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Expression of NMDA Neuroreceptors in Experimental Ischemia

M. U. Gappoeva*, G. A. Izykenova, O. K. Granstrem, and S. A. Dambinova

Department of Neurology and Neurosurgery, Pavlov State Medical University, ul. Tolstogo 6/8 , St. Petersburg, Russia; E-mail: gappoeva@mail.ru; gizyken@aol.com

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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. Expression of NR2A and NR2B receptor mRNAs up-regulated in cortex after 3 h of reperfusion following middle cerebral artery occlusion (MCAo). This effect was accompanied by an increase in NR2A and NR2B immunoreactivity. At six hours of reperfusion, drastic activation of NR2A mRNA expression was observed in the penumbra that returned to the control level at 24 h of reperfusion. The monitoring of NR2A autoantibodies in the blood of the experimental rats showed its reliable increase to the 5-6th day of reperfusion that maintained elevated to the 20th day of the experiment. The data indicate that NR2A and 2B receptor subunits and NR2A autoantibodies are biochemical markers of the neurotoxicity underlying cerebral ischemia.

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

NMDA receptors make up 80% of all excitatory receptors. These receptors are heteromeric pentamers or tetramers of NR1 and NR2 subunits that form functionally active channels. There are four NR2 subunits: NR2A, NR2B, NR2C, and NR2D that are responsible for regulation of Ca^{2+}-permeability and neurotoxicity underlying cerebral ischemia [1, 2]. It was shown by *in situ* hybridization that NR1 mRNA is present in almost all brain structures, while NR2 mRNAs are distributed through the local regions of the forebrain [3].

The alteration of expression of the key factors regulating metabolism in the ischemia-affected brain region leads to the imbalance of excitation–inhibition processes, to involvement of the neuroreceptive mechanisms in brain cell interactions and compromises blood–brain barrier (BBB) increasing its permeability. In addition, thrombin activated serine proteases can cause the cleavage of synaptic NMDA receptors. As a result, peptides from cleavage of NMDA receptors may enter the bloodstream at increased levels and activate the immune system generating autoantibodies [4].

Massive release of glutamate and aspartate due to cerebrovascular deficit activates NMDA receptors [4-6] and leads to the increase in intracellular Ca^{2+} [7]. Abnormally increased calcium concentration induces irreversible changes, such as edema, lysis, and finally neuronal death [1].

NMDA receptors are thought to be a major contributor to the post-ischemic elevation of Ca^{2+} permeability. This is supported by the observation that NMDA-triggered toxicity in neuronal cultures is dependent on the presence of extracellular Ca^{2+} because prior application of neuroprotective NMDA receptor antagonists block NMDA triggered increase in Ca^{2+} permeability [8].

The study of molecular events occurring in cerebral tissue under the toxic effect of glutamate and aspartate leading to cerebral ischemia allows, on one hand, for early diagnosis of cerebral insults, and, on the other hand, for choosing the strategy of brain neuroprotection during cerebrovascular deficit [9].

The results of modulation of global ischemia in experimental animals are contradictory: the expression of NR2A mRNA in CA1 neurons decreases [10], increases [11], or does not change [12]. Therefore, it is interesting to determine what crucial changes occur in the infarction-damaged brain regions and how that reflects the brain damage–immune system response.

To study the role of NMDA receptors in molecular mechanisms of neurotoxicity we used the models of global and focal cerebral ischemia in rats. The alterations in expression, immunoreactivity of NR2 subunits of NMDA neuroreceptors, and the dynamics of NR2A autoantibody

* To whom correspondence should be addressed.

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accumulation in blood of the experimental animals were investigated.

MATERIALS AND METHODS

Experimental animals. Male Wistar rats ($n = 24$, 350 ± 15 g) were used in the experiments. The rats were anesthetized by intraperitoneal injection of chloral hydrate (0.4 g/kg body weight), the right MCA and the common carotid arteries (CCA) were ligated with 10-0 surgical suture [13]. After 30 min of ischemia, the sutures were removed from the CCA to allow 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 group. The arterial blood pressure was measured during surgery and 8 h of following reperfusion through a femoral catheter (PE-50, Dural Plastics and Engineering, USA). Rectal temperature was maintained at 37°C throughout the surgical procedure. After recovery from anesthesia the animals were returned to their home cages to allow reperfusion of blood to the ischemic brain area. The animals had free access to water and food. Animals that did not show neurological deficit in the behavioral test of Bederson et al. [14] were selected.

Measurement of the infarction area. After 24 h of reperfusion rats with MCAo ($n = 6$) were anesthetized and perfused +4°C neutral buffered saline. Brains were removed and fixed at 10% paraformaldehyde. The chilled brain was cut into 2.0 mm-slices and was incubated with 2% triphenyltetrazolium chloride (TTC) for 30 min at 37°C. The area of infarction in each slice was measured with a digital scanner (Fuji, Japan).

RT-PCR analysis. The animals with MCA occlusion were decapitated after 0, 3, 6, and 24 h of reperfusion. The frozen brains were sectioned in the coronal plane in a cryostat at −18°C into 700 μm serial sections. Samples (20-30 mg) of infarction-damaged cortex, adjacent penumbra, and control cortical samples from the left undamaged hemisphere were microdissected and stored at −70°C.

Total RNA was isolated from the cerebral samples using an RNAgents kit (Promega, USA) according to method [15]. Reverse transcription (RT) reaction of total RNA was carried out as follows: 33.5 μl of RNA (10 μg) was incubated at 55°C for 10 min, chilled to 4°C, and 1 μl of oligo-dT was added. The reaction mixture was heated to 42°C, and 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 mixture was incubated for 50 min. The primers specific to N-terminal fragments of NR2A, NR2B, NR2C, and NR2D receptor subunits were used in polymerase chain reaction (PCR) (table) [10, 11]. PCR reactions were performed in sterile tubes in final volume 50 μl containing 100 nmol of cDNA, 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. The mixture was incubated at 48°C for 45 min, then 2 min at 94°C with following 40 cycles (94°C for 30 sec, 60°C for 1 min, 68°C for 2 min) using a programmable 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 Tina software.

All PCR reactions were performed in the exponential phase of amplification. The alteration of NR2 cDNA expression (320-400 bp) was assessed by comparison with that for a standard, β-actin (540 bp, sense 5′-TGT GAT GGT GGG AAT GGG TCA G-3′; antisense: 5′-TTT

Sequences of the primers used 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'	

GAT GTC ACG CAC GAT TTC C-3′). The expression of β-actin cDNA 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 protein was precipitated by 500 μl of isopropanol. The mixture was incubated for 10 min at 25°C and then centrifuged for 10 min at 4°C and 12,000 *g*. Then protein pellets were washed from phenol with 95% ethanol containing 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 according to 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 Transfer Unit (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 rinsing in TTBS buffer the protein bands were developed using 3,3′-diaminobenzidine tetrahydrochloride (Sigma Fast^{TM}).

Blood sample withdrawal and analysis. Blood samples (1.5 ml) were withdrawn 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 until analyzed.

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 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 Student's method at 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 damage in global and focal ischemia provide evidence about the 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 permanent occlusion of the right carotid artery that resulted in a clear-cut infarction with significant loss of neurons in the cortex of both hemispheres [19]. An elevated level of autoantibodies to NR2A subtype of NMDA receptors was detected in serum of the experimental animals to the 6th day of reperfusion in comparison to that for control which tended to decrease to the 24th day of the experiment (Fig. 1). The immunostaining of cortical samples from rats with global ischemia using autoantibodies isolated from blood revealed a protein band with molecular weight 190 kD that corresponds to NMDA receptor complex (Fig. 2).

For detailed study of NR2-NMDA receptor involvement in the molecular mechanisms of neurotoxicity, we used a well-known model of MCA occlusion. The MCAo and reperfusion resulted in the local infarct

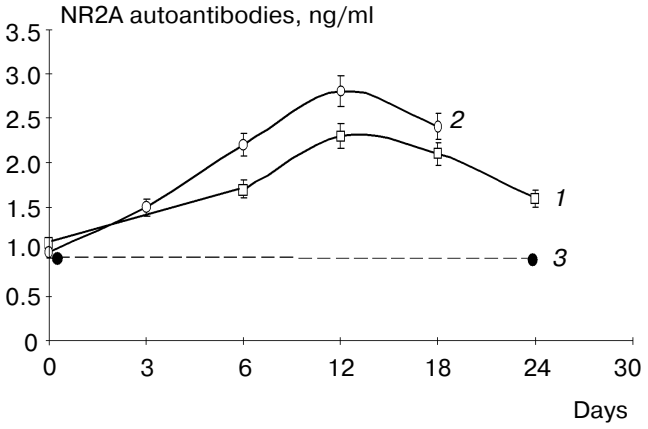

Fig. 1. Measurement of NR2A autoantibodies in blood serum of rats with global (*1*) and focal (*2*) ischemia in comparison to control animals (*3*).

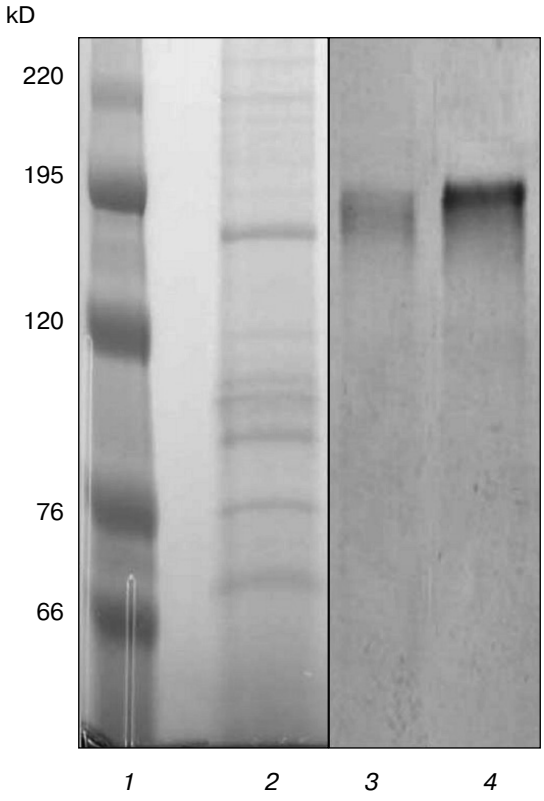

Fig. 2. SDS electrophoresis of synaptic membranes isolated from the cerebral cortex of rats with global ischemia: *1*) protein standards; *2*) membranes solubilized in 1% SDS and stained with Coomassie. Western blot of cortical samples with antibodies isolated from blood of the same animals: *3*) in control animals; *4*) in rats with global ischemia.

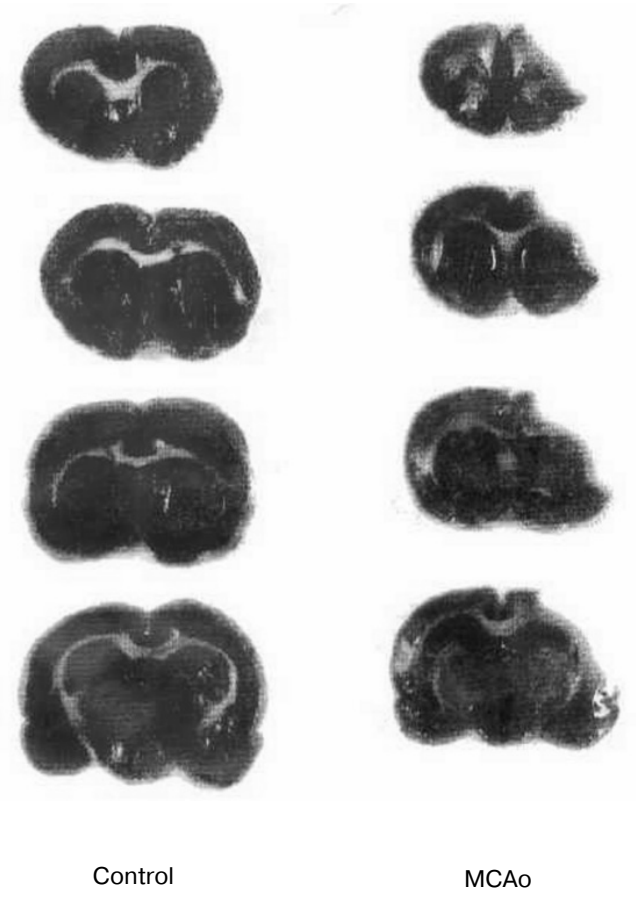

Fig. 3. Triphenyltetrazolium chloride (TTC) staining of cerebral slices from control rats and MCAo rats.

of the right cerebral cortex in all operated animals (n = 24). The volume of infarct defined by TTC staining of the 2-mm frontal cerebral sections (Fig. 3) was 102.9 ± 12.1 mm^3 [20]. In addition, MCA ligation caused insignificant cerebral edema developed on the borders of the infarct. This region represented 7.5 ± 0.3% of the whole infarct region. There were no symptoms of neurological deficit manifested in left-sided hemiparesis and motor abnormalities that are typical for dysfunctions of sensomotor cortex and the basal ganglia of the right hemisphere [21].

The alteration of expression of NR2 subunits in the cortex at 3, 6, and 24 h of reperfusion was studied by quantitative RT-PCR. The comparison of mRNA expression of NR2A, NR2B, NR2C, and NR2D revealed the increase in NR2A mRNA expression that initiated from the first hours of reperfusion in infarction-damaged cortex and in the penumbra, healthy tissue surrounding the infarct, as well (Fig. 4). NR2A expression was increased up to 100% in the area of infarction and was almost 4-fold

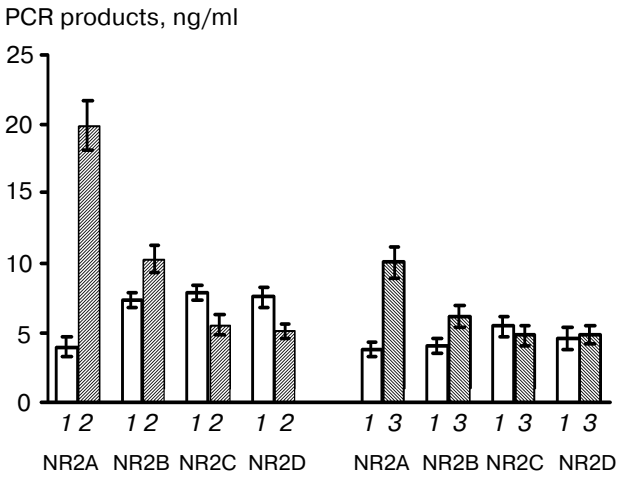

Fig. 4. Expression of NR2-NMDA receptors in cortical samples from MCAo rats: *1*) control samples; *2*) area of infarction; *3*) penumbra.

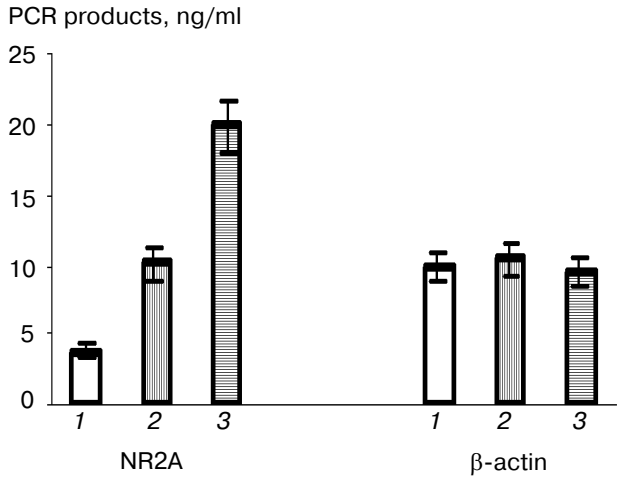

Fig. 5. Quantitative analysis of mRNA expression of NR2A subunit in cerebral cortex of rats with focal ischemia in: *1*) control samples; *2*) area of infarct; *3*) penumbra.

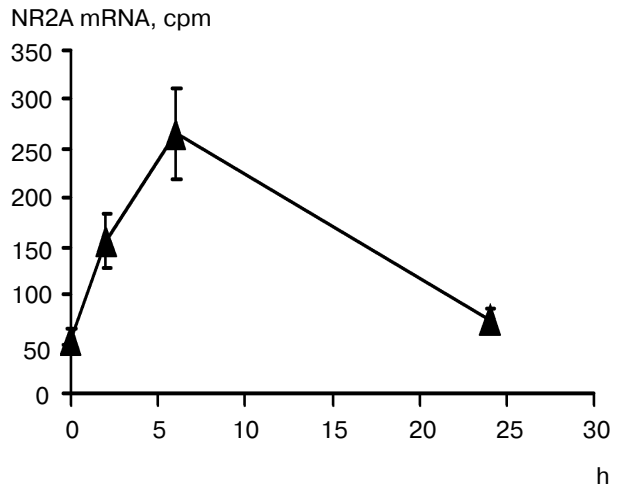

Fig. 6. Dynamics of NR2A mRNA expression in the infarction-damaged cortex of MCAo rats.

higher in the penumbra. Significant elevation of NR2B mRNA expression (40 and 47%, respectively) compared to that for control was observed at 6 h after surgery, while NR2C(D) synthesis varied insignificantly in all cortical samples during all the time studied.

Figure 5 depicts results of the quantitative expression of mRNAs encoding the NR2A subunit and corresponding β-actin, the standard structural component of cytoskeleton expressed on the same level in infracted area, penumbra, and control cortical samples. The maximal NR2A expression was observed in penumbra, while the expression of the standard was stable in all analyzed samples. Monitoring of NR2A mRNA levels within 24 h after surgery showed its maximal values at 6 h of infarction development with reduction to the control level at 24 h of reperfusion (Fig. 6).

The immunoreactivity of NR2A/B, NR2C, and NR2D subunits of NMDA receptor in the cortex of rats with induced focal ischemia was detected by Western blot using corresponding commercial antibodies. This method allows the assessment of relative contents of these subunits in cortical samples. Experimental animals exhibited strong NR2A-NR2D immunoreactivities at the end of the first week of reperfusion in penumbra and infarction-damaged area compared with that for control samples isolated from the left hemisphere (Fig. 7). NR2A/B subunit immunoreactivity was twice as high when compared with amounts for the NR2C and NR2D subunit.

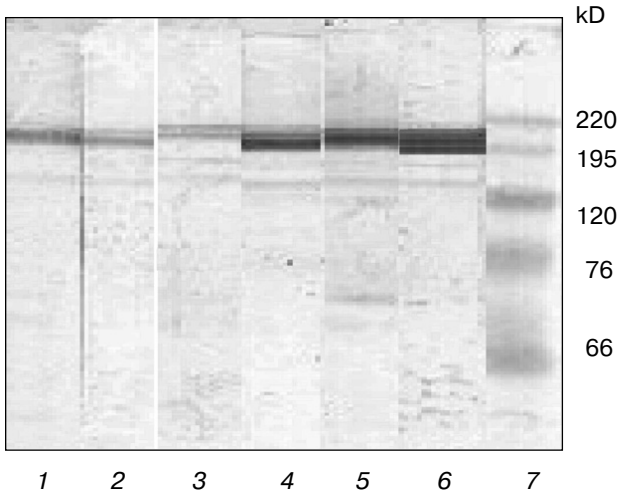

Fig. 7. Western blot assay of solubilized synaptic membranes isolated from control samples (*1-3*) and the area of infarct (*4-6*) with commercial polyclonal antibodies to: *1*, *4*) NR2D; *2*, *5*) NR2C; *3*, *6*) NR2A/B; *7*) protein standards.

The accumulation profiles of NR2A autoantibodies in rats with focal and global ischemia appeared to be similar (Fig. 1). Significantly elevated levels of NR2A autoantibodies were noted from the 6th day of reperfusion in experimental rats compared with that for sham operated rats. These levels reached maximum to the 14th day and tended to reduce to the 18th day of the experiment.

These experiments support our hypothesis that the alterations in NR2A synthesis and immunoreactivity occur on the early stages of ischemia and NR2A is the most sensitive to neurotoxicity and neuronal damage component of NR2-NMDA receptors. The fragments of damaged receptors pass the BBB and activate the immune system generating autoantibodies. Thus, NR2A

subtype of glutamate receptors might serve as a marker of neurotoxicity, and NR2A autoantibodies reflect the processes in brain under cerebral ischemia.

DISCUSSION

Presently much attention is given to the potential role of NMDA receptors in mechanisms of neurotoxicity underlying cerebral ischemia. Glutamate receptors and, in particular, the receptors of NR2-NMDA subtypes regulate intracellular Ca^{2+}-flows. Therefore, changes of properties and quantities of these receptors might lead to the alteration of the intracellular concentrations of calcium ions in cortical neurons. The current research was devoted to the investigation of molecular changes of NR2 receptor subtypes on the early stages of ischemia (less than 24 h) using the models of global and focal cerebral ischemia.

However, it was impossible to control the infarction development in each animal with global induced ischemia. Therefore, we used further a model of focal ischemia induced by MCA occlusion providing a clear-cut area of infarct in the right cortex with controlled volume of infarct and the minimal manifestation of cerebral edema [13]. In addition, this model allows the generation of transient ischemic conditions in rats with insignificant manifestation of motor deficit.

The present studies demonstrated: 1) the up-regulation of NR2A subtype of NR2-NMDA receptors, in particular in penumbra; 2) increased receptor expression accompanied with growing immunoreactivity of NR2A/B; 3) altered expression and immunoreactivity of NR2A followed by appearance of elevated levels of NR2A autoantibodies in blood of the experimental animals.

Interestingly, the expression of NR2A mRNA was elevated in penumbra, where processes of recovery and adaptation to damage are activated by ischemia. Perhaps the up-regulated NR2A mRNA expression is directed towards the regeneration of neuronal membranes affected by neurotoxicity.

The expression of NR2A and NR2B mRNAs is closely associated with alteration of immunoreactivity in cortex under the ischemia. The significant increase in the expression of these subunits within 3-6 h of reperfusion was accompanied by the elevation of NR2A/B proteins detected in cortical areas of infarction and its penumbra. The latter might reflect the time of the possible "therapeutic window", when cerebral functions can be supported by neuroprotective therapy [9].

In other research performed in rats with global ischemia the down-regulated expression of NR2A and NR2B mRNAs in hippocampus accompanied by decreased immunoreactivities of corresponding receptor subunits was found [10]. The possible redistribution of NR2A/B receptor subunit syntheses occurs between the cortex and hippocampus activating the expression in cortical infarct and penumbra and decreasing or not altering it in hippocampus [12].

The elevated expression of NR2A mRNA and NR2A immunoreactivity in turn lead to the appearance of high levels of NR2A autoantibodies in blood of rats with induced ischemia. The accumulation of autoantibodies in the blood indicates receptor damage in cerebral cortex and may reflect the volume of the infarcted region. It was noted that the concentrations of autoantibodies were higher in blood of rats with focal ischemia compared with that in animals with vast infarct. We observed a similar difference in the levels of NR2A autoantibodies earlier in patients with transient ischemic attack and acute cerebral ischemic stroke [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 key markers of neurotoxicity underlying cerebral ischemia, and autoantibodies to NR2A trace processes occurring in the ischemia-damaged brain. The present data extend our knowledge about molecular mechanisms of ischemia involving interaction between immune and nervous systems.

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