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EXPERIMENTAL ARTICLES

The Effect of the Synthetic Neuroprotective Dipeptide Noopept on Glutamate Release from Rat Brain Cortex Slices

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Abstract—The level of spontaneous and K^+-stimulated release of endogenous glutamate was studied in experiments on slices of brain cortex of Wistar rats. Pronounced spontaneous release of the neuromediator and its increase under conditions of stimulation were registered by high-performance liquid chromatography with electrochemical detection. The effect of the nootropic and neuroprotective dipeptide Noopept (GVS-111) on release of glutamate was investigated. The peptide in concentrations of 10^{-5} and 10^{-6} M caused a statistically significant decrease in spontaneous and K^+-stimulated glutamate release. This effect could be the basis of the neuroprotective action of the peptide, suggesting that further studies of Noopept as neuroprotector are very promising.

Key words: glutamate release, brain cortex slices, Noopept, neuroprotective properties

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INTRODUCTION

Glutamate is the main excitatory neurotransmitter in mammalian brain. NMDA is the most studied among the known subtypes of the glutamate receptors. It is widely distributed in the brain, particularly in the frontal cortex, hippocampus and basal nuclei [1]. This amino acid is important for cognitive functions and is involved in numerous biochemical reactions (e.g., the Krebs cycle and fatty acid biosynthesis) [2]. At present glutamate is considered to be a key mediator in the pathogenesis of brain neurotoxic damages in the course of such states as hypoxia, hypoglycemia, and neurodegenerative disorders (e.g., Alzheimer's and Parkinson diseases along with several others).

Massive glutamate release into the intercellular space was observed in experiments on ischemic stroke and brain injury of animals using a microdialysis technique [3]. It is well established that the uptake of glutamate by nervous tissue is disturbed under conditions of glucose–oxygen deprivation. This is accompanied by an increase in extracellular concentration of the neuromediator [4]. Thus, conditions for the excessive stimulation of postsynaptic glutamate receptors, in particular those of the NMDA subtype, appear. This process is associated with increased calcium influx into neurons. At the same time, the overproduction of free radicals develops. All these events result in the damage and further death of neurons. Diminishing the neurotoxic effect of glutamate could serve as one of the most important mechanisms of neuroprotection.

It is well known that some nootropic agents are capable of both restoration of impaired cognitive functions and exhibition of a neuroprotective effect. Neurochemical studies of the effects of pyracetam, which is an ancestor of this class of drugs, gave ambiguous results for such parameters as reverse uptake, release and metabolism of glutamate in the brain cortex [5]. The insufficiently high activity of pyracetam and the lack of convincing data on the mechanisms of its effects stimulated a search for new nootropic agents. One of the most promising directions of such a search is the synthesis of neuropeptide analogues. The original approach elaborated in the Institute of Pharmacology of the Russian Academy of Medical Sciences during the previous 20 years consists in the synthesis of dipeptides imitating the structure of the non-peptide agent and the structure of the peptide active site with the corresponding neurotrophic activity [6]. On the basis of the structure of the $AVP_{4–9}$ active fragment of the basic metabolite of vasopressin, and the non-peptide pyracetam prototype, Gudasheva et al. [7, 8] have synthesized a series

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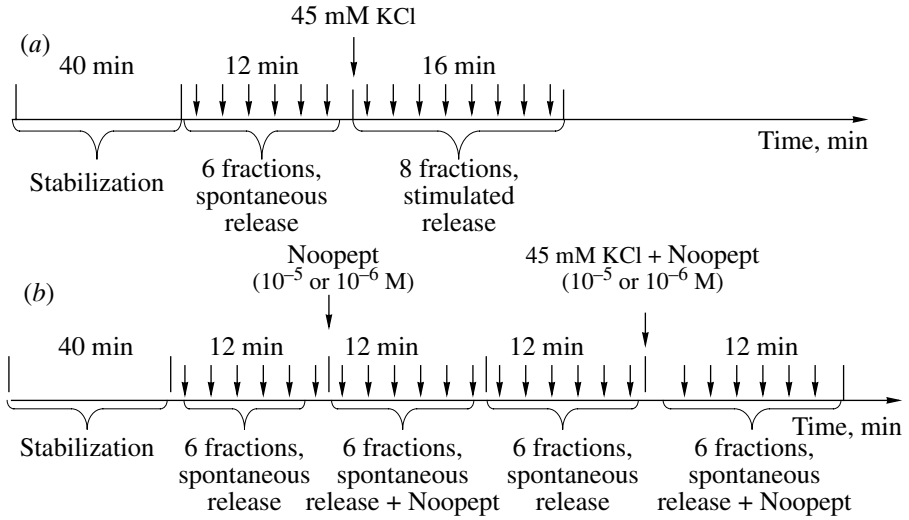

Fig. 1. Sequence of Preparation of Perfusates in (*a*) control experiment and (*b*) the experiment with addition of Noopept.

of *N*-acyl derivatives of proline among which agents with nootropic activity were found. The highest activity was observed for the ethyl ether of *N*-phenylacetyl-*L*-prolylglycine, called Noopept (GVS-111) [9]. In addition to its capacity for improving cognitive functions, Noopept exhibited a neuroprotective effect on a model of photo induced thrombosis of the prefrontal cortex [10], chemically in cultures of cerebellar granular neurons subjected to glucose–oxygen deprivation [11] and in cultures of neurons from aborted fetuses with diagnosed Down's syndrome [12].

Previously, Noopept was shown to increase the survival of granular neurons in cell cultures of the cerebellum in response to cytotoxic glutamate action [11]. One of the possible mechanisms of suppression of the neurotoxic glutamate effect could be the inhibition of its presynaptic release. It is known that agents affecting glutamate release (Lyubelisyl, BW619C89) are effective neuroprotectors [13, 14].

This study is aimed at investigation of the effects of Noopept at the level of spontaneous and stimulated glutamate release by slices of rat brain cortex as one of the possible mechanisms of its neuroprotective effects.

EXPERIMENTAL

Reagents of "Chemically Pure" grade or higher were used in the study. Noopept was synthesized under the supervision of prof. T. A. Gudasheva in the Chemistry Departments of the Zakusov Research Institute of Pharmacology of the Russian Academy of Medical Sciences. All the solutions were prepared using water of the highest degree of purity (18.2 MGOm).

Experiments were performed on Wistar male rats (from the "Stolbovaya" nursery of the Russian Academy of Sciences) with body weights of 180–220 g, which were kept under standard conditions with free access to water and food.

Pentobarbital-anesthetized rats (50 mg/kg intraperitoneally) were decapitated and the brain was removed. Slices of the frontal brain cortex (300 μm) were prepared using a Lancer Vibratome Series 1000 vibratome (United States). These slices were placed into 400 μl perfusion chambers. A constant rate of fluid flow through these chambers (200 μl/min) was provided using a peristaltic pump (Zaling, Poland). Superfusion of brain slices was carried out at 37°C in Krebs-Ringer solution containing 124 mM NaCl, 5 mM KCl, 1.25 mM NaH_2PO_4, 2 mM $MgSO_4$, 2 mM $CaCI_2$, 26 mM $NaHCO_3$, 15 mM HEPES and 10 mM glucose [15]. In the first series of experiments (the control), the level of spontaneous and stimulated glutamate release was measured. After a 40 minute stabilization period, during which the samples were not collected for analysis, we collected six 2-minute fractions for the subsequent measurements of the level of spontaneously released glutamate. In the studies of stimulated glutamate release, composition of the perfusion media was changed as follows: The KCl concentration in the incubation media was increased from 5 to 45 mM with an equimolar decrease in the NaCl concentration. Later, eight 2-minute fractions were collected (i.e., stimulated release of neurotransmitter in response to membrane depolarization) (Fig. 1*a*).

In the second series of experiments, the effect of Noopept on the level of spontaneous and stimulated glutamate release was studied. For this purpose, after 40 minutes of stabilizing and collection of six 2-minute fractions with spontaneous glutamate release, a peptide was added to the Krebs-Ringer solution at a concentration of 10^{-5} or 10^{-6} M, and six 2-minute fractions were

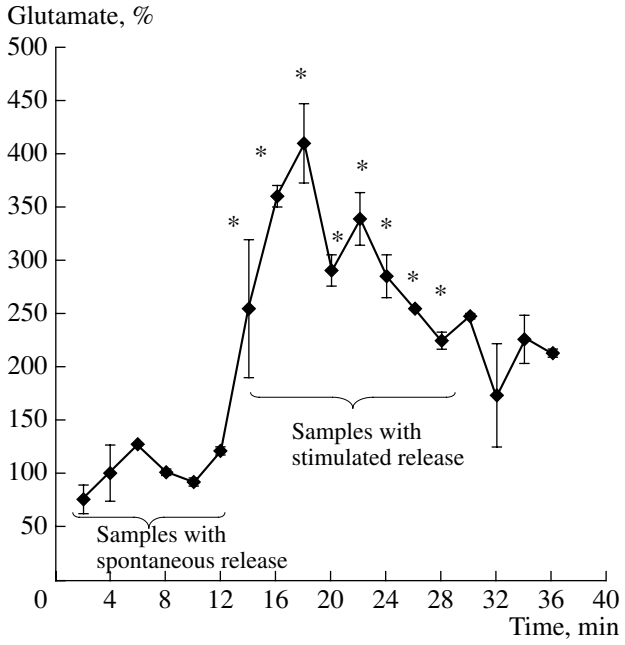

Fig. 2. Curve of the levels of glutamate release in control samples. Y axis shows glutamate concentration, % of the level of spontaneous release.

*$p < 0.05$ compared to the level of spontaneous release.

collected. Following this, the perfusion media was replaced by Krebs-Ringer solution without Noopept, and six 2-minute fractions with spontaneous glutamate release were collected. At the final stage, the concentration of KCl was increased to 45 mM in Krebs-Ringer solution simultaneously with peptide addition at a concentration of 10^{-5} or 10^{-6} M. Six 2-minute fractions were collected (Fig. 1*b*). Then, 1 N $HClO_4$ (50 µl) was added to each sample of perfusate at a ratio of 1 : 10 of the total volume. The samples were frozen and kept at –20°C for further measurement of glutamate concentration by high performance liquid chromatography with electrochemical detection (HPLC/ED) [16].

An LC-4B electrochemical detector (BAS, USA) was used with a potential of +850mV using a carbon–glass electrode against a Ag/AgCl comparison electrode. A 0.05 M sodium phosphate buffer containing 0.025 mM EDTA and 5% acetonitrile served as a mobile phase. *L*-homoserine internal standard (0.01 mg/ml) in 0.2 N NaOH and *o*-phthalaldehyde sulphite reagent (10 µl) in 0.1 M borate buffer (pH 9.5) were added to the perfusate (25 µl) for amino acid derivatization. A solution of glutamate (0.2 mg/ml) in 0.1 N $HClO_4$ was used as a standard. After 15 minutes of incubation at 37°C, the mixture (25 µl) was applied onto an Agilent Hypersil ODS column (5 µm, 4.6 × 250 cm, loop volume of 5 µl) of an Agilent 1100 chromatograph (United States). Glutamate concentration was calculated using "Chemstation Agilent" software (USA). The final result was expressed as nM/mg of the tissue per 2 minutes.

Statistical analysis was performed using the Biostat computer program and nonparametric tests (Wilkoxon-Mann Whitney U-test). Results were presented as mean ± S.E.M.

RESULTS AND DISCUSSION

The curve of sequential measurement of the level of glutamate release in control samples is shown in Fig. 2. Our data are generally in a good agreement with the results of the other studies performed under similar conditions [17, 18].

Noopept at a concentration of 10^{-5} and 10^{-6} M significantly decreased spontaneous glutamate release. The data presented in Fig. 3*a* demonstrate that the level of spontaneous release decreased by 27% ($p < 0.05$) at a Noopept concentration of 10^{-6} M, while the decrease was also significant and equal to 22% at a concentration of 10^{-5} M. In the presence of 45 mM KCl, the release level was 360% of the spontaneous level (Fig. 3b, $p < 0.01$). Noopept significantly decreased the level of stimulated glutamate release in the cortex slices. This level was 200% at a concentration of 10^{-5} M ($p < 0.05$ compared to stimulated release in the control samples) and 165% at 10^{-6} M ($p < 0.05$ compared to stimulated release in the control samples and $p < 0.05$ compared to the samples with peptide at a concentration of 10^{-5} M).

Thus, Noopept significantly decreased the level of both spontaneous and stimulated glutamate release. The extent of this decrease depended on the peptide concentration. It was significantly higher at a concentration of 10^{-6} M than at 10^{-5} M. The observed decrease in the peptide effect at its higher concentration suggests a bell-shaped curve of the dose-effect relationship, which is characteristic of a number of peptides, including Noopept [19].

It is interesting to compare the effects of Noopept and other nootropic agents on the glutamatergic system. Noopept is a dipeptide analog of the active fragment of vasopressin [7], which not only positively affects memory, but exhibits a neuroprotective activity as well [20]. Thus, the ability of this peptide to decrease K^+-stimulated glutamate release in the supraoptical nucleus [21] should also be noted. As for the effects of the non-peptide prototype of Noopept, pyracetam the studies of Nickolson et al. [5] demonstrated the absence of its effect on the reversed uptake, release, and metabolism of glutamate in the brain cortex. The study of Bering et al. [22] revealed the pyracetam capacity for binding to glutamate receptors; however this effect was observed at pyracetam concentration of 10^{-3} M, a level which is impossible to achieve through its systemic injection in therapeutic

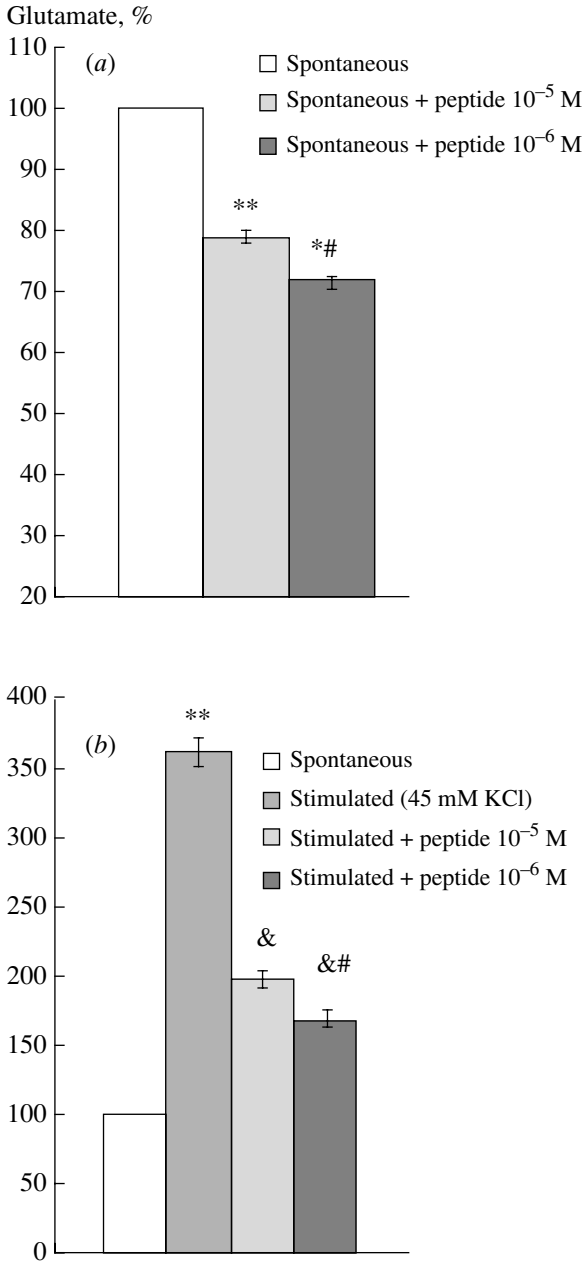

Fig. 3. The effect of Noopept in the concentrations of 10^{-5} and 10^{-6} M on the level of (*a*) spontaneous or (*b*) stimulated glutamate release by rat brain cortex slices.
Spontaneous, the level of spontaneous release.
Stimulated, the level of K^+–stimulated release.
*$p < 0.05$ compared to the level of spontaneous release.
**$p < 0.01$ compared to the level of spontaneous release.
& $p < 0.05$ compared to the level of stimulated release.
# $p < 0.05$ compared to peptide at concentration 10^{-5} M.
The data is presented as percentage of the level of spontaneous release.

doses. In the study of Dr. N.A. Andreeva, pyracetam, in contrast to Noopept, did not decrease glutamate toxicity [11]. Another agent with nootropic and neuroprotector properties, Bilobil, (Bilobalide, the active substance of Ginkgo Biloba) inhibited the stimulated glutamate release by brain slices in a dose dependent manner. This effect of Bidobil resembled that of Noopept [18]. However, the nootropic effect of this agent was reached only at higher doses than that of Noopept.

Analyzing the possible mechanisms of the Noopept effects described above, it is necessary to note that glutamate release is a calcium dependent process [23]. We demonstrated previously that Noopept blocks potential-dependent calcium channels [24] and voltage-dependent calcium-dependent potassium channels [25]. We suggest that Noopept's ability to inhibit glutamate release in cortex slices is associated with its inhibition of the functional activity of these channels. Thus, we demonstrated that the Noopept inhibition of presynaptic glutamate release, which is considered to be one of factors of cell neurotoxicity, may be the basis of the neuroprotective effect of this peptide.

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