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EFFECT OF GLUTAMATE ANTAGONISTS ON NITRIC OXIDE PRODUCTION IN RAT BRAIN FOLLOWING INTRAHIPPOCAMPAL INJECTION

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Abstract – Stimulation of glutamate receptors induces neuronal nitric oxide (NO) release, which in turn modulates glutamate transmission. The involvement of ionotropic glutamate NMDA and AMPA/kainate receptors in induction of NO production in the rat brain was examined after injection of kainate, a non-NMDA receptor agonist; kainate plus 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), a selective AMPA/kainate receptor antagonist; or kainate plus 2-amino-5-phosphonopentanoic acid (APV), a selective NMDA receptor antagonist. Competitive glutamate receptor antagonists were injected with kainate unilaterally into the CA3 region of the rat hippocampus. The accumulation of nitrite, the stable metabolite of NO, was measured by the Griess reaction at different times (5 min, 15 min, 2 h, 48 h, and 7 days) in hippocampus, forebrain cortex, striatum, and cerebellum homogenates. The used glutamate antagonists APV and CNQX both provided sufficient neuroprotection in the sense of reducing nitrite concentrations, but with different mechanisms and time dynamics. Our findings suggest that NMDA and AMPA/kainate receptors are differentially involved in nitric oxide production.

Key words: APV, CNQX, excitotoxicity, kainate, neuroprotection, oxidative stress, nitrite, NO

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INTRODUCTION

Excitatory amino acids act on the CNS through various receptors, which are classified into two groups: ionotropic and metabotropic. Ionotropic receptors act on cationic-specific ion channels and comprise N-metyl-D-asparate (NMDA), alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA), and kainate (KA) receptors (V a r j u et al., 2001). Mammals possess six NMDA receptor subunits, four AMPA receptor subunits and five KA receptor subunits (J a n s s e n s et al., 2001). Kainic acid (KA), a pyrrolidine excitotoxin isolated from the seaweed *Digenea simplex,* acts on glutamate receptors, which leads to neurotoxic damage resembling the alterations observed in some neurological disorders (C a n d e l a r i o – J a l i l et al., 2001). Glutamate receptors are the primary excitatory neurotransmitter receptors in the vertebrate brain and are of critical importance to a wide variety of neurological processes. Recent reports suggest that ionotropic glutamate receptors may have a unique transmembrane topology not shared by other ligand-gated ion channels. The ionotropic receptors open a cationic channel that allows the passage of Na^+, K^+, and Ca^{2+}. Neocortical AMPA and KA receptors show little permeability to Ca^{2+}, except in the case of a subpopulation of interneurons. The NMDA receptor, in addition to allowing passage of Na^+ and K^+, is the main calcium ionophore of the cerebral cortex. This receptor differs from the other glutamate receptors by being both ligand-gated and voltage sensitive (K a c z m a r e k et al., 1997).

Stimulation of glutamate KA receptors induces neuronal nitric oxide (NO) release, which in turn modulates glutamate transmission (A l a b a d i et al.1999; N a k a k i et al., 2000). Nitric oxide is a highly reactive signal molecule in the CNS. It is a unique messenger molecule that serves diverse physiological functions throughout the body. Nitric oxide is synthesized from L-

arginine by nitric oxide synthase (NOS). The agent is a gaseous chemical messenger that acts on interneuronal communications, synaptic plasticity, memory formation, receptor function, intracellular signal transmission and mediator release (B r o w n et al., 1999; H e a l e s et al., 1999; L e i et al., 1999). However, pathological conditions may occur when higher fluxes of these mediators are generated, such as during the process referred to as excitotoxicity, i.e., the excessive activation of glutamate KA receptors. This is a condition common to both acute and chronic neurological diseases (S a n g p i e l et al., 1998; B r o r s o n et al., 1999; C i r i o l o et al., 2001). Excitotoxicity produced by glutamate is initiated by a sustained increase of intracellular Ca^{2+}. Influx of Ca^{2+} serves as a signal for activation of Ca^{2+}-calmodulin dependent and protein kinase C-regulated NOS. Activation of NOS generates NO, which can produce oxidative damage. In addition, elevated cytosolic free Ca^{2+} can activate phospholipase A_2, leading to subsequent generation of arachidonic acid. Metabolism of arachidonic acid can than produce free reactive oxygen species (ROS) and lipid peroxidation (P a t e l et al., 2003). Because NO is a reactive free radical, it has many potential targets to initiate neurotoxic cascades. It is well known that NO toxicity may be amplified by the presence of superoxide radical, the one-electron reduction product of oxygen, since these species react at a diffusion-limited rate to form peroxynitrite, a potent oxidant. Thus, oxidative stress plays a critical role in excitotoxicity (G u n a s e k a r et al., 1995).

In view of the above, the present study was undertaken to examine whether the production of NO after receipt of intracerebral KA injections can be modulated by pretreatment with competitive glutamate receptor antagonists; namely, CNQX, a selective AMPA/KA receptor antagonist; and APV, a selective NMDA receptor antagonist.

MATERIALS AND METHODS

Animals

Adult rats of the Wistar strain *(Rattus norvegicus)* of both sexes, with body weight 200 ± 30 g, were used for experiments. Groups of two or three rats per cage (Erath, FRG) were housed in an air-conditioned room at room temperature of 23 ± 2 °C with 55 ± 10% humidity and lights on 12 h/day (07.00-19.00). The animals were given a commercial rat food and tap water *ad libitum*. These animals were anesthetized by giving intraperitoneal injections of pentobarbital sodium (0.0405 g/kg b.w.) and placed in a stereotaxic frame.

Experimental procedure and intracerebral injection of drugs

The rats were divided into five basic groups (drug-treated: KA, KA+CNQX, and KA+APV; and control: intact and sham-operated animals), each basic group consisting of five different subgroups (according to survival times) of eight animals each. The drug-treated, groups received a unilateral injection of antagonist: only KA (Sigma Chemical Co. U.S.A., 0.5 mg/ml, dissolved in 0.1 M saline, pH 7.2; 1 µL total volume); KA plus CNQX (Wak-Chemie Medical GMBH, Tocris, 0.5 mg/ml, dissolved in DMSO, pH 7.2; 1 µL total volume); and KA plus APV (Sigma Chemical Co. U.S.A., 0.5 mg/ml, dissolved in 0.1 M saline, pH 7.2; 1 µL total volume) into the CA3 region of the hippocampus (coordinates from bregma: anteroposterior: -3.3 mm, dorsoventral: 3.2 mm, and lateral: 3.0 mm) using a Hamilton microsyringe with a beveled tip. The control group received the same volume (1 µL) but only saline solution (sham-operated), white the group of intact animals served as a control for mechanical injection. The animals were allowed to survive for 5 min to seven days (5 min, 15 min, 2 h, 48 h and 7 days). All animals were anesthetized and decapitated, after which the brains were immediately removed. The ipsi- and contralateral hippocampus, forebrain cortex, striatum, and cerebellum from individual animals were quickly isolated and homogenized in ice-cold buffer containing 0.25 M sucrose, 0.1 mM EDTA, and 50 mM K-Na phosphate buffer, pH 7.2. Homogenates were centrifuged twice at 1580*g* for 15 min at 4°C. The supernatant obtained by this procedure was then frozen and stored at -70°C.

Nitrite measurement

Nitrite and nitrate determinations in biological material are increasingly being used as markers of NO production. We detected nitrite in the rat brain homogenates by the Griess method (G u e v a r a et al., 1998). Nitric oxide production was quantified by measuring nitrite, a stable oxidation end product of NO (G r e e n et al., 1982). Briefly, nitrite production was determined by mixing 50 µL of the assay buffer with 50 µL of Griess reagent [1.5 % sulfanilamide in 1 M HCl plus 0.15 % N-(1-naphthyl) ethylenediamine dihydrochloride in distilled water, v:v]. After 10 min of incu-

bation at room temperature, the absorbance at 540 nm was determined and nitrite concentrations were calculated from the sodium nitrite (Sigma) standard curve. All measurements were performed in triplicate.

Protein concentration measurement

The content of protein in rat brain homogenates (hippocampus, striatum, forebrain cortex, and cerebellum, ipsilateral and contralateral) was measured by the Lowry method (L o w r y et al., 1951) using bovine serum albumin (Sigma) as standard. All measurements were performed in triplicate.

Materials

Chemicals were purchased from Sigma (St. Louis, MO, U.S.A.). Other chemicals were of analytical grade. All drug solutions were prepared on the day of the experiment. Animals used for procedures were treated in strict accordance with the Ethical Committee of the Serbian Association for Animal Science (SLASA).

Data presentation and analysis

All experiments were done with $n = 8$. Each assay was performed at least twice under identical conditions. Data are expressed as means ±SD. The statistical significance of differences between groups was assessed by Student's *t*-test (paired and unpaired) for individual comparisons and regression analysis for overall significance (with $p < 0.05$ as significant and $p < 0.01$ as very significant).

RESULTS

The results presented in Figs. 1-4 show the nitrite levels (mM/mg proteins) in hippocampal, cortical, striatal, and cerebellar homogenates, respectively. Comparison of nitrite levels in the intact group and sham-operated animals shows the effect of mechanical injection in rat brain. There was no significant difference between nitrite levels in these two groups. This means that mechanical injection only is not sufficient to trigger oxidative stress and/or excitotoxicity. We therefore used sham-operated animals as controls. In the control group, nitrite levels showed no significant differences between the left and right hemispheres in only of the tested structures. Also, there was no significant difference between mean nitrite levels obtained from each hemisphere after antagonist treatment in any of the tested brain structures, although the injection site was in the ipsilateral hippocampus.

Intrahippocampal KA injection resulted in generally higher levels (according to the Student *t*-test; $p<0.05$) of nitrite production in all tested brain structures. The obtained levels of nitrite production were highest in the hippocampus (Fig. 1). Rapid increase in nitrite production was found at 5 min after KA injection and these

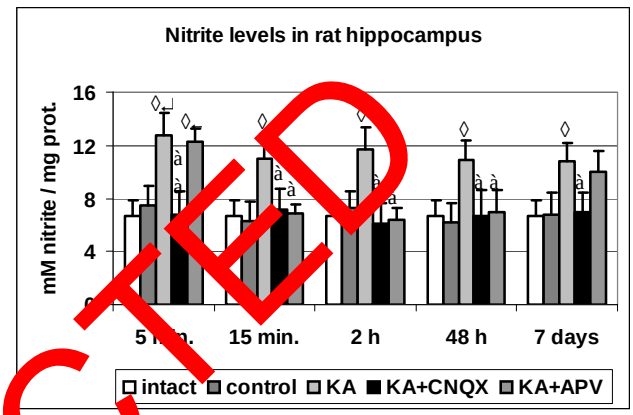

Fig. 1. Effect of intrahippocampal injection of kainate (KA), kainate plus APV (KA+APV), and kainate plus CNQX (KA+CNQX) on nitrite levels (mM NO^-_2/mg prot.) in the rat hippocampus at different survival times. Data are means ± S.D. * and ** indicate statistically significant ($p<0.05$) and very significant ($p<0.01$) differences between glutamate antagonist-treated and control (sham-operated) animals. * and ** indicate statistically significant ($p<0.05$) and very significant ($p<0.01$) differences between glutamate antagonist-treated and KA-treated animals.

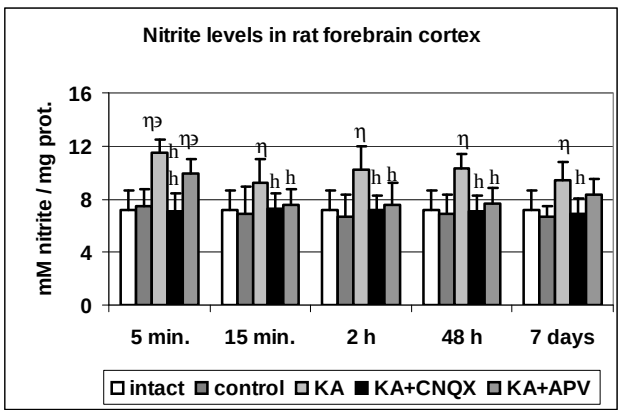

Fig. 2. Effect of intrahippocampal injection of kainate (KA), kainate plus APV (KA+APV) and kainate plus CNQX (KA+CNQX) on nitrite levels (mM NO^-_2/mg prot.) in the rat forebrain cortex at different survival times. Data are means ± S.D. * and ** indicate statistically significant ($p<0.05$) and very significant ($p<0.01$) difference between glutamate antagonists treated and control (sham-operated) animals. * and ** indicate statistically significant ($p<0.05$) and very significant ($p<0.01$) difference between glutamate antagonists treated and KA-treated animals.

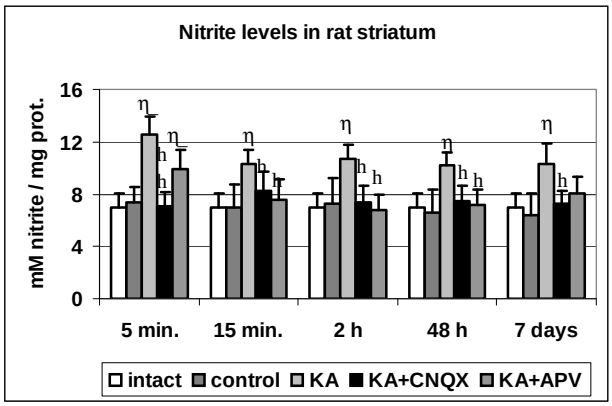

Fig. 3. Effect of intrahippocampal injection of kainate (KA), kainate plus APV (KA+APV), and kainate plus CNQX (KA+CNQX) on nitrite levels (mM NO^-_2/mg prot.) in the rat striatum at different survival times. Data are means ± S.D. * and ** indicate statistically significant (p<0.05) and very significant (p<0.01) differences between glutamate antagonist-treated and control (sham-operated) animals. * and ** indicate statistically significant (p<0.05) and very significant (p<0.01) differences between glutamate antagonist-treated and KA-treated animals.

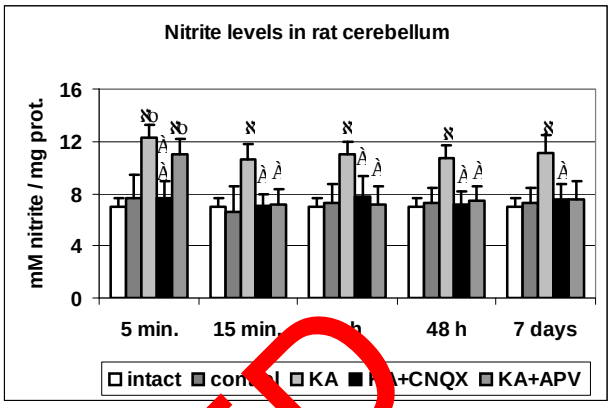

Fig. 4. Effect of intrahippocampal injection of kainate (KA), kainate plus APV (KA+APV), and kainate plus CNQX (KA+CNQX) on nitrite levels (mM NO^-_2/mg prot.) in the rat cerebellum at different survival times. Data are means ± S.D. * and ** indicate statistically significant (p<0.05) and very significant (p<0.01) differences between glutamate antagonist-treated and control (sham-operated) animals. * and ** indicate statistically significant (p<0.05) and very significant (p<0.01) differences between glutamate antagonist-treated and KA-treated animals.

higher levels continued to be above normal at all tested times (with 7 days as the final time point) in all tested brain structures (Figs. 1-4). At 5 min after KA injection nitrite measurements in the hippocampus (12.6 ± 1.63 µM NBT/mg protein), in the forebrain cortex (11.45 ± 1.00 µM NBT/mg protein), in the striatum (12.5 ± 1.54 µM NBT/mg protein) and in the cerebellum (12.26 ± 1.00 µM NBT/mg protein) showed statistically very significant differences (p<0.01) compared with the equivalent control group (Figs. 1-4). The results obtained for the contralateral hippocampus, forebrain cortex, striatum and cerebellum were similar (data not presented).

Intrahippocampal KA plus CNQX injection resulted in a reduction of nitrite levels back to control values in all tested brain structures (Figs.1-4). Thus, there was a significant decrease in nitrite levels only in comparison to KA-treated animals (p<0.05). Analogous to the excitotoxic effect obtained with KA-injected animals, statistically the most significant decrease was obtained at 5 min (6.74 ± 1.83 µM NBT/mg protein in the hippocampus, 7.07 ± 1.33 µM NBT/mg protein in the forebrain cortex, 7.03 ± 1.11 µM NBT/mg protein in the striatum and 7.61 ± 1.29 µM NBT/mg protein in the cerebellum, p<0.01; Figs. 1-4).

Intrahippocampal KA plus APV injection resulted in decrease of nitrite levels in all tested brain structures as compared with the equivalent group of KA-treated animals, but with different time dynamics (Figs.1-4). The effect of this antagonist was interesting because at 5 min after injection, nitrite levels in all tested brain structures were still high in comparison with the control group (12.28 ± 1.00 µM NBT/mg protein in the hippocampus, 9.87 ± 1.16 µM NBT/mg protein in the forebrain cortex, 9.89 ± 1.50 µM NBT/mg protein in the striatum, and 10.96 ± 1.17 µM NBT/mg protein in the cerebellum, p<0.05; Figs.1-4).

DISCUSSION

The role of NO in cerebral insult remains controversial. While numerous studies have used ischemia, hypoxia and status epilepticus models, few have examined NO in the KA model of excitotoxicity. Animals exposed to KA-induced status epilepticus display a striking pattern of selective neuronal vulnerability in the hippocampus. Neurons in the hilus/CA3 and CA1 subfields appear particularly sensitive, whereas dendate gyrus granule cells are resistant (B e c k e r et al., 1999; L e r e et al., 2002), which is likely due to the high concentration of KA receptors on their membranes. Regional distribution of NMDA and AMPA/KA receptors of the rat brain was found to be highest in deep layers (layer 5) of the forebrain cortex, the cerebellar granule cell layer, and the caudate putamen (C a r r o l l et al., 1998; B a i l e y et al., 2001), which is why we tested these particular brain regions: hippocampus, forebrain cortex, striatum, and

cerebellum.

In the present study, an appropriate dose of KA (0.5 mg/ml) was used to cause slight brain damage in the ipsilateral, but not contralateral, hippocampus; there were no behavioral or epileptic effects. It was previously shown that NO formation occurs in different regions of the rat brain during KA-induced seizures (M u l s c h et al., 1994; Y a s u d a et al., 2001). In our experiments, nitrite levels were measured at various times following intrahippocampal KA injection in the above-indicated four rat brain structures. Cortical areas such as the pyriform and entorhinal cortices are known to contain the highest packing densities of nNOS-positive interneurones (B i d m o n et al., 1999), suggesting that neurotransmission and probably cognitive information processing in normal animals would be affected by the pharmacological modulation of NO production.

We have shown that NO end-product levels in the rat brain increased immediately after KA injection and continued to increase gradually throughout the experiments. Under conditions of normal behavior in the rat the damage was localized mainly in the CA3 region of hippocampus, where neuronal loss occurred.

Agonist-triggered Ca^{2+} influx may constitute a key link between glutamate receptor activation and subsequent neurodegeneration. In cortical culture, brief periods of activation of NMDA channels, which are highly Ca^{2+}-permeable are capable of triggering widespread neurodegeneration. In contrast, much more prolonged periods of activation of AMPA/KA receptor-gated channels are required before comparable neurotoxicity develops. This may reflect the fact that most AMPA/KA channels are poorly permeable to Ca^{2+} and likely cause secondary Ca^{2+} influx via the depolarization and activation of voltage-sensitive Ca^{2+} channels. Multiple factors have been hypothesized to contribute to the differences in toxicity that result from NMDA and AMPA/KA receptor activation (C a r r i e d o et al., 1996; N i c h o l l s et al., 2000).

In this study, we detected different effects of the NMDA antagonist APV and the AMPA/KA antagonist CNQX on nitrite levels after intrahippocampal injection with KA. The effect of KA on nitrite production was blocked by the glutamate antagonists. Intrahippocampal injection of KA plus CNQX resulted in decrease of nitrite production to around control levels in all tested brain structures. Thus, significant decrease in nitrite levels was found only in comparison to KA treated animals, i.e., the overall effect of a selective AMPA/KA receptor antagonist was a decrease of KA-induced excitotoxicity. The accent effect of intrahippocampal injection of KA plus APV also resulted in decrease of nitrite production. However, this effect was detected 15 min after injection, suggesting the existence of an NMDA receptor-mediated component of basal nitrite production in physiological conditions and differences of mechanisms and time dynamics between CNQX and APV. The used glutamate receptor antagonists of showed the same pattern in all tested brain structures.

From the data presented it is obvious that increase of nitrite levels in KA-induced neurotoxicity is not dependent on activation of only one class of ionotropic glutamate receptors. We hypothesize that by selectively blocking AMPA receptors with CNQX, we reduced nitrite production but did not inhibit several other cellular pathways of NO generation (H a l a s z et al., 2004). A possible explanation is that KA enhances hippocampal NO generation (K a s h i k a r a et al., 1998), while KA injection results in differential regulation of nNOS mRNA and NO formation in the rat hippocampus (K a s h i k a r a et al., 2000). It was previously reported that inhibition of nNOS by 7-nitroindazole can effectively lower NO production at early testing times (from 5 min to 2 h) in the rat brain following intracerebral KA injection (R a d e n o v i ć et al., 2003).

Published results implicate neuronal NO generation in the pathogenesis of both direct and secondary excitotoxic neuronal injuries *in vivo*. The precise cellular mechanisms that lead to neurotoxicity under these conditions still remain unclear. Although NMDA receptors likely contribute critically to neuronal injury in various acute conditions, several observations support the hypothesis that AMPA/KA receptors may be of greater importance to the neurodegenerative process (C a r r i e d o et al., 1998, 2000). Considerable evidence supports a link between Ca^{2+} influx and glutamate receptor-mediated neurodegeneration. Brief periods of activation of highly Ca^{2+}-permeable NMDA channels can result in substantial intracellular Ca^{2+} accumulation and widespread neuronal injury (H y r c et al., 1997; L u et al., 1996; T s e n g et al., 2003). Mitochondria can buffer these large Ca^{2+} loads but they do so at the expense of triggering injurious ROS production (P e n g et al., 1998). Additionally, the extremely rapid interconversion of ROS within the cell can make it difficult to identify the originating species.

We previously reported differential roles of NMDA and AMPA/KA receptors in superoxide production and mitochondrial MnSOD activity in the rat brain (Radenović et al., 2004).

In contrast to NMDA receptors, AMPA/KA receptors are generally Ca^{2+} -impermeable and trigger injury more slowly, with prolonged periods of activation needed before significant neuronal injury occurs (Koh et al., 1990). Subpopulations of central neurons, however, are highly vulnerable to AMPA/KA receptor-mediated injury, likely attributable in part to the existence of large numbers of AMPA/KA channels with high Ca^{2+} permeability (Weiss et al., 2001).

The used glutamate antagonists APV and CNQX both provided sufficient neuroprotection in sense of decreasing nitrite levels, but with different mechanisms and time dynamics.

In conclusion, the increase of NO production in distinct brain regions functionally connected via afferents and efferents suggests that these regions are affected by the injury. Furthermore, the data point to differential roles of NMDA and AMPA/KA receptors during this neuropathological condition.

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ЕФЕКАТ АНТАГОНИСТА ГЛУТАМАТА НА СТВАРАЊЕ АЗОТ ОКСИДА У МОЗГУ ПАЦОВА

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Стимулација глутаматских рецептора доводи до стварања азот оксида (NO) у неуронима мозга што доводи до модулације глутаматске неуротрансмисије. Испитивана је улога глутаматских NMDA и AMPA/каи-

натних рецептора у стварању NO у мозгу пацова после интрацеребралне апликација каината, агониста AMPA/каинатних рецептора, каината са 6-циано-7-нитрокиноксалин-2,3-дионом (CNQX), селективним антагонистом AMPA/каинатних рецептора или каината са 2-амино-5-фосфонопентаноиском киселином (APV) селективним антагонистом NMDA рецептора. Антагонисти глутамата су аплицирани унилатерално у селективно осетљив CA3 регион хипокампуса. Стварање NO је праћено преко акумулације нитрита, стабилних метаболита NO, Griess-овом методом. Мерења су вршена у хипокампусу, кортексу, стиатуму и церебелуму мозга пацова 5 min, 15 min, 2 x, 48 x и 7 дана након апликације. У свим праћеним можданим структурама неуропротективно је деловала примена CNQX и APV у смислу смањења продукције NO, али са очигледном разликом у механизму дејства и временској динамици. Резултати нашег истраживања доказују да су глутаматски NMDA и AMPA/каинатни рецептори различито укључени у процес продукције NO.

Кључне речи: APV, CNQX, ексцитотоксичност, каинат, неуропротекција, оксидативни стресс, нитрите, NO.