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GENETICS

Effect of Pharmacological Modulation of Activity of Metabotropic Glutamate Receptors on Their Gene Expression after Excitotoxic Damage in Hippocampal Neurons

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Microinjection of kainic acid into rat hippocampus causes excitotoxic neuronal damage predominantly in the CA3 and CA1 fields. These lesions can be significantly reduced by simultaneous administration of MPEP, a negative allosteric modulator of type 5 metabotropic glutamate receptors, and LY354740, an agonist of type 2 metabotropic glutamate receptors. The decrease in neuronal death in the hippocampus during pharmacological modulation was paralleled by adaptive changes in gene expression. In the hippocampus, gene expression of type 5 postsynaptic metabotropic glutamate receptor was close to the control level, and in the frontal cortex expression of the gene of α_1-subunit of the $GABA_A$ receptor returned to normal. In the frontal cortex, a reciprocal relationship was observed for type 2 metabotropic glutamate receptor: expression of the corresponding gene decreased in response to pharmacological activation.

Key Words: *kainic acid; metabotropic glutamate receptors; MPEP; LY354740; gene expression*

Excessive or prolonged glutamate receptor activation in the brain leads to excitotoxic death of neurons. Excitotoxicity develops in case of exogenous administration of glutamate receptor agonists (ibotenic and kainic acids, NMDA), epileptic activity, ischemia of brain structures, and numerous neurodegenerative diseases [3,10]. Metabotropic glutamate receptors (MGR) turned out to be promising targets for neuroprotection from excitotoxic damage [1]. We have previously shown that combined modulation of presynaptic MGR-2 activity with specific agonist LY354740 and postsynaptic MGR-5 activity by specific non-competitive antagonist MPEP reduces the kainate-induced neuronal death in hippocampus [7]. The damaging effect of kainate at the initial stages was most pronounced in the hippocampal field CA3, but the damaged area increased with time, and after 4 weeks, a significant decrease in the number of neurons was observed in the CA1 field as well [4]. However, MGR modulators, even when administered several days after kainate microinjection, significantly reduced hippocampal damage and protected hippocampal CA1 neurons [7]. Kainate-induced damage to the hippocampus includes many mechanisms at different CNS levels, including gene expression [2,5,6,11]. Assuming, that adaptive rearrangements at the level of gene expression contribute to compensation of structural and functional hippocampal disorders caused by excitotoxicity, it is important to assess changes in the expression of genes

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TABLE 1. Primers for Analysis of the Expression of the Corresponding Genes

Gene	Forward primer	Reverse primer
grm2	CTATGCCACCCACAGTGATG	GCACAGTGCGAGCAAAGTAATC
grm5	AGTTCGTGAGCAATATGGGATT	GATCCATCTACACAGCGTACCA
gabra1	CCGTGTCAGACCACGATATG	TACAGCAGTGTGCCATCCTC
GAPDH	ACAGCAACAGGGTGGTGGAC	TTTGAGGGTGCAGCGAACTT

directly related to regulation of excitation-inhibition processes in the brain.

The aim of this study was to assess MGR gene expression under conditions of excitotoxicity reduction by postsynaptic MGR-5 inhibition and presynaptic MGR-2 activation [7].

MATERIALS AND METHODS

Experiments were carried out on male Wistar rats weighing 180±200 g. The animals were kept and used in accordance with the Directive 86/609/EEC on the Protection of Animals Used for Experimental and Other Scientific Purposes. The rats were divided into 3 groups: one control and two experimental groups. Rats of both experimental groups were stereotaxically injected with 0.2 μg of kainic acid (Sigma) under pentobarbital anesthesia (30 mg/kg). Kainate was dissolved in isotonic NaCl and injected in a volume of 1 μl into the left and right dorsal hippocampus (coordinates: AP: -3.0, ML: ±3.0, V: -3.0). Within 2-4 h after kainic acid administration, transient limbic seizure (head and forelimbs twitching, shaking off, *etc*.) developed, but no convulsions were observed later. The animals of the control group received injection of isotonic NaCl solution in the hippocampus.

To reduce excitotoxicity, rats of one experimental group were treated with non-competitive MGR-5 antagonist MPEP (2-methyl-6-(phenylethynyl)-pyridine, Tocris) in a dose of 5 mg/kg 24 h and 48 h after kainate microinjection. In addition, positive allosteric MGR-2 modulator LY354740 — (1*S*,2*S*,5*R*,6*S*)-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylic acid (synonym — Eglumegad) in a dose of 2 mg/kg was administered 2-5 days later to rats of this group. The substances were dissolved in isotonic NaCl (with addition of NaOH, as recommended by the manufacturer) and injected intraperitoneally once a day in a volume of 1 ml. Animals of the control group received isotonic NaCl solution at the same time.

Four weeks after kainate microinjection, the animals of the control and both experimental groups were decapitated, and brain structures (frontal cortex and hippocampus) were isolated and homogenized in a denaturing buffer containing guanidin isothiocyanate (Sigma). Total RNA was isolated from the brain structure homogenates by phenol—chloroform extraction. RNA was purified from possible contamination with genomic DNA by DNase according to the standard protocol, recommended by the manufacturer (New England Biolabs). The concentration of isolated RNA was measured by spectrophotometry, the quality of the obtained RNA was evaluated by electrophoresis in 1% agarose gel.

The reverse transcription reaction was performed according to the standard protocol, developed by the reverse transcriptase manufacturer (Fermentas). Quantitative real-time PCR was carried out in the DT-322 detecting amplifier (DNA-Technology), using SYBR Green dye (Invitrogen). The *GAPDH* gene was used as a reference (Table 1). The amount of mRNA in the hippocampus and frontal neocortex was estimated after determining the threshold cycle, recorded by the amplifier, and subsequent calculation according to the $2^{-\Delta\Delta Ct}$ method. The quality and molecular weight of the PCR products were assessed by electrophoresis in 3% agarose gel.

Statistical data processing was carried out in the Microsoft Excel application using Mann—Whitney *U* test.

RESULTS

Expression of the MGR genes, as well as of the $GABA_A$ receptor α_1-subunit in the hippocampus and frontal cortex after kainate microinjection and subsequent pharmacological treatment differed from the expression level in control rats and in rats treated with kainate alone (Table 2).

In the hippocampus, expression of MGR-5 gene (*grm5*) increased under the influence of kainate. Activation of transcription of this gene may be indicative of increased glutamatergic transmission, which implements excitotoxicity. In the frontal cortex, where kainate-induced cell death was not observed, *grm5* expression was unaltered. However, in this brain area expression of the $GABA_A$ receptor α_1-subunit gene (*gabra1*) was significantly activated, which can be interpreted as strengthening of inhibitory influences aimed at compensation of hippocampal hyperexcit-

TABLE 2. Gene Expression in Rats of the Eperimental Groups in Comparison with the Control Group (*M*±*m*)

Group	Hippocampus			Frontal cortex		
	grm5	grm2	gabra1	grm5	grm2	gabra1
Control (*n*=5)	1	1	1	1	1	1
Kainate (*n*=10)	2.0±0.8*	1.0±0.2	0.8±0.1	1.0±0.1	1.7±0.7	2.6±0.9*
Kainate+MPEP+LY354740 (*n*=10)	1.2±0.4	0.8±0.2	0.8±0.1	1.1±0.1	0.5±0.3*	1.0±0.1

Note. *$p<0.05$ in comparison with the control.

ability. The level of expression of the MRG-2 (*grm2*) gene under the influence of kainate was altered neither in the hippocampus, nor in the frontal cortex.

The pharmacological modulation of MGR activity used for weakening of the hippocampus excitotoxic damage not only reduced neuronal death in the hippocampus, but also affected receptor mRNA. In the hippocampus, *grm5* expression decreased and reached the control value; at the same time, *grm2* expression was not altered. In the neocortex, other trends were observed: in the absence of changes in *grm5* expression, *grm2* expression significantly decreased. The increased *gabra1* expression decreased to the control level after pharmacological correction (Table 2).

Kainate inducing progressive neuronal death in the hippocampus and alters expression of many genes associated with excitotoxicity, neuroinflammation, impaired calcium metabolism, energy metabolism, autophagy, *etc*. [3,5,6,11]. The very fact of participation of genetic processes in the implementation of effects of the neurotoxin is not surprising, since its action is followed by significant changes in brain cellular elements and neural networks. Pharmacological correction of pathological processes in the brain is also accompanied by changes at the genome level. These changes are based on adaptive cellular processes, produced by the effects of injected substances as well as by excitotoxicity and neurodegeneration. The revealed changes in the *gabra1* gene expression in the neocortex can be considered as an example of adaptation to the disturbed excitation/inhibition balance in the brain. This subunit is a part of the majority of $GABA_A$ receptors [9], and normalization of its expression after the action of MGR ligands can be considered as cessation of inhibitory control by the neocortex due to suppression of excitotoxic excitation in the hippocampus. On the other hand, changes in *grm5* and *grm2* expression are produced not only by kainate, but also by the pharmacological effect on the activity of these receptors. Normalization of *grm5* expression in the hippocampus is especially important, since its maintenance at the control level is an important condition for optimal functioning of glutamate and GABAergic neurotransmission. It has been shown that these receptors are involved in learning and memory mechanisms [12] and are localized on the postsynaptic membranes of not only glutamate, but also GABAergic synapses of the hippocampus. Concerning the presynaptic MGR-2, it can be noted that their mRNA level was not significantly altered by kainate, but after pharmacological treatment it significantly decreased in the frontal cortex. In this case, the reciprocal relationship between selective activation of the receptor and expression of its gene became apparent. Indeed, this relationship was also revealed in our experiments with TCN 238 — the selective positive allosteric MGR-4 modulator [13]. Systemic (4-time) administration of this substance did not produce changes in animal behavior, but caused adaptive inhibition of the MGR-4 gene expression in the hippocampus 5 days after the injections. The same reciprocal relationship between the activity of neurotrophic factors and expression of their genes was shown under conditions of spinal cord injury [8].

The observed changes in gene expression in frontal neocortex of rats after intra-hippocampal kainate administration and pharmacological excitotoxicity suppression seem to be important. Reorganization of functionally important neural networks that occurs after hippocampus damage with excitotoxin, may be the reason for these changes. Obtained results confirm the close connection of these brain regions also at the level of gene expression, which is manifested under conditions of hippocampal damage.

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