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Expression of NMDA Neuroreceptors in Experimental Ischemia

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Abstract—The role of NMDA receptors in molecular mechanisms of neurotoxicity was investigated using rat models of global and focal cerebral ischemia. The expression of NR2A and NR2B receptor subtypes increased 3 h after reperfusion in the basin of the middle cerebral artery (MCA). This effect was accompanied by an increase in NR2A and NR2B immunoreactivity in cerebral cortex of rats with focal ischemia. Six hours after reperfusion we observed drastic transient activation of the expression of NR2A receptor subunit in the penumbra that returned to the control level 24 h post reperfusion. The monitoring of NR2A autoantibodies in the blood of the experimental rats showed a reliable increase in their content on the 5-6th day of reperfusion that remained elevated even on the 20th day of the experiment. The data indicate that NR2A receptor subunit and NR2A autoantibodies are biochemical markers of the developing neurotoxicity that represents one of the mechanisms of cerebral ischemia.

Key words: neurotoxicity, NR2-NMDA receptors, RT-PCR, autoantibodies

NMDA receptors constitute 80% of all receptors of excitation. These receptors are heteromeric pentamers or tetramers of NR1 and NR2 subunits that form functionally active channels. The NR2 subunit consists of NR2A, NR2B, NR2C, and NR2D that are responsible for membrane permeability for calcium ions and regulation of neurotoxicity. The latter forms the basis for cerebral ischemia [1, 2]. It was shown by hybridization *in situ* that NR1 mRNA is present in almost all brain structures, while NR2 mRNA is distributed through the local regions of the forebrain [3].

The alteration of expression of the crucial factors that regulate metabolism in the affected cerebral region leads to the imbalance between excitation and inhibition processes, involvement of the neuroreceptive mechanisms in cooperation of cellular interactions in cerebral tissue and to the alteration of blood–brain barrier (BBB) function. Receptor fragments penetrate through the affected BBB in the blood stream and activate the immune system that produces antibodies to the host cerebral antigens [4].

Massive release of glutamate and aspartate during alteration of cerebral circulation activates NMDA receptors [4-6] and leads to the increase of concentration of intracellular Ca^{2+} [7]. The pathologic increase of calcium concentration induces irreversible changes, such as edema, lysis, and finally death of a neuron [1].

It is suggested that NMDA receptors are the main regulators of the post-ischemic increase of permeability for Ca^{2+}. This hypothesis is also confirmed by the following data: NMDA-activated mechanism of neurotoxicity in neurons in culture depends on the concentration of the extracellular Ca^{2+}; the preceding application of antagonists of NMDA receptors blocks the increased permeability for these ions [8].

The elicitation of the succession of the molecular events occurring in cerebral tissue under the toxic effect of glutamate and aspartate that lead to cerebral ischemia allows, on one hand, for early diagnostics of cerebral thrombosis, and, on the other hand, for determination of the strategy of brain neuroprotection during alteration of circulation in brain [9].

The results of modulation of global ischemia in experimental animals are contradictory: the expression of NR2A subunit in CA1 neurons decreases [10], increases [11], or does not change [12]. Thus, it is interesting to determine what crucial changes occur in the infarcted cerebral regions during ischemia and whether they have any effect on the systematic level.

To study the role of NMDA receptors in molecular mechanisms of neurotoxicity we used the models of global and focal cerebral ischemia in rats. We monitored the

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variations in expression, immunoreactivity of NR2 subunits of NMDA receptors, and the dynamics of accumulation of NR2A autoantibodies in blood of the experimental animals.

MATERIALS AND METHODS

Experimental animals. The experiments were carried out on male rats of Wistar line ($n = 24$, 350 ± 15 g). The rats were anesthetized by intraperitoneal injection of chloral hydrate (0.4 g/kg body weight), the right MCA and the common carotid arteries (*arteriae carotis communis*, ACC) were tied with 10-0 surgical suture [13]. Ligatures were taken off from ACC 30 min after reperfusion. The global ischemia was induced in animals ($n = 8$) by applying a ligature on the right carotid artery for up to one month. Sham-operated animals ($n = 6$) were used as a control. The arterial blood pressure was measured during operation and 8 h of reperfusion by femoral catheter (PE-50, Dural Plastics and Engineering, USA) together with rectal temperature that was maintained at 37°C. After rehabilitation after anesthesia the animals were put back into the cages for the restoration of circulation in the ischemic cerebral region. The animals had free access to water and standard food. Animals that did not perform the neurological deficit in the test of Bederson et al. [14] were selected.

Measurement of the infarcted region. The animals with MCA occlusion were anesthetized and perfused 24 h after the end of operation. Then the brain was isolated and incubated 5 min in 0.9% physiologic solution. The chilled brain was cut into 2.0 mm-sections that were stained with 2% triphenyltetrazolium chloride (TTC) for 30 min at 37°C and fixed in 5% formaldehyde solution. The region of infarct was measured in each section using a digital camera (Fuji, Japan).

RT-PCR analysis. The animals with MCA occlusion were decapitated after 0, 3, 6, and 24 h of reperfusion. The brain was put on ice. Samples weighting 20-30 mg were taken from the cerebral cortex in the region of infarct and adjacent to infarct (penumbra); the control samples of cortex were taken from the left hemisphere. The samples were homogenized and stored at −70°C.

Total RNA was isolated from cerebral samples using an RNAgents kit (Promega, USA) by a published method [15]. Reverse transcription (RT) was carried out as follows: 33.5 μl of RNA (10 μg) was heated to 55°C for 10 min, chilled to 4°C, and then 1 μl of oligo-dT was added. The reaction mixture was heated to 42°C, then master buffer containing 5 μl of 10× buffer, 5 μl of $MgCl_2$ (12.5 mmol), 2 μl of DTT, 2.5 μl of dNTPs (10 mmol), and 1 μl of reverse transcriptase was added and the reaction was carried out for 50 min. The primers used in polymerase chain reaction (PCR) (table) were specific to N-terminal fragments of NR2A, NR2B, NR2C, and NR2D receptor subunits [10, 11]. One microliter (100 nmol) of cDNA was mixed in sterile tubes with 5 μl of 10× buffer, 1.25 μl of corresponding primer pair (40 nmol), 0.25 μl of Taq DNA polymerase, 1 μl of dNTPs, 0.5 μl of 2.5 μCi [^{32}P]α-dCTP, and 39.75 μl of DNAse/RNAse-free water to the final volume of the reaction mixture 50 μl. The tubes were incubated at 48°C for 45 min, then 2 min at 94°C and 40 cycles (94°C for 30 sec, 60°C for 1 min, 68°C for 2 min) in the programmed termocycler DNA Engine PTC-200 Peltier (DNA Research, USA). Amplified products (5 μg) were analyzed by electrophoresis in 7.5% polyacrylamide gel and stained with ethidium bromide. The gels were dried and radioautographed at −20°C for 15 days. The DNA bands were scanned in a Fuji Imager (Fuji, Japan) using the Tina program.

All PCR reactions were carried out in the exponential phase of amplification. The alteration of NR2 recep-

Sequences of the primers for RT-PCR

NMDA receptor	Primer		Reference
NR2A	Sense:	5'-GCGCGCAGCACGCCCCATTGCATCC-3'	[10]
	Antisense:	5'-GGGCCACAGCCTCCTGGTCCGTGTCA-3'	
NR2B	Sense:	5'-CCCAGCATCGGCATCGCTGTGATCCTC-3'	[10]
	Antisense:	5'-CATGATGTTGAGCATGACGGAAGCTTG-3'	
NR2C	Sense:	5'-CTGGACCTGCCTCTGGAGATCCAGCCA-3'	[11]
	Antisense:	5'-GCGGTCCGCGACGGCGCGCACGCC-3'	
NR2D	Sense:	5'-GCGGCAGAGGCGGCGCGCTTGGGCCC-3'	[11]
	Antisense:	5'-GCCTGGGGCACGCGTGGTCACTGCCAC-3'	

tor expression (320-400 bp) was assessed in comparison to the expression of a standard, β-actin (540 bp, sense 5′-TGT GAT GGT GGG AAT GGG TCA G-3′; antisense: 5′-TTT GAT GTC ACG CAC GAT TTC C-3′). β-Actin expression was stable in all RNA samples.

Western-blotting analysis. The protein fraction was isolated from phenol–ethanol supernatant obtained after RNA precipitation from cerebral cortex of rats with MCA occlusion (6 h of reperfusion). The proteins were precipitated by addition of 500 μl of isopropanol. The mixtures were incubated for 10 min at 25°C and then centrifuged for 10 min at 4°C and 12,000*g*. The protein pellets were washed from phenol with 95% ethanol with 0.3 M guanidine hydrochloride for 20 min at 25°C and centrifuged 5 min at 7500*g*. Proteins were diluted in 1% SDS (1 : 1 w/v) and protein content was measured by the modified micromethod of Lowry [16].

The resulting proteins were separated in 10% SDS-polyacrylamide gel and transferred to Hybond-C nitrocellulose membrane (Amersham, USA) using a TE 70 SemiPhor apparatus (Pharmacia Biotech, Sweden). After washing in TTBS buffer (Tris-buffer containing 0.05% Tween 20, 100 mM Tris, 0.9% NaCl), the membranes were incubated in 5% BLOTTO and then with antibodies (0.15 mg/ml) to NR2A/B, NR2C, NR2D (Chemicon, USA) for 1 h at 25°C in TTBS. The membranes were washed in TTBS and incubated with rabbit antibodies (1 : 20,000) labeled with horseradish peroxidase (Sigma, USA) for 1 h. After washing in TTBS buffer the protein bands were stained with 3,3′-diaminobenzidine tetrahydrochloride (Sigma Fast™, USA).

Selection and analysis of blood samples. Blood samples (1.5 ml) were taken from *vena caudata* of the experimental animals on the 0th, 3rd, 6th, 12th, 18th, 24th days of reperfusion. Serum was obtained by centrifugation for 5 min at 4°C at 4000*g*. The samples were stored at –70°C.

A peptide fragment of NR2A subunit of NMDA receptor (21 amino acid residues) was used as an antigen for the analysis of the level of autoantibodies in blood. This peptide was synthesized by the solid-phase method on an NPS-400 semi-automatic synthesizer (Neosystem Laboratory, France) and was purified by reverse-phase HPLC on a C18 column [17].

The concentration of NR2A autoantibodies was measured in blood serum samples by the ELISA method [18]. Diluted blood serum (1 : 20, 100 μl) and NR2A polyclonal antibodies (0.01-400 ng/ml, Chemicon) were applied on the 96-well immunological plate containing NR2A peptide (1 μg/well). The plate was incubated 1 h at 25°C and washed with PBS containing 0.05% Tween 20, pH 7.4. Peroxidase-labeled rabbit antibodies (1 : 1000, Sigma) were added to each well and incubated for 1 h at 25°C. The plate was washed for 15 min with PBS and then 100 μl of the substrate solution (tetramethylbenzidine dichloride, TMB, Sigma) was added to each well. The reaction was terminated by addition of 2 N H_2SO_4, and the plate was scanned at 450 nm on a Microplate Reader (Bio-Rad, USA). The concentration of autoantibodies was calculated using a standard curve for NR2A antibodies.

Statistical analysis was carried out by the method of Student using the level of significance $p < 0.05$ that is common for the majority of biomedical studies.

RESULTS

The growing number of studies devoted to the investigation of the mechanisms of cellular destruction in global and focal ischemia provide evidence for activation of glutamate receptors, in particular of NR2-NMDA receptors that are sensitive to Ca^{2+}-cellular permeability. Therefore, it was interesting to determine what subunit of the NR2 complex is the most sensitive to the early manifestations of neurotoxicity during ischemia.

In the preliminary experiments, we used the model of global ischemia induced by tying of the right carotid artery that leads to infarct with significant loss of neurons in the cortex of right and left cerebral hemispheres [19]. An elevated level of autoantibodies to NR2A subtype of NMDA receptors in comparison to the control was found in the blood serum of the experimental animas to the 6th day of reperfusion with lowering tendency to the 24th day of the experiment (Fig. 1). Staining of samples of the cerebral cortex of rats with induced global ischemia using autoantibodies that were isolated from blood allowed for the isolation of a protein band with molecular weight 190 kD that corresponds to NMDA receptor complex (Fig. 2).

To study the involvement of NR2-NMDA receptors in the molecular mechanisms of neurotoxicity in details,

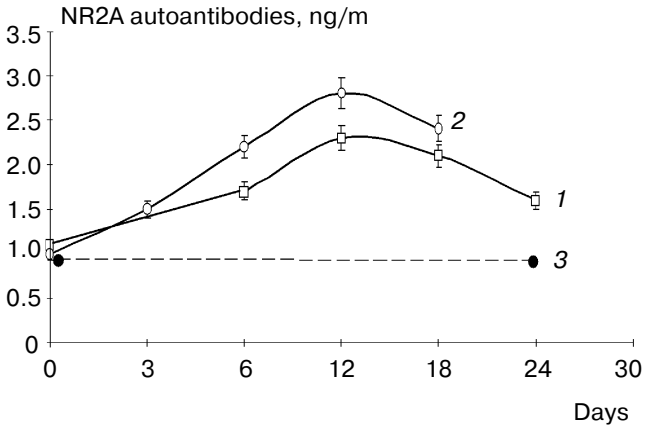

Fig. 1. Measurement of NR2A autoantibodies in blood serum of rats with global (*1*) and focal (*2*) ischemia in comparison to control animals (dotted line).

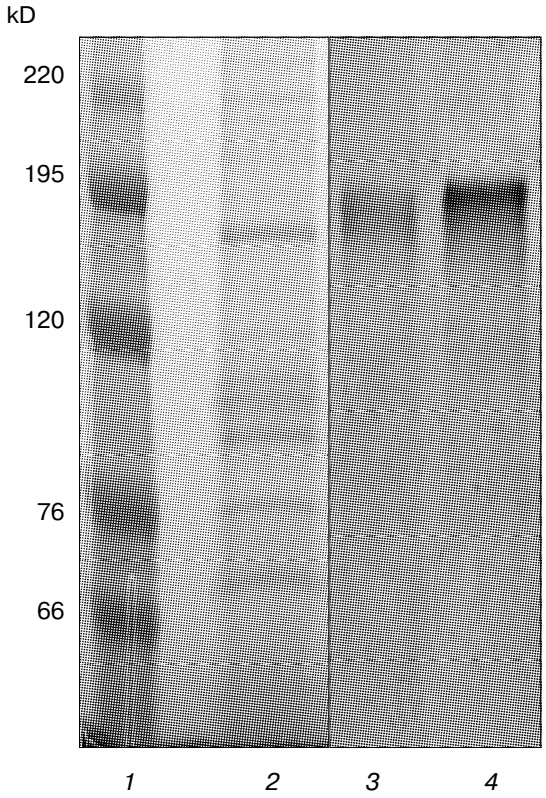

Fig. 2. Electrophoresis in 10% SDS-polyacrylamide gel and Western blot of synaptic membranes from the cerebral cortex of rats using antibodies isolated from blood serum of rats with global ischemia: *1*) standard; *2*) electrophoresis of membranes solubilized in 1% SDS and stained with Coomassie; *3*) cortical samples of the control animals, region of infarct; *4*) cortical samples from the region of infarct.

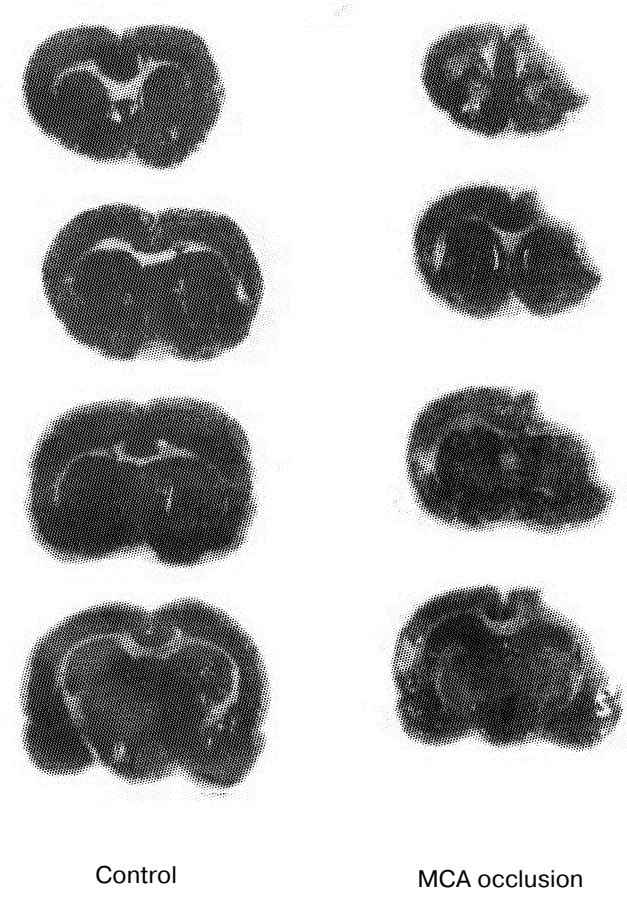

Fig. 3. Triphenyltetrazolium chloride (TTC)-stained cerebral sections from the control rats and rats with MCA occlusion.

we used a well-known model of MCA occlusion. The latter resulted in the local infarct of the cerebral cortex of the right hemisphere in all operated animals ($n = 24$). The volume of infarct after TTC staining of the 2-mm frontal cerebral sections (Fig. 3) was 102.9 ± 12.1 mm^3 [20]. In addition, MCA tying led to insignificant cerebral edema that was formed on the borders of the infarct. This region represented 7.5 ± 0.3% of the whole infarct region. None of the operated animals had symptoms of neurological deficit in the form of left-side hemiparesis and motor disorders that are typical for the functional alterations of the sensory motor cortex and the basal ganglia of the right hemisphere [21].

The alteration of expression of NR2 subunits in the cortex 3, 6, and 24 h after reperfusion was studied by quantitative RT-PCR. Comparison of expression of NR2A, NR2B, NR2C, and NR2D revealed that the increase in NR2A synthesis starts during the first hours of reperfusion both in the cortex regions affected by infarct and in the penumbra, healthy tissue adjacent to the

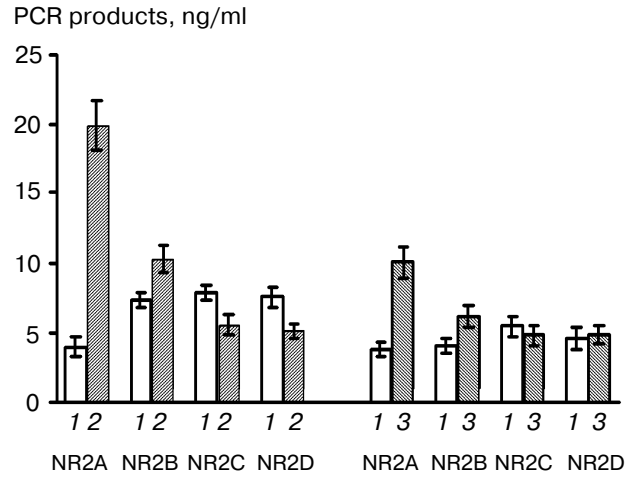

Fig. 4. Expression of NR2-NMDA receptors in cerebral cortex samples from rats with MCA occlusion: *1*) control (healthy cortex); *2*) region of infarct; *3*) penumbra region.

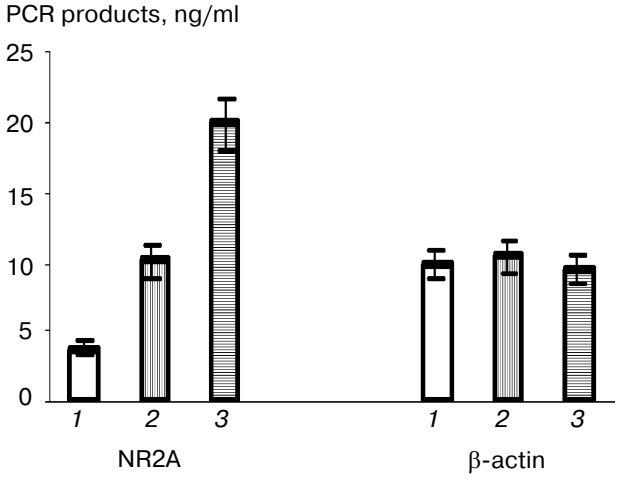

Fig. 5. Quantitative analysis of expression of NR2A subunit in cerebral cortex of rats with focal ischemia: *1*) control (healthy cortex); *2*) region of infarct; *3*) penumbra.

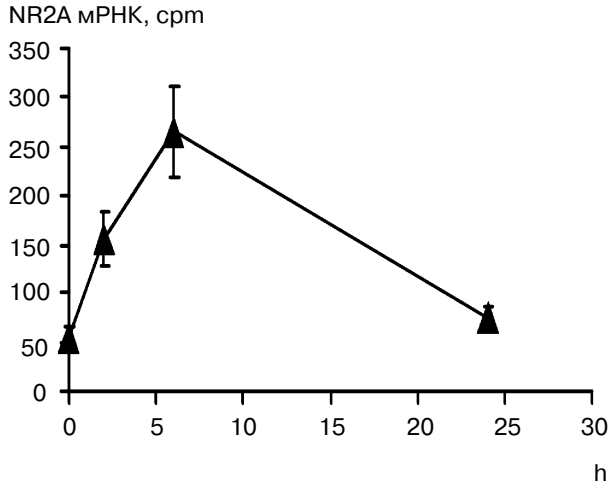

Fig. 6. Dynamics of NR2A mRNA expression in the region of infarct in cortex of rats with focal ischemia.

infarct border (Fig. 4). NR2A expression was 100% higher in the infarct zone and almost 4-fold higher in penumbra. Reliable (in comparison to the control) increase of NR2B synthesis (40 and 47%, respectively) was observed 6 h after operation, while NR2C(D) synthesis varied insignificantly during all the studied time periods and in all cortex regions.

Figure 5 presents the results of the quantitative expression of mRNA coding for NR2A fragment and of β-actin mRNA (standard, the structural cytoskeleton component in infarcted, penumbra, and healthy cerebral cortex zones). The maximal NR2A expression was observed in penumbra, while the expression of the standard was stable in all analyzed samples. Monitoring of the level of NR2A mRNA during the following 24 h after operation showed the maximal values 6 h after the formation of the infarct region with lowering to the normal level at 24 h of reperfusion (Fig. 6).

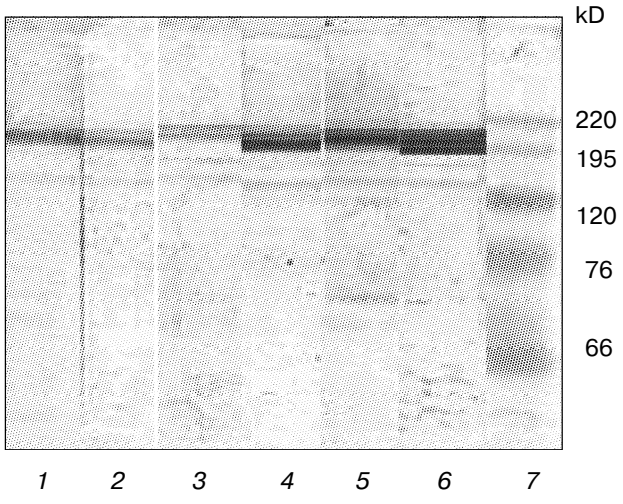

Fig. 7. Western blotting of solubilized synaptic membranes isolated from penumbra and the region of infarct with commercial polyclonal antibodies to subunits of NMDA receptors: *1*, *4*) NR2D; *2*, *5*) NR2C; *3*, *6*) NR2A/B; *7*) standard.

The Western-blotting method with commercial polyclonal antibodies was used to study the immunoreactivity of NR2A/B, NR2C, and NR2D subunits of NMDA receptor in the cortex of rats with induced focal ischemia. This method allows for the estimation of the relative contents of these subunits. Increased immunoreactivity of all analyzed subunits was found in experimental animals at the end of the first week of reperfusion in penumbra and infarct-affected regions in comparison to the healthy cortex regions isolated from the left hemisphere (Fig. 7). NR2A/B fragment showed elevated immunoreactivity (2-fold higher) in comparison to NR2C and NR2D subtypes.

The profile of accumulation of autoantibodies to the peptide fragment of NR2A in rats with focal ischemia was analogous to that of the animals with global ischemia (Fig. 1). It was shown that the level of autoantibodies in the blood of experimental rats reliably exceeded the control level starting from the 6th day of reperfusion. These levels were maximal on the 14th day and lowered to the 18th day of the experiment.

The results of the experiments confirm our suggestion that in the early stages of ischemia alteration of synthesis and immunoreactivity of NR2A occurs, and NR2A is the most sensitive to neurotoxicity and neuronal damage component of NR2-NMDA receptors. The frag-

ments of destroyed receptors penetrate through the BBB and activate the immune system that produces antibodies. Thus, NR2A subtype of glutamate receptors may be used as a marker of neurotoxicity, and autoantibodies to NR2A reflect the processes that occur in brain during cerebral ischemia.

DISCUSSION

Today much attention is given to the potential role of NMDA receptors in neurodegenerative processes, the consequences of cerebral ischemia and developing on its background neurotoxicity. Glutamate receptors and, in particular, the receptors of NR2-NMDA subtype regulate intracellular Ca^{2+}-flows. Hence, changes of properties of these receptors and of their quantity might lead to the alteration of the intracellular concentrations of calcium ions in cortex neurons. The current investigation was devoted to the study of the molecular changes of NR2 receptor subtype on the early stages of ischemia (less than 24 h) using the models of global and focal cerebral ischemia.

However, the control of the development of the affected cerebral region and of the homogeneity of manifestations of infarct from one animal to another is difficult during modulation of global ischemia. Therefore, we used further a model of focal ischemia induced by MCA tying. This model provides a local region of infarct in the cortex of the left hemisphere with controlled volume of infarct and the minimal manifestation of cerebral edema [13]. In addition, this model provides a possibility to obtain an early form of infarct in rats, in which the symptoms of changes in motor activity are not yet developing.

The principal results obtained in this study are as follows: 1) we observed increased expression of NR2A subtype of NR2-NMDA receptors, in particular in penumbra; 2) we simultaneously demonstrated the increase in immunoreactivity of NR2A/B protein; 3) altered expression and immunoreactivity of NR2A led to high concentrations of autoantibodies to NR2A in blood of the experimental animals.

Interestingly, we showed in this study that the expression of NR2A subunit of NMDA receptor is elevated in penumbra, where energetic metabolism is conserved and in which ischemia activates repair and adaptive processes. Perhaps the activated expression of this subunit is directed towards the regeneration of neuronal membranes that are affected by neurotoxicity.

The expression of NR2A and NR2B mRNAs is closely associated with alteration of immunoreactivity of the cortical proteins encoded by these mRNAs during ischemia. Significant increase in the expression during 3-6 h of reperfusion was accompanied by the increase in quantity of NR2A/B proteins detected in cortical regions of infarct and its penumbra. It appears that this fact reflects the time that is needed for the formation of the "therapeutic window", when cerebral functions can be maintained by immediate neuroprotective therapy [9].

The analogous association between NR2A and NR2B mRNA expression and immunoreactivity of the corresponding proteins was demonstrated in hippocampus of rats with global ischemia [10]. However, the authors of this study observed the decreased expression of corresponding NR2A and NR2B mRNAs and their immunoreactivities in hippocampus. The possible redistribution of activation of synthesis of NR2A/B receptor subunits occurs between the cortex and hippocampus activating the expression in the regions adjacent to infarct and decreasing or not altering it in hippocampus [12].

The elevated expression of NR2A mRNA and NR2A immunoreactivity in its turn lead to the increase in concentration of NR2A autoantibodies in blood of rats with ischemia. The accumulation of high concentrations of autoantibodies in blood is a sign of the processes of receptor destruction in cerebral cortex and may reflect the volume of the infarcted region. It should be noted that the concentrations of autoantibodies were higher in blood of rats with focal ischemia than in animals with vast infarct. We observed a similar difference in the levels of NR2A autoantibodies earlier in patients with transient alteration of cerebral circulation and vast cerebral ischemic thrombosis [6, 22].

Thus, the results of our studies indicate the primary dysfunction of NMDA receptors in brain affected by cerebral ischemia. It is shown that NR2A receptors are crucial markers of neurotoxicity developing under the influence of ischemia, and autoantibodies to NR2A are the witnesses of the processes occurring in the ischemic brain. The data presented here allow for a broader vision of the molecular mechanisms of ischemia occurring under active interaction of immune and nerve systems.

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