Praca oryginalna *Original paper*

Glutamate as a neural factor for the *ex vivo* release of catecholamines from the rabbit hippocampus*

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Summary

The aim of the research was to confirm the hypothesis about the direct influence of different concentrations of glutamate (Glu; 5, 50 and 200 μM) on the release of catecholamines (CA's) from hippocampal slices incubated for 90 min. The hippocampus is the central structure of the motivational system and, along with the hypothalamus, amygdala, and medial prefrontal cortex (mPFC), is responsible for memory, learning, and the initiation and course of the reaction to stressoric factors. In our research, we focused on the direct effect of different Glu concentrations on CA release without the involvement of other neural cerebral structures and humoral responses. The effect of Glu added to the incubation fluid was found to be inconsistent. When added at the lowest concentration, Glu inhibited the release of CA from the incubated slices, and at higher concentrations tended to increase the release of CA. Notably, Glu significantly increased the release of epinephrine (E) from incubated slices of the hippocampus. These effects may suggest that physiological concentrations of Glu inhibit CA release from hippocampal neurons *in vitro* and therefore enhance its excitatory effects *in vivo*.

Keywords: hippocampus; glutamate; release of catecholamines; rabbit

The hippocampus is one of the constituent elements of the brain's limbic system that controls memory. It is a small, seahorse-like structure located in the parietal lobe of the cerebral cortex of the telencephalon (*cerebrum*). It plays an important role in the consolidation (transfer) of information from short-term to long-term memory (especially during sleep) and spatial orientation. Damage to the hippocampus hinders learning ability (1).

The hippocampus is made up of a *fascia dentata*, *hillus* and *cornu Ammonis*. There are four areas CA1, CA2, CA3 and CA4 in the hippocampus. The hippocampus itself can produce new neurons throughout the whole body's life span (2). Excessive stress results in post-traumatic stress disorder (PTSD), activates the hypothalamus-pituitary-axis (HPA), which in turn causes an increased release of cortisol damaging the hippocampus (28). Other glucocorticosteroids have similar effects, reducing the volume and impairing the function of this brain structure (2-4). This is also how an increase in oxidative stress affects the dopaminergic (DA-ergic) and neurotensinergic (NTS-ergic) systems (5).

The hippocampus (CA1 group cells) belongs to the limbic system. It is located underneath the cerebral cortex. It is located underneath the cerebral cortex and is made of *Ammon's horn* and the *dentate gyrus*. Neurogenesis does not stop there following birth. It plays an important role in managing descriptive (declarative) memory, especially episodic (from short-term to long-term memory). In humans, hippocampus is located in the dorsal part of the *temporal lobe* and borders with the *amygdala* on the medial side. Through connections it acts on the structures of the limbic system and plays a role in controlling impulse and emotional activities (2).

Hypoxia, ischemia, injury, or diseases, as well as prolonged stress, can adversely affect the functioning of the hippocampus. In Cushing's disease, for example, in which the adrenal glands produce large amounts of the stress hormone cortisol, it stimulates the HPA stress axis, which can lead to a decrease in the size of the hippocamp (atrophy). Changes in the hippocampus

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can results in the development of Alzheimer's disease, Huntington's disease, epilepsy, schizophrenia, depression, or general amnesia, as well as other neurodegenerative diseases (15). Some scientists believe that the hippocampus is the "heart of the brain," affecting the integration of different areas of the cerebral cortex. Initially, it was thought that the hippocampus demonstrates only high-frequency activity (5).

It is now argued that in rodents low-frequency hippocampal activity causes subcortical activity extending beyond the hippocampus. In addition, it stimulates the formation of connections between the hippocampus and various areas of the cerebral cortex. Pharmacological inactivation of the hippocampus, in turn, weakens its association with other brain regions; hence the "brain-heart" analogy (7).

By lowering the release of corticotropin-releasing hormone (CRH) from the hypothalamus, the hippocampus inhibits stress reactions. In case of very high levels of glucocorticosteroids in the blood it means that the hippocampus not only does not inhibit but also activates the glucocorticosteroid cascade. This leads to a persistent stress response and hippocampal damage. Under the influence of a strong stressor, the damaged hippocampus loses its ability to inhibit the hypothalamus activity (8).

Most of the branched glutamatergic (Glu-ergic) fibers are found in the hippocampus. Most L-glutamate (L-Glu) is in the end posterior area of commissural fibers and in Schaffers collaterals (9, 10). Excess Glu can cause neurotoxic or neurodegenerative effects leading to degenerative and intensified release of various transmitters, including CAs, in the adrenergic pathways of the brain *in vivo*. The *ex vivo* method made it possible to exclude any other effects of the intracerebral neural systems on the changes in CA's concentrations obtained after applying different concentrations of Glu as a strong stressoric factor. The research hypothesis suggests a possible modulation of catecholamine release from the hippocampal tissue under the influence of Glu. The aim of the research was to confirm the hypothesis about the direct *in vitro* influence of different concentrations of glutamate (Glu; 5, 50 and 200 μM) on the release of catecholamines (CA's) from hippocampal slices incubated for 90 min.

Material and methods

The experiment was conducted on Popielno White female rabbits of 12 weeks of age and 2 ± 0.75 kg average body weight. All the animals assessed before the experiment were in good health condition. Before the experiment, 24 rabbits (i.e., 24 determinations) were divided randomly into four groups (n = 6 in each group). They were kept in individual batteries of cages at a neutral temperature (18-20°C) under a photoperiodic regime of 14L : 10D and were fed *ad libitum*. All experimental procedures had been approved by the 2nd Local Ethics Committee at the Pharmacology Institute in Krakow (No. 116/2019). After decapitation, the brains were removed and placed in 0.9% NaCl and brain structures were dissected. The pieces of hippocampal tissue (about 50 mg) were isolated from the brain (according to the "Atlas of the rabbit brain and spinal cord" – Shek et al., 1986), and then cut with scissors into tiny slices which were placed in incubation wells (cell culture; Sigma-Aldrich, St. Louis, USA) containing 1 ml of Krebs incubation medium (phosphate buffer containing 0.3% glucose and 0.1% bovine serum albumin – BSA) without (control group) or with (study group) three doses of L-Glu (G1626; Sigma-Aldrich, USA) in concentrations of I – 5, II – 50, or III – 200 μM in a medium volume of 1 mL. Every 30 minutes, each slice of the hippocampal tissue was placed in the next well with the medium. Incubation was carried out at 39°C in an atmosphere of 95% air and 5% CO_2 in a Sanyo incubator (MCO-18AIC, Japan). The medium collected after 30, 60, and 90 minutes of the experiment was used for dopamine (DA), norepinephrine (NE), and epinephrine (E) radioimmunoassay (RIA) measurement in accordance with the instructions provided by the company (DRG, Germany). The experiments were performed according to protocols described in previous publications (6, 11). The radioactivity of the samples was measured in a gamma counter „Wizard" (LKB, Austria). The lowest limits of sensitivity and mean recoveries for E and NE assays were 19 $pg \cdot mL^{-1}$ and 0.2 $pg \cdot mL^{-1}$, respectively. The intra- and inter-assay coefficients of variation for E and NE analyses were 10.1% and 12.3%, and 5.7% and 10.9%, respectively. For DA it was 0.10 pg/mL with intra-run error of 12.3% and the inter-run error of 22.7%, respectively. The results were converted for 1 $ng \cdot mg^{-1}$ of hippocampal tissue.

The results were analyzed statistically using two-way analysis of variance for repeated measurements. The significance of differences between mean values was determined by Duncan's test. The calculations were carried out using SigmaStat 2.03 software (SPSS Science Software GmbH, Erkrath, Germany). A probability of $P < 0.05$ or $P < 0.01$ indicated statistically significant or highly statistically significant differences, respectively, between the mean values. Figures were prepared using Grapher 12 (Golden Software Inc., Golden, CO, USA).

Results and discussion

***In vitro* effect of L-Glu on DA release from rabbit's hippocampus slices.** Figure 1 shows the amount of DA released into the incubation medium from the control groups hippocampus tissue and all three experimental groups treated with different doses of Glu. In the control secretion, after the first 30 minutes of the experiment, the value of 0.050 ± 0.01 $ng \cdot mg^{-1}$ of tissue was found, after another 30 minutes, the amount of DA released into the medium decreased to the value of 0.010 ± 0.006 $ng \cdot mg^{-1}$ of tissue ($P < 0.01$), the last measurement of the amount of released DA showed 0.020 ± 0.002 $ng \cdot mg^{-1}$ of tissue. After using Glu for incubation of the hippocampus tissue at a concentration of 5 μM after 30 min of its duration, a value of

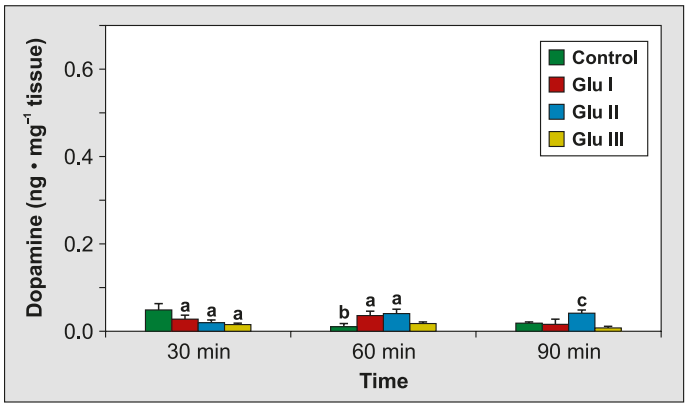

Fig. 1. *In vitro* effect of different glutamate concentrations (5, 50 and 200 µM) pretreatment on dopamine secretion from rabbit hippocampal streaps cells
Explanations: Each value represents the mean ± SEM from 24 determinations. Values marked with different letters differ significantly at P < 0.05-0.01; two-way ANOVA followed by Duncan's *post hoc* test

0.025 ± 0.005 ng·mg^{-1} of tissue was recorded after 60 min. The value did not change (0.027 ± 0.005 ng·mg^{-1} tissue: P > 0.05) and then decreased to 0.018 ± 0.005 ng·mg^{-1} tissue (P < 0.05) after another 30 min. The higher dose of Glu 50 µM used to incubate the hippocampal tissue did not change the amount secreted DA into the medium (0.020 ng·mg^{-1} of tissue) compared to the value found in the Glu 5 µM group, after another 30 minutes of the experiment the value was found to be 0.030 ± 0.040 ng·mg^{-1} of hippocampal tissue and it did not change until 90 minutes of the experiment. The highest dose of Glu 200 µM applied to the greatest extent reduced the DA release in comparison with the control groups tissue (0.014 ± 0.002 ng·mg^{-1} of tissue; P < 0.01), after the next 30 minutes it did not change significantly (0.016 ± 0.002 ng·mg^{-1} of tissue), then it decreased to a value of 0.010 ± 0.002 ng·mg^{-1} tissue after 90 minutes of the experiment, in comparison to the second groups tissue (P < 0.05).

***In vitro* effect of L-Glu on NE release from rabbit's hippocampus slices.** The mean control concentrations of NE released into the medium from hippocampal slices decreased over the 90 min of incubation from 0.5 after 30 min to 0.2 ng·mg^{-1} tissue after 60 and 90 min of incubation, i.e., by 60% respectively. L-Glu added to the medium at a concentration of 5 µM inhibited the release of NE from the hippocampal slice after 30 and 90 min by 60% and 24%, respectively. On the other hand, at 60 min of observation, it caused an increased release of NE from 0.25 to 0.43 ng·mg^{-1} of tissue (Fig. 2).

L-Glu added to the medium at a concentration of 50 µM inhibited the release of NE from hippocampal slices after 30 min. from 0.5 to 0.2 ng·mg^{-1} and after 90 min from 0.18 to 0.04 ng·mg^{-1} tissue. L-Glu added to the medium at a concentration of 200 µM significantly decreased the concentration of NE released from hippocampal slices after 30 min of incubation from 0.4 to 0.2 ng·mg^{-1} and from 0.18 to 0.04 ng·mg^{-1} of tissue after 90 min of incubation (Fig. 2).

During the 90-minute incubation of rabbit hippocampus tissue in the incubation medium with three doses of Glu, it showed no significant effect on the amount of NE released.

***In vitro* effect of L-Glu on E release from rabbit's hippocampus slices.** The mean concentration of E released into the medium from hippocampal slices in the control groups increased during 90 min of incubation from 0.12 to 0.14 and 0.30 ng·mg^{-1} tissue: i.e., by 16% and 150%, respectively. L-Glu added to the medium at a concentration of 5 µM significantly decreased the release of E from hippocampal strips from 0.3 to 0.15 ng·mg^{-1} tissue in 90 min of incubation (Fig. 3).

L-Glu added to the medium at a concentration of 50 µM decreased the release of E from hippocampal homogenates in 30 and 60 min of incubation, from 0.12 to 0.4 ng·mg^{-1} and from 0.14 to 0.3 ng·mg^{-1}, respectively, and decreased from 0.3 to 0.2 ng·mg^{-1} of tissue in 90 min of incubation. L-Glu added to the medium at a concentration of 200 µM increased the release of E from hippocampal homogenates in 30 min

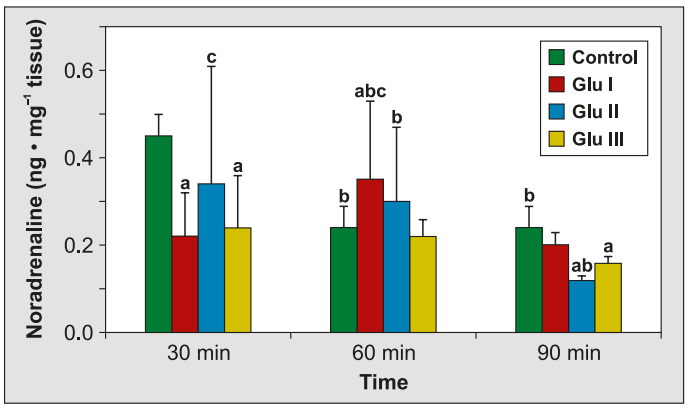

Fig. 2. *In vitro* effect of different glutamate concentrations (5, 50 and 200 µM) pretreatment on noradrenaline secretion from rabbit hippocampal streaps cells
Explanations: as in Fig. 1

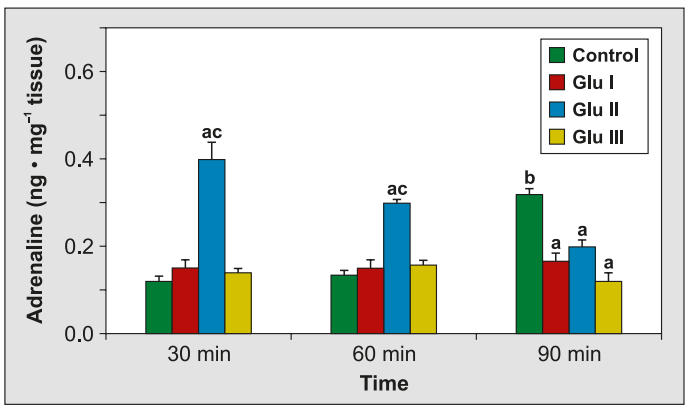

Fig. 3. *In vitro* effect of different glutamate concentrations (5, 50 and 200 µM) pretreatment on adrenaline secretion from rabbit hippocampal streaps cells
Explanations: as in Fig. 1

from 0.12 to 0.16 ng · mg^{-1} in 60 min from 0.13 to 0.16 ng · mg^{-1} and decreased in 90 min from 0.3 to 0.12 ng · mg^{-1} of tissue (Fig. 3). In the case of E, a temporary E release, an increasing effect was demonstrated only in the Glu 50 μM group after 30 and 60 min of the experiment ($P < 0.01$; Fig. 3). The amount of NA released, and especially A, into the medium from rabbit hippocampus tissue turned out to be significantly higher than the DA values found.

It has already been established (31) that in 90 minutes of incubating slices of animal brain structures – in our case the rabbit hippocampus – the release of the two main catecholamines – DA and NE – is reduced by an average of 70% and 53%, respectively. Paradoxically, the concentrations of spontaneously released E increased successively by 17 (after 30 min) and 150% (after 90 min). Perhaps, this surprising phenomenon could be due to the intensification of the metabolic rate on the DA-E pathway, which led to an increase in the concentration of CA released from hippocampal slices (from 0.12 to 0.14 and 0.3 ng · mg^{-1} tissue, respectively). The obtained results also prove the presence of all 3 examined CAs in the hippocampus structures and confirm the fact that this structure is dominated by the NE-ergic over DA-ergic system, as the observed concentrations of the control NE release were 0.5 ng · mg^{-1}, DA – 0.05 ng · mg^{-1} and E – 0.11 ng · mg^{-1} of the tissue.

The hippocampus is a part of the motivational system responsible for learning, memory, plasticity, and emotions. In rats this structure is characterized by a large density of Glu-ergic fibers, ionic (iGluR) and metabotropic (mGluR) receptors. It contains high concentrations of Glu itself, which is the strongest agonist of both groups of Glu-ergic receptors and the most widely distributed transmitter in the whole brain (11-13). Distribution of Glu in individual layers of lyophilized slices of rat hippocampus was demonstrated in the vicinity of the commissural fiber tips and *Schaffer collaterals* (14). Later, Glu was found as a transmitter of the commissural system of the hippocampus (2). Moreover, the hippocampal-cortical connecting pathways were found, and the fact that the concentration of Glu in the hippocampus was increased at the sites of Glu-ergic fiber proliferation was demonstrated. Synaptic Glu-ergic transmission in the commissural collateral pathway of the rat hippocampus was increased because of cerebral ischemia (14). More recent publications indicate different Glu concentrations in the rabbit hippocampus, from 10.5 to 22 μM (17). Glu injected intra peritoneally (*i.p.*) caused a significant reduction in the concentration of glutamic acid decarboxylase (GAD) from 0.13 to 0.05 ng · mg^{-1} of rabbit hippocampus tissue (16). The 15 min episode of ongoing cerebral ischemia as a strong stressoric factor in the rabbit caused a 3-fold increase in extracellular Glu concentration in the extracellular hippocampal dialysate and a twice higher concentration of aspartate (another transmitter stimulating Glu-ergic receptors) (5, 18, 19).

The uptake of Glu is mediated by its Na^+ dependent transporters, preventing its release from the synapse. The astrocyte transporters are responsible for the majority of Glu uptake (20). Unregulated Glu uptake may lead to neuronal hyperstimulation, which may in turn induce neurotoxicity, as it uses up intra-neuronal stores and leads to atrophy and even apoptosis. Hence it is already known that excess Glu acts as a stressoric (noxious) factor. Long-lasting hyperstimulation in the structures of the motivational system triggers the stimulation of the HPA axis with a massive release of glucocorticosteroids and CAs, including cerebral CAs. Does the same thing happen in the hippocampus on an isolated structure slice model? We decided to investigate the hippocampus as a component of the post-stress axis reaction in this study. Rabbit hippocampus slices were used to check the correlation between different Glu concentrations and the release of DA, NE, and E over a 90-minute period from the application of Glu (21).

Since the ascending NE catecholaminoergic system representing the afferent arc of the central sympathetic system projects into the forebrain and the cerebellum, CA-ergic neurons conduct nerve excitations to the centers of the so-called integrators (hypothalamus, limbic cortex, and its nuclei) and influence the activity of the HPA axis. The descending NE-ergic system from the *locus coeruleus* and *subcoeruleus* pathway fields projects into the spinal cord encompassing sympathetic preganglionic neurons in the thoracic segments. These neurons represent the efferent reflex arc of the central sympathetic system, which affects sympathetic transmission and the activity of the peripheral sympathetic nervous system (21).

Almost all forebrain areas (*cortex*, *olfactory bulb*, *hippocampus*, *striatum*, *lower forebrain*, *cerebellum*) are innervated by *locus coeruleus* neurons (23, 24). Most NE-ergic fibers belong to the descending CA-ergic system as the dorsal NE-ergic bundle and together with the ascending ventral NE-ergic bundle innervate the cortex, hippocampus, basal ganglia, thalamus, and part of the hypothalamus.

The hippocampus is a very important center in the stress response, especially in adapting to repeated stressful experiences (25), as it inhibits the HPA axis (26). Excitatory (mainly Glu-ergic) signals to the paraventricular nucleus (PVN) region of the ventral hippocampus are transmitted in nucleus *intrastitialis striae terminalis* (NIST). Stimulation of hippocampal neurons leads to a reduction in basal and stress-induced plasma glucocorticosteroid levels. In contrast, damage to the hippocampus or its abdominal seizure or cleavage of the fimbria-fornix pathway results in increased expression of CRH and vasopressin mRNA in PVN

(27, 28). The hippocampus is one of the critical targets for the negative feedback of glucocorticosteroids. High concentrations of circulating glucocorticosteroids reduce the activity of the HPA axis, acting at the level of the hippocampus (25).

Most of the CA1 area neurons simultaneously contain gamma aminobutyric acid receptor ($GABA_BR_1$, $GABA_BR_2$) subunits, vesicles of Glu mRNA, calcitonin gene-related peptide (CGPR) and proenkephalin mRNA transporters (29). Epinephrinergic fibers run together with DA-ergic fibers in the noradrenergic dorsal bundle.

DA of the mesocortical and mesolimbic pathways is involved in memory, learning and emotional processes (30). Stress increases DA concentrations in the frontal cortex, local changes in DA after stressors in limbic cortex fields may be associated with changes in behavior in response to stress stimuli (17, 31).

Glu inhibited the release of CA from the hippocampus incubated slices, and at higher concentrations tended to increase the release of CA. Notably, Glu significantly increased the release of E from incubated slices of the hippocampus. These effects may suggest that physiological concentrations of Glu (5 μM) inhibit CA release from hippocampal neurons *in vitro* and therefore enhance its excitatory effects *in vivo*. The results of the experiment described in this study clearly indicate that NA is the dominant catecholamine in the hippocampus, as evidenced by certain values. The authors' suggestion regarding very low dopamine values is the fact that it is used for the next stages of catecholamine synthesis. The presented results relate to preliminary *in vitro* studies, which suggests the need for further *in vivo* studies to define the direct effect of Glu on catecholamine release from the hippocampus more precisely.

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