anesthesia using the coordinates: AP = + 0.5 mm, ML = + 3 mm, DV = −5.5/4.5 mm [16]. The striatum was used because this structure is among the first damaged brain structures after ischemia. For example, the 30-min MCAO by Koizumi method in adult rats damaged only striatum, the 2-h MCAO caused damage not only in the striatum but in the neocortex [24].

RNA-seq. Twenty-four hours after MCAO or LPS administration, the rats were sacrificed by rapid decapitation. Hippocampi were rapidly isolated, placed on cold ice-blocks, and next were transferred to a RNAlater solution (Life Technologies, USA) and stored at −70°C. RNA-seq was carried out at JSC Genoanalytica (https://genoanalytica.ru, Russia). Total RNA was purified from 3 hippocampi of each control and experimental group using Trizol reagent (Thermo Fisher Scientific, USA) according to the manufacturer instructions [25]. Quality and quantity of total RNA was determined using an RNA 6000 Nano Kit (Agilent Technologies, USA) with a BioAnalyzer (Agilent Technologies). Poly-A fraction was purified from total RNA using an oligo-dT magnetic beads from the Dynabeads mRNA Purification Kit (Thermo Fisher Scientific). Next, libraries for NGS sequencing were constructed from poly-A RNA using a NEBNext® Ultra™ II RNA Library Prep (NEB, USA) according to the provided instructions. RNA concentration in the libraries was determined using a Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific) with a Qubit 2.0 fluorometer (Thermo Fisher Scientific). Fragment length distribution in the libraries was determined with an Agilent High Sensitivity DNA Kit (Agilent Technologies). Sequencing was carried out using a HiSeq1500 system (Illumina, USA) generating at least 10 million of short reads per sample with 50 bp length. Reads were aligned to the Rnor_6.0 reference genome using STAR aligner. Differential expression analysis was carried out using the DESeq2.0 software package.

Real-time PCR. Expressions of some genes were verified with real-time PCR using TaqMan technology with a VIIA7 amplifier (Thermo Fisher Scientific). Total RNA extraction, assessment of its quality, and cDNA synthesis were carried out according to the protocols described previously [25]. Gene expression levels: *Casp3* (Rn00563902_m1), *IL1b* (Rn00580432_m1), *Slc1a2* (Rn00691548_m1), *Gria2* (Rn00568514_m1), *Glul* (Rn01483107_m1), *Gad2* (Rn00561244_m1) were determined in two repeats in RNA samples after NGS sequencing with additional samples from the same experimental group to gather eight samples per group. All reactions were carried out according to the manufacturer instructions, relative gene expression levels were determined using ΔΔCt method using the housekeeping β-actin gene (Rn00667869_m1) as a reference gene.

Statistics. Changes in gene expression determined with RNA-seq were considered significant according to the Bonferroni adjusted multiple correction p-value (adjusted p-value – padj) padj < 0.05. Significance of the data obtained after real-time PCR verification was evaluated with the Student's t-test in Statistica 6.0 software.

RESULTS

Genes associated with cell death. Signs of cell death were clearly observed in the hippocampus one day after MCAO-ML. Significant increase in the expression of the executive protease caspase-3 gene (*Casp3*) (Fig. 1a) was detected by RNA-seq and confirmed by real-time PCR ($p < 0.05$) (Fig. 1b). After all exposures (MCAO-ML, MCAO-MK, and LPS), *Casp4* expression was also increased. In addition, significant increases of expression of the *Casp1* gene (padj < 0.05) and the death receptor gene *Fas*, and an increasing trend for the *Casp8* gene (padj = 0.0946) were detected after LPS application.

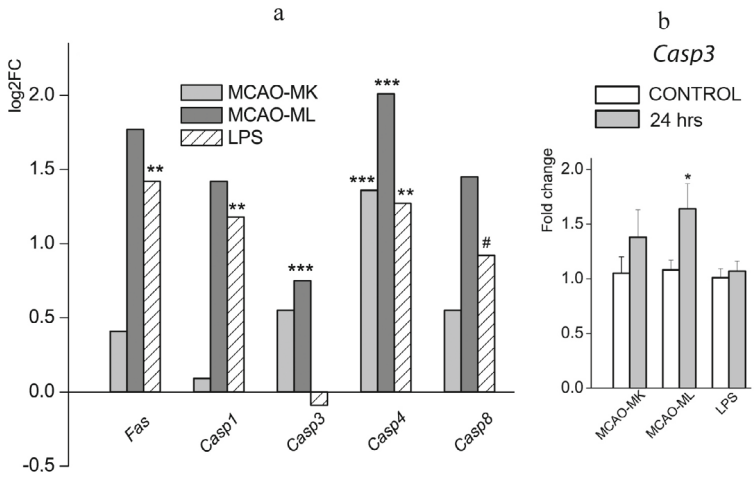

Fig. 1. Expression of the genes associated with cell death after two types of ischemic exposure and central administration of LPS. a) According to the results of sequencing; ** padj < 0.01, *** padj < 0.001, # padj < 0.1. b) Real-time PCR verification; * $p < 0.05$.

According to the Gene Ontology analyses, all these genes were associated with biological processes, including "apoptotic process", "positive regulation of neuron apoptotic process", "positive regulation of apoptotic process", "positive regulation of cell death".

It should be noted that a strong criterion was used to assess significance of the differences in gene expression for the sequencing results: differences were considered significant only at padj < 0.05. However, according to the opinions of numerous researchers, this can lead to the loss of genes that actually changed, but did not reach the required level of significance. The data from Fig. 1 shows that such "lost" genes for the MCAO-ML could include *Fas*, *Casp1*, and *Casp8*. Their expression significantly changed compared to the corresponding control values, but only at the level of *p*-value < 0.05, whereas the padj value did not reach required threshold.

Genes of inflammatory response. Expression of the *Gfap* gene encoding marker of astrocytes was significantly increased in the hippocampus one day after both ischemic exposures (Fig. 2a). The level of expression of *Aif*, the gene encoding microglial protein marker Iba-1, was increased only after central administration of the direct pro-inflammatory stimulus (LPS). According to the RNA sequencing results, MCAO-MK did not affect any of the key pro-inflammatory cytokine genes, MCAO-ML increased the expression level of two genes (*Il1b* and *Il6*), while LPS exposure increased the expression level of a single gene (*Il1b*). Analysis of the *Il1b* mRNA levels by real-time PCR (Fig. 2b) confirmed increase in the *Il1b* gene expression after MCAO-ML and administration of LPS. Moreover, the results of real time PCR showed significant increase in the *Il1b* gene expression also after MCAO-MK, while for the RNA-seq data the level of significance was only at the level of *p*-value of 0.0021 (padj > 1). The list of pro-inflammatory markers also included the *Mmp9* gene, expression of which was significantly increased one day after MCAO-ML and LPS administration.

Genes of the glutamatergic system. Changes in the expression of glutamatergic genes, encoding transporters and receptors, are shown in Fig. 3 (a – results of RNA-seq, b – results of real-time PCR analysis).

None of the five currently known glutamate transporter genes changed their expression in the hippocampus one day after MCAO-MK or MCAO-ML. Central administration of LPS resulted in the significant increase in the *Slc1a2* expression (Fig. 3a), this effect was confirmed by the results of PCR analysis ($p < 0.05$; Fig. 3b). Ischemic exposure did not affect expression of the vesicular glutamate transporters in the hippocampus, while a significant decrease in the expression of the *Slc17a6* gene was observed after LPS exposure. Gene expression of two neutral amino acid transporters for glutamine and glutamate (*Slc1a4* and *Slc1a5*) was significantly increased after MCAO-MK and LPS administration. MCAO-ML significantly increased expression of the *Slc1a5* gene. On the contrary, expression of the other neutral amino acid transporter gene, *Slc38a5*, was decreased after MCAO-MK. These results indicate possible change in the metabolism of glutamine and glutamate in the hippocampus after both ischemic and direct pro-inflammatory exposures. This possibility is consistent with the observed increase in gene expression of Glul (enzyme catalyzing synthesis of glutamine from glutamate) after MCAO-MK, which was confirmed by the results of real-time PCR analysis ($p < 0.05$).

Glutamate receptors in mammals include ionotropic and metabotropic subtypes. Ionotropic receptors (NMDA, AMPA, and kainate) are heterotetrameric transmembrane channels that, after being activated by a neurotransmitter, allow calcium or sodium cations to enter the cell. Each subunit is encoded by a separate gene, and functional properties of the receptor are determined by the subunit composition of the tetramer. Metabotropic glutamate receptors, each encoded by a separate gene, belong to the GPCR superfamily of G-protein-coupled receptors.

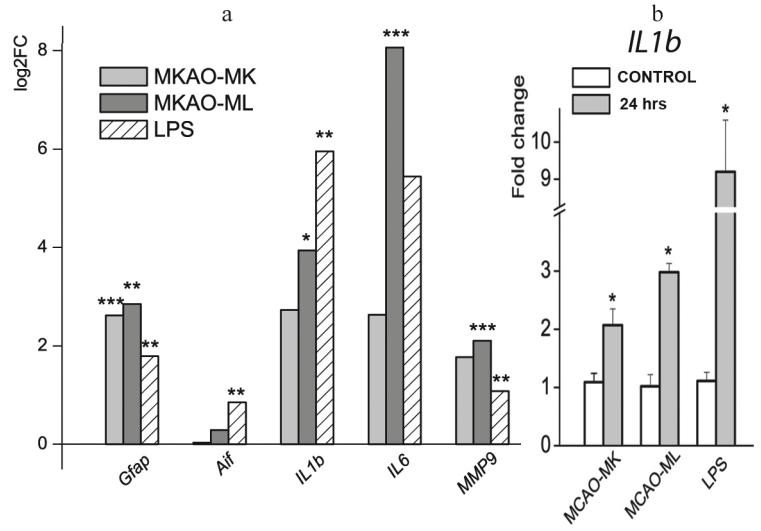

Fig. 2. Expression of the genes associated with neuroinflammation after two types of ischemic exposure and central administration of LPS. a) According to the results of sequencing; * padj < 0.05, ** padj < 0.01, *** padj < 0.001. b) Real-time PCR verification; * $p < 0.05$.

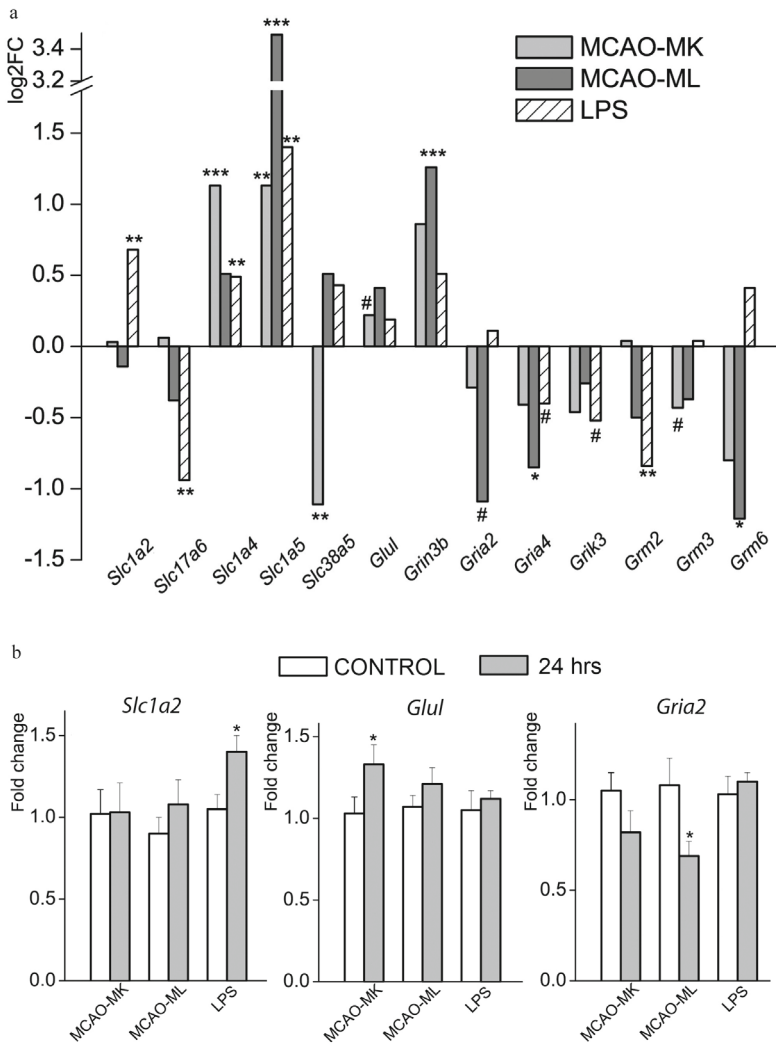

Fig. 3. Expression of the genes associated with glutamatergic neurotransmission after two types of ischemic exposure and central administration of LPS. a) According to the RNA-seq results; * padj < 0.05, ** padj < 0.01, *** padj < 0.001, # padj < 0.1. b) Real-time PCR verification; * $p < 0.05$.

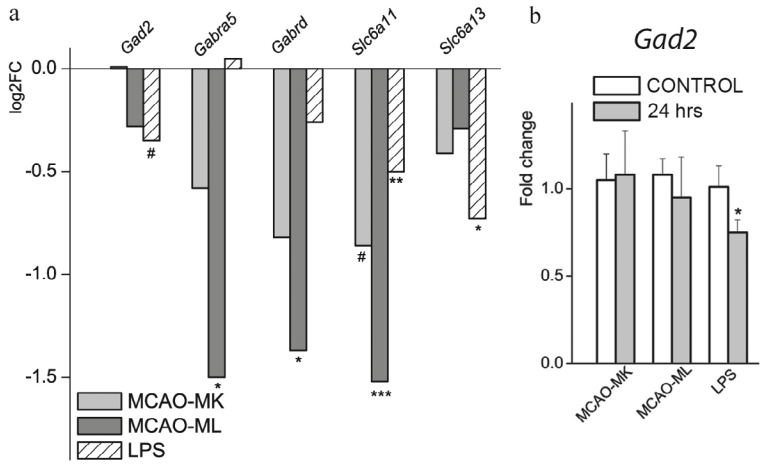

Fig. 4. Expression of the genes involved in GABAergic neurotransmission after two types of ischemic exposure and central administration of LPS. a) According to the results of sequencing; * padj < 0.05, ** padj 0.01, *** padj < 0.001, # padj < 0.1. b) Real-time PCR verification; * $p < 0.05$.

After MCAO-MK, only a decreasing trend (padj = = 0.0679) in the expression of *Grm3* encoding the metabotropic receptor was found. MCAO-ML resulted in the changes of expression of the genes of both ionotropic and metabotropic receptors within 24 h. According to the results of RNA-seq, there was a significant decrease in the expression of *Gria4*, subunit of the ionotropic AMPA receptors. Expression of *Gria2*, another AMPA receptor subunit, was found to be decreased according to the RNA sequencing data, but only insignificantly ($p = 0.0272$), however, the real-time PCR data demonstrate significant decrease in the expression. In contrast to the decrease of expression of the genes encoding subunits of the AMPA receptors, expression of the *Grin3b* gene, subunit of the ionotropic NMDA receptor, was shown to be significantly increased. In addition to the ionotropic AMPA receptors, significant decrease in the expression of metabotropic receptor Grm6 gene was also observed after MCAO-ML.

Expression of the *Gria4* gene encoding the AMPA receptor subunit decreased significantly (padj < 0.05) after MCAO-ML, and at the trend level (padj = 0.0531) after LPS administration. Expression of the *Grik3* gene encoding the subunit of kainate receptors was reduced also at the trend level (padj = 0.0734) after LPS administration. Decrease in the expression of *Grm2* gene encoding metabotropic receptor was significant after LPS administration.

Genes of the GABAergic system. Expression of the genes of GABAergic system are shown in Fig. 4a. After all treatments, expression of the *Slc6a11* gene decreased in the hippocampus: at the trend level (padj = 0.0932) after MCAO-MK and significantly (padj < 0.05) after MCAO-ML and LPS administration. Expression of the *Slc6a13* gene encoding another GABA transporter, as well as the *Gad2* gene encoding the GABA synthesis enzyme moderately decreased (padj = 0.0871) only after LPS exposure. The change in *Gad2* expression after LPS administration was confirmed by the results of real-time PCR (Fig. 4b). Expression of the GABAergic receptor genes, *Gabra5* and *Gabrd*, significantly decreased after MCAO-ML (Fig. 4a).

Genes of calcium/calmodulin-dependent protein kinases. Ca^{2+}/calmodulin-dependent kinases, or CaM kinases, play an important role in the activity of glutamatergic and GABAergic receptors. Expression of the *Camkk1*, *Camkk2*, *Calm1*, and *Camk4* genes decrease after MCAO-ML in the hippocampus according to the RNA-seq data (Fig. 5). MCAO-MK led to the decrease of expression of only one of these genes, *Camkk1*. Central LPS administration did not change expression of any genes encoding calcium/calmodulin-dependent protein kinases.

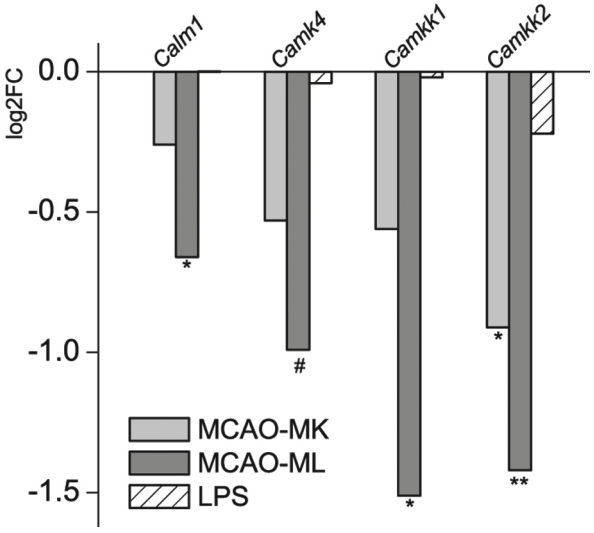

Fig. 5. Expression of the genes related to calcium/calmodulin-dependent protein kinases after two types of ischemic exposure and central administration of LPS (according to the RNA-seq results). * padj < 0.05, ** padj < 0.01, # padj < 0.1.

DISCUSSION

Previously we have suggested that cerebral ischemia can cause a secondary damage in such remote from the lesion site brain region as hippocampus through the mechanisms that still remain obscure. Delayed hippocampal damage that is linked to the development of post-ischemic pathology including cognitive impairment, was shown to be associated with glucocorticoid and inflammatory changes [26, 27]. Indeed, hippocampus seems to be specifically affected by the stress and inflammatory stimuli during post-ischemic period [3].

MCAO-ML and MCAO-MK are the most commonly used preclinical stroke models. However, meta-analysis of the few comparative studies of these models in rodents shows that the data about similarities and differences between these models are contradictory and depend on the species used (mice, rats), duration of ischemia, and presence or absence of reperfusion [28]. This applies to the data on the infarct volume, mortality, and neurological deficits. In our previous studies on these models with rats, we were not able to find significant differences in the volume of cerebral infarction assessed 72 h after MCAO by the standard staining with mitochondrial dye 2,3,5-triphenyl tetrazolium chloride (TTC) [20]. There were also no significant differences in the development of neurological deficits in the first 14 days after MCAO and in survival of animals [20, 23]. Absence of the differences in neurological deficits between the two models indirectly confirms similar volumes of cerebral infarction, since there is a direct relationship between these parameters [29]. Despite the fact that MCAO-ML and MCAO-MC modeling in rats limit the ischemic focus to the areas of the neocortex and striatum [20], it has been experimentally shown that there are significant differences in the binding of corticosterone in the hippocampus and the frontal cortex (outside the area of primary ischemic damage), not only in the acute period [20], but even 3 months after MCAO [23]. It is important to note that in the clinical prospective study, we were not able to show any association between the development of post-stroke cortisol-dependent cognitive and depressive disorders and neurological deficits (an indirect measure of the size of ischemic damage), which reflect development of the primary ischemic infarction in the region of the middle cerebral artery [30].

In the present study, signs of apoptosis such as increase in expression of the caspase-3 gene, a key protease for execution of the programmed cell death, were observed in the hippocampus already one day after MCAO-ML. These data confirm the previously reported manifestation of apoptosis during the first days after ischemia/reperfusion [19, 31, 32]. Death of neuronal cells leads to activation of inflammation, which, in turn, could additionally trigger the processes of apoptosis [33]. Therefore, it is not surprising that the direct exposure to a pro-inflammatory stimulus, bacterial endotoxin LPS, led to the increase in expression of not only the *Aif* gene, encoding the Iba-1 marker protein of activated microglia, but also of the *Fas* death receptor gene in the hippocampus one day after LPS administration.

Neuroinflammation is associated with activation of glial cells and increased production of pro-inflammatory cytokines. The most rapid activation after ischemia was noticed in the astrocytes: the two-hour MCAO in rats significantly increased the number of activated astrocytes in the hippocampus one day later, whereas no activation of microglial cells was detected at this time point yet [34]. Similarly, we observed increase in the expression of the *Gfap* gene, the astrocyte marker protein, but not of the *Aif* gene, in the hippocampus 24 h after both types of MCAO. Ischemia/reperfusion increases permeability of the blood–brain barrier leading to the entry of lymphocytes from the peripheral blood into the brain and enhanced production of pro-inflammatory cytokines [35]. Interestingly, after the MCAO by the Longa et al. method, an increase in gene expression of the matrix metalloproteinase-9 (*MMP9*), which is involved in disturbances of the barrier permeability [36], was accompanied by the more pronounced increase in the expression of pro-inflammatory genes than after the MCAO-MK. According to the sequencing data, gene expression of both key pro-inflammatory cytokines, *Il1b* and *Il6*, was significantly increased after MCAO-ML, while increase in the expression of only one of them, *Il1b*, was detected after MCAO-MK by PCR analysis. These data generally confirm the results of Smith et al. [18] who reported significantly more pronounced pro-inflammatory activation in the mice after MCAO-ML than after MCAO-MK.

Glutamatergic excitotoxicity associated with glutamate accumulation in the extracellular space and hyper-activation of glutamate receptors are regarded as main causes of ischemia-induced neurodegeneration [5, 37, 38], and therefore, attenuation of the glutamatergic transmission was suggested to be important for mitigating cell death [39]. Indeed, experimental induction of ischemia by the common MCAO method causes rapid, within the first minutes, increase in the level of extracellular glutamate in the hippocampus [40, 41]. However, the elevated level of extracellular glutamate is normalized quickly, apparently, via reuptake of the neurotransmitter through the cell membrane by specific transporters. Currently, five types of such carriers are known, which transfer the released glutamate to astrocytes, where it is converted into glutamine by glutamate synthase [42]. Then, the glutamine carrier proteins on the plasma membrane of astrocytes and neurons mediate transfer of glutamine from astrocytes to neurons, where it is converted back to glutamate by glutaminase and loaded by the vesicular glutamate transporters into the synaptic vesicles for further use. It is believed that more than

half of glutamate is formed as a result of such a glutamate-glutamine cycle between the neurons and astrocytes [43]. Despite the rapid normalization of the acute increase in the level of extracellular glutamate, cerebral ischemia could cause long-term changes in the regulators of glutamatergic neurotransmission, which, in turn, could result in the delayed changes in both activity of neurotransmission and related functions. MCAO in both models caused increase in the hippocampal gene expression of the neutral amino acid transporters, *Slc1a4* and *Slc1a5*, which likely indicates a change in the metabolism of glutamine and glutamate in the hippocampus after ischemia. Increase in the *Slc1a5* gene expression is consistent with the previously published changes observed after LPS administration and MCAO according to Koizumi et al. [16], and in the present study, this effect of ischemia was also confirmed in the Longa et al. MCAO model. The suggestion that the increased expression of neutral amino acid transporters is associated with the possible change in the metabolism of glutamine and glutamate is indirectly confirmed by the increase in the expression of the *Glul* (glutamine synthetase) gene together with the changes in the expression of both transporters after MCAO-MK. Direct pro-inflammatory stimulation by central administration of LPS resulted in the significant increase in expression of the *Slc1a2* gene encoding transporter for glutamate reuptake from the synaptic cleft, and decrease in expression of the vesicular transporter gene (*Slc17a6*). Gene expression levels of these types of transporters were not changed in either of the MCAO models.

The action of glutamate is mediated by ionotropic and metabotropic glutamate receptors, and gene expression of some of them was altered by the challenges applied. The changes were more pronounced after the MCAO-ML and LPS exposure with higher pro-inflammatory activation than after the MCAO-MK. Localization of the glutamate receptors on the glial cells [44] provides additional evidence for interaction of responses of the glutamatergic and pro-inflammatory systems. After MCAO-ML and LPS administration, but not after MCAO-MK, decrease in the expression of the *Gria4* gene, a subunit of the AMPA receptor, was observed in the hippocampus. These results are consistent with the decrease in expression of the AMPA receptor protein in the hippocampus of rats one day after the permanent MCAO, standard or aggravated by diabetes [19]. In this study, a difference was also found in the responses of glutamate receptors to the type of ischemic injury: no changes in the expression of the AMPA receptor protein in the hippocampus of rats were detected 24 h after transient ischemia.

Increase in the expression of the *Grin3b* gene, subunit of the NMDA receptors, was observed after MCAO-ML, but not after MCAO-MK or LPS administration. This effect after the MCAO-ML may reflect either development of post-traumatic stress disorder in animals [45] or, on the contrary, earlier activation of the recovery processes in this model. Regarding the second possibility, it should be noted that excitotoxic death of the neurons after ischemic injury is associated primarily with hyperactivation of NMDA receptors; however, clinical trials of antagonists of these receptors have shown not only improvement, but even aggravation of brain damage [10]. Unlike antagonists, treatment with agonists during the acute period after a stroke may be more "useful" for recovery through modulating of the "glycine site" in NMDA [11]. *Grin3b* mRNA is widely abundant in the adult rat brain [46], and combination of the NR1/NR3B subunits (Grin1/Grin3b) may represent a type of the excitatory glycine receptor [47]. However, elucidation of the role of the Grin3b subunit of NMDA receptors in the post-ischemic period requires special investigation.

All three experimental approaches used in the present study induced decrease in the expression of metabotropic receptor genes. After MCAO-MK expression of the *Grm3* gene changed, after MCAO-ML – of the *Grm6*, and after LPS – of the *Grm2*. Proteins encoded by the *Grm3* and *Grm2* genes belong to the second group of metabotropic receptors, *Grm6* – to the third group. Stimulation of metabotropic receptors of the third group expressed on microglial cells shifts microglia to a neuroprotective phenotype, while stimulation of the receptors of the second group, specifically mGluR2 (Grm2), – to a neurotoxic phenotype, releasing the Fas-ligand and triggering apoptosis via activation of caspase-3 [45]. The Grm2 knockout mice showed a smaller lesion volume and accelerated behavioral recovery after MCAO [48]. Different effects of MCAO-MK and MCAO-ML on the expression of components of the glutamatergic system may be associated with different accumulation of corticosterone in the hippocampus [20, 23], since glucocorticoid hormones control most components of this system [49] and also may induce the development of hyperglutamatergic transmission [50] leading to excitotoxicity.

In addition to the enhancement of glutamatergic signal, disruption of the inhibitory action of GABA on neuronal excitability contributes to the deteriorating effects of ischemia [51]. The experimental models used in our study affected expression of the genes of GABAergic system markers. Common response to all exposures was decrease in the expression of the GABA transporter gene *Slc6a11*. MCAO-ML, specifically, reduced the expression of two GABAergic receptor genes, *Gabra5* and *Gabrd*.

Although the clinical trials of drugs targeting NMDA receptors as neuroprotective agents have failed, there is some evidence of the possibility to alleviate their negative side effects through the influence on the signaling cascade downstream of the receptor stimulation [52]. Another promising route may involve modulating the GABAergic receptor signaling pathways and regulators

Levels of gene expression in the hippocampus after MCAO-MK, MCAO-ML, and central administration of LPS by adjusted *p*-value (padj)

Process	Gene	MCAO-MK	MCAO-ML	LPS
Apoptosis	*Fas*	≈	≈	↑**
	Casp1	≈	≈	↑**
	Casp3	≈	↑***	≈
	Casp4	↑***	↑***	↑**
	Casp8	≈	≈	↑#
Neuroinflammation	*Gfap*	↑***	↑***	↑**
	Aif (IBA1)	≈	≈	↑**
	Il1b	≈	↑*	↑**
	Il6	≈	↑***	≈
	MMP9	≈	↑***	↑**
Glutamatergic system	***Slc1a2***	≈	≈	↑**
	Slc17a6	≈	≈	↓**
	Slc1a4	↑***	≈	↑**
	Slc1a5	↑**	↑***	↑**
	Slc38a5	↓**	≈	≈
	Glul	↑#	≈	≈
	Grin3b	≈	↑***	≈
	Gria2	≈	↓#	≈
	Gria4	≈	↓*	↓#
	Grik3	≈	≈	↓#
	Grm2	≈	≈	↓**
	Grm3	↓#	≈	≈
	Grm6	≈	↓*	≈
GABAergic system	***Gad2***	≈	≈	↓#
	Gabra5	≈	↓*	≈
	Gabrd	≈	↓*	≈
	Slc6a11	↓#	↓***	↓**
	Slc6a13	≈	≈	↓*
CaM kinases	*Calm1*	≈	↓*	≈
	Camk4	≈	↓#	≈
	Camkk1	≈	↓*	≈
	Camkk2	↓*	↓**	≈

Designations. ↑↓, Significant increase or decrease of gene expression; ≈, absence of significant changes of gene expression; * padj < 0.05, ** padj < 0.01, *** padj < 0.001, # padj < 0.1.

Genes with expression verified by real-time PCR are marked in bold.

of synaptic plasticity, which include, in particular, the protein products of the *Camkk1* and *Camkk2* genes that change expression after MCAO-ML. In the recent study, it was shown that restoration of the GABA receptors expression reduced by ischemia by blocking the mechanism of their interaction with CaMKII phosphorylated by Camkk1 and Camkk2 inhibited excitotoxic death of neurons [53]; this can significantly expand pharmacological possibilities to inhibit progressive neuronal death after ischemic stroke.

CONCLUSION

In general, the obtained results show both differences and similarities between the gene responses to the influences used in the present study (table). Many more genes associated with activation of apoptosis and neuroinflammation, glutamate reception, and markers of the GABAergic system changed their expression after the MCAO-ML and LPS administration than after the MCAO-MK. Compared with MCAO-ML, MCAO-MK and LPS induced changes in the expression of a larger number of genes involved in glutamate transport. The most pronounced difference between the responses was the changes in the expression of calmodulin and calmodulin-dependent kinases genes that were observed after MCAO, especially MCAO-ML, but not after LPS administration. The revealed specific features of the hippocampal gene expression responses to two types of MCAO and to a pro-inflammatory stimulus can contribute to further understanding of the molecular mechanisms underlying diversity of post-stroke consequences, both in the model studies and in the clinic.

Limitation. The main aim of our study was to study early responses of the hippocampal gene expression to the occlusion of middle cerebral artery using two MCAO models. For this purpose, the hippocampi were isolated 24 h after ischemia or sham surgery. Design of the experiment did not allow direct assessment of the infarct volume in the experimental animals and intergroup comparisons. However, the scores of neurological deficits may be used as an indirect confirmation of the cerebral infarction. The data of neurological deficit scores correlate with the ischemic volume as has been shown previously [29].

Contributions. Project conceptualization and management: G.T.S. and N.V.G.; Research: T.S.K., D.A.L., E.V.S., M.V.O., Y.V.M.; Discussion and original draft preparation: N.V.G., G.T.S., T.S.K., N.N.D; Writing the paper: G.T.S.; Review and editing: N.V.G., T.S.K., N.N.D.

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