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Participation of glutamic acid in the release of catecholamines from the amygdala of rabbits*

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Summary

Glutamic acid (Glu), as the most stimulating neurotransmitter, occurs in almost all structures of the central nervous system (CNS). Especially high concentrations are found in the structures of the motivational system (*hypothalamus*, *hippocampus*, *amygdala*, *medial prefrontal cortex* – mPFC) and the motor system (*striatum*, *caudate nucleus*, *pallidum*). Glu is also found in the autonomic nervous system (ANS), as well as in peripheral tissues and organs (in the adrenal glands, especially important in stress; the hypothalamic-pituitary-adrenal axis – HPA axis). Catecholamines (CA), including dopamine (DA), norepinephrine (NE), and/or epinephrine (E), are major neurotransmitters that mediate various CNS functions, such as motor control, cognition, emotion, memory processing, pain, stress, and endocrine modulation. The aim of the study was to investigate the *in vitro* effect of different concentrations of Glu (5, 50, and 200 µM in a volume of 1 ml of Krebs medium) on CA release from rabbit amygdala sections collected after decapitation of 12-week-old female rabbits. The same piece of tissue was transferred every 30 minutes to successive incubation wells containing Glu in an appropriate dose. The medium collected from the wells after 30, 60, and 90 minutes of incubation was frozen until DA, NE, and E analyses were performed by the RIA method. The results showed that Glu differently affected CA release from the amygdala. Generally, an inhibitory effect of Glu on CA release from the amygdala was observed. It was surprising to find that the concentrations of E in the amygdala were higher than those of NE and DA.

Keywords: amygdala, glutamate, catecholamine release, rabbit

The amygdala (*corpus amygdaloideum*), a subcortical brain structure found exclusively in mammals, is a fundamental component of the *limbic system* alongside such structures as the hypothalamus, hippocampus, and mPFC of the brain. It plays a key role in emotional learning and memory processes, adaptation to stress, and coordination of sexual and nutritional behavior. It has a modulating effect on the functions of the ANS, as well as on the endocrine functions of the CNS (8, 20, 26). The amygdala is responsible mainly for triggering negative emotions, aggression, anxiety, or fear, and defensive reactions by stimulating the sympathetic nervous system (25, 47). Thus, it plays a central role in anxiety responses to stress and in situations of emotional arousal (49).

Both pharmacological studies and those using lesion imaging (fMRI, PET) or hybridization (12) prove that stimulating these structures causes anxiogenic effects, and their inhibition causes anxiolytic symptoms (25). Information received by the amygdala is directed to the lateral ganglia and then to the basal ganglia complex. They analyze, transform, and confront that information with previously established patterns of emotional memory (1, 33).

The amygdala nuclei also have two-way connections to the centers governing neuromodulation: the cholinergic nuclei of the forebrain (*nuclei basales*), dopaminergic nigra substance (*substantia nigra*), and noradrenergic locus coeruleus (*locus coeruleus*). Thus, many neurotransmitters and stress mediators can regulate the behavioral expression of anxiety or fear, acting on the amygdala nuclei (40). The amygdala is closely related to the various brain structures through

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the neural pathways that organize the expression of anxiety and stress (3). These pathways are monoaminergic, cholinergic, GABA-ergic, and Glu-ergic. Glu plays an important role among the transmitters that excite the amygdala, saturating specific glutamate receptors of a ionotropic N-Methyl-D-Aspartic receptor (iNMDAR) and metabotropic Glu-ergic receptor ($mGluR_{1-8}$) nature (21, 24). The greatest concentration of Glu-ergic receptors occurs in the motivational structures: the hippocampus, basal ganglia, septum, hypothalamus, amygdala, and mPFC of the brain. They are also found in the ganglia of the vegetative nervous system, spleen, adrenal glands, and digestive tract. Their stimulation plays a key role during neuroplasticity of neurons, memory, learning, and remembering, and their permanent stimulation or an excessive accumulation of Glu in the synaptic gaps of neurons may lead to excitoneurotoxicity and degenerative diseases. Gamma-aminobutyric acid (GABA) remains a biogenic antagonist of Glu, inhibiting its activity in brain structures pre- and postsynaptically (15).

The amygdala receives Glu-ergic afferents from various brain regions, including the cerebral cortex and thalamus (28). The action of Glu in rapid neurotransmission and processes related to long-term synaptic plasticity builds on extracellular Glu as an important mediator of the stress effect on the functioning of the amygdala (1). Microdialysis performed on rats has shown that acute stress caused by immobilization of an animal increases the extracellular concentration of Glu in the nuclei complexes of the basolateral (BLA) and central parts of the amygdala (37, 38, 43). Glu, in turn, stimulates the HPA axis (9, 14). Glu release from the amygdala also occurs in other types of stress, and it is modulated by the fear response. In rats, fear expression was shown to occur when shock induced a sharp increase in Glu concentration in the BLA (48).

Neuropeptides released immediately after HPA stimulation regulate the activity of the amygdala and other structures, among which the amygdala plays a major role in inducing stress reactions (9). Exposure to stress increases the release of neurotransmitters from the amygdala, including Glu, GABA, NE, OXY, and serotonin (5-HT). This immediately stimulates signaling pathways that weaken the molecular cascade involving the amplification of postsynaptic neurons, which, in turn, leads to an immediate regulation of specific genes involved in neuroplasticity processes (1, 5).

Various stress factors, including those of a physical and mental nature, increase the concentration of NE released in various regions of the brain, including the amygdala. Stress factors stimulate the ascending noradrenergic transmission system (30, 34) and cause densification of innervation around the amygdala (31). Microdialysis has shown that, under stress, the release of NE in the BLA, MeA (medial amygdala), and CeA (central amygdala) increases (2, 10, 22, 27, 29, 32, 36, 45). Thus, noradrenergic transmission is associated with the onset of negative emotions, such as anxiety and fear, in individuals exposed to stress (19, 44). The increase in NE released is prevented or suppressed by benzodiazepines (BDAs). MeA is innervated by noradrenergic neurons, reaching it mainly from the *locus coeruleus* (27, 39, 41). Microdialysis also proved that stress provoked by immobilization of an animal caused a threefold increase in the concentration of NE released from MeA (27). On the other hand, the administration of α_1- or β-adrenergic receptor antagonists directly into MeA reduced the adrenocorticotropic hormone (ACTH) response to immobilizating stress (27). This proved that an increased release of NE from MeA occurs mainly through ACTH receptors, facilitating the activation of the HPA axis in response to acute stress (27). Stress stimulation of noradrenergic activity in MeA by *bed nucleus stria terminalis* (BNST) projections and the preoptic cortical field is one possible mechanism by which MeA modulates stress-induced activation of the HPA axis. The effects of MeA stimulation by increased concentrations of plasma glucocorticoids are partially blocked by lesions of the preoptic field or BNST alone, but inhibited to a greater extent by lesions of both structures and completely blocked by bilateral end band lesions (6).

Considering the above facts, this study aimed to determine the *in vitro* effect of various concentrations of L-Glu, the primary excitatory amino acid/transmitter in the CNS, on CA release from rabbit amygdala slices. Glu concentrations amounting to 5, 50, and 200 times its physiological concentration were used. It is known that excess Glu in brain neurons causes a strong depolarization of neural presynaptic terminals and intensifies the release of various transmitters, including CA, in the adrenergic pathways of the brain (35, 46).

Material and methods

The experiment involved 8 Popielno White female rabbits aged 12 weeks. Animals were kept in special wooden cages from birth to the start of the trial. At first, they stayed with their mothers, and, after weaning on day 35, they were housed in individual battery cages. Water and feed (DeHeus complete commercial mixture appropriate for does' age) were available *ad libitum*. The lighting schedule was 10 D : 14 L.

The procedure consisted of gently holding each rabbit by the hind limbs and vigorously stunning the back of the head at the height of the medulla so as not to damage the skull, and then bleeding the animal by cutting the two carotid arteries. Consent No. 116/2019 to conduct the experiment was issued by the 2nd Local Ethical Committee for Animal Experiments at the Institute of Pharmacology in Kraków.

After decapitation of each rabbit, the skin was removed from the skull, the meninges were removed using a skull trepanation kit, and the whole brains were isolated (4, 11). The amygdala obtained from each rabbit was first placed

on ice and dried on filter paper. Then it was placed in a physiological saline solution in Petri dishes on ice. Subsequently, each amygdala was cut into smaller fragments of similar weight (approx. 50 mg). Tissue sections were placed in incubation wells (cell culture Sigma) containing 1 ml of the incubation medium (Krebs phosphate buffer – 0.3% glucose and 0.1% bovine albumin – BSA). Tissue incubation was carried out in the atmosphere of carbogen (95% O_2 and 5% CO_2) at a temperature of 38°C in a Sanyo incubator MCO-18AIC. After 10 minutes of stabilization, sections of brain tissue were transferred to subsequent wells containing pure Krebs medium and three different doses of glutamic acid (L-glutamic acid monosodium salt hydrate; *Sigma*): I – 5 µM, II – 50 µM, and III – 200 µM in a volume of 1 ml of Krebs medium, and the same tissue section was then transferred to successive incubation wells containing glutamic acid incubation medium every 30 minutes. The medium collected from the wells after 30, 60, and 90 minutes of incubation of the tissue of the analyzed rabbit brain structure was frozen until the analyzes were performed.

The amount of DA, NE, and E released into the medium after 30, 60, and 90 minutes of incubation was measured by the RIA method in accordance with instructions provided by the producer's (DRG, Germany). In the case of NE, the sensitivity of the method was 42 pg·mg^{-1}, the within-run error was 10.9%, and the outside-run error was 12.3%. These values for A were 19.0 pg·mg^{-1}, 10.6%, and 9.5%, respectively, and for DA they were 29 pg·ml^{-1}, 23.5% and 18.0%, respectively. The concentration values obtained for all three catecholamines were converted into ng·mg^{-1} of amygdala tissue.

The data were analyzed statistically by a two-way ANOVA, followed by Duncan's Multiple Range test. Log transformation was performed to maintain homogeneity of variance and normality. Differences in values were significant at $P < 0.05$. Calculations were performed using Sigma Stat 2.03 (SPSS Science Software GmbH, Germany). The results are presented as means ± SE.

Results and discussion

The amount of DA released into the incubation medium from the amygdala slices of the control group was estimated at 0.07 ± 0.01 ng·mg^{-1} of tissue after the first 30 minutes of the experiment (Fig. 1). During the next 30 minutes, the amount of this catecholamine released into the medium decreased to 0.04 ± 0.01 ng·mg^{-1} of tissue ($P < 0.01$), and there was no change during the last 30 minutes (0.04 ± 0.01 ng·mg^{-1} of tissue). The addition of Glu I to the incubation medium resulted in a significant reduction in the amount of dopamine released into the medium compared to the values found at the same time in the control group (0.02 ± 0.001 ng·mg^{-1} tissue; $P < 0.01$). Unexpectedly, after 60 minutes of incubation of amygdala tissue, there was a significant increase in dopamine secretion (0.08 ng·mg^{-1}; $P < 0.01$), and after 90 min of the experiment, the amount of dopamine secreted decreased to the level of the control group (0.04 ± 0.007 ng·mg^{-1} of tissue). The addition of 50 µM Glu to the incubation

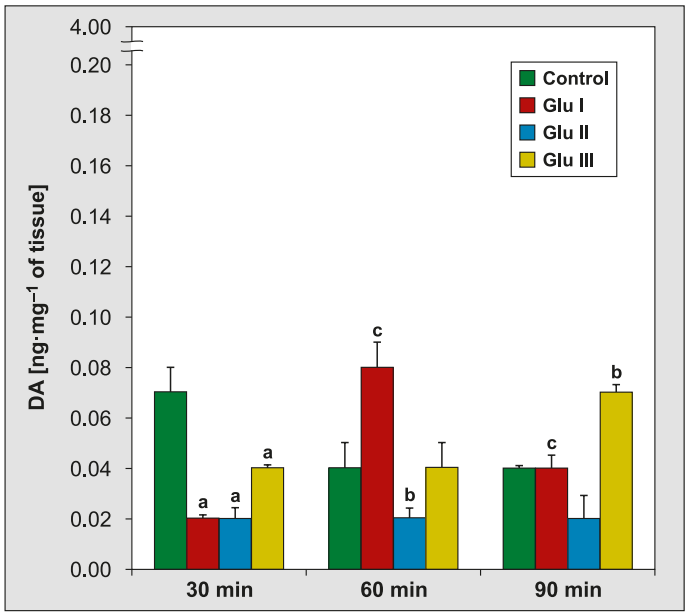

Fig. 1. Changes in the concentration of dopamine (DA) released from rabbit amygdala tissue after 30, 60, and 90 minutes of incubation in the presence of glutamate (L-Glu) at concentrations of 5 (Glu I), 50 (Glu II), and 200 (Glu III) µM (n = 12)

Explanations: a – significant differences ($P \leq 0.05$-0.01) compared with the values in the control group; b – significant differences ($P \leq 0.05$-0.01) between the Glu doses; c – significant differences ($P \leq 0.05$-0.01) compared with the values observed after 30 minutes of incubation

medium also reduced dopamine secretion from rabbit amygdala tissue to 0.02 ± 0.004 ng·mg^{-1} tissue, and it remained unchanged over the next 30 minutes of the experiment (0.02 ± 0.005 ng·mg^{-1} tissue; Fig. 1). The highest dose of Glu III inhibited the release of DA from the amygdala tissue during incubation. After the first 30 minutes, the value recorded was 0.04 ± 0.001 ng·mg^{-1} of tissue, which was significantly lower than the values found after this period of incubation in the control group ($P < 0.01$). Then it gradually increased to 0.07 ± 0.003 ng·mg^{-1} of tissue after 90 minutes of incubation, which was the highest value recorded in all groups (Fig. 1).

The mean control concentrations of NE released into the medium from amygdala homogenates increased after 60 min of incubation (1.99 ± 0.33 ng·mg^{-1} of tissue) compared to the value found after the first 30 min of the experiment (1.30 ± 0.20 ng·mg^{-1} of tissue; $P < 0.05$), and a significant decrease in the amount of NE was noted after 90 min (0.32 ± 0.09 ng·mg^{-1} of tissue; $P < 0.01$; Fig. 2). In all experimental groups, the amounts of NE released into the incubation medium after the first 30 minutes of the experiment were significantly smaller than the amount found in the control group after that period: 0.16 ± 0.09 ng·mg^{-1} of tissue in the Glu I group ($P < 0.01$), 0.69 ± 0.07 ng·mg^{-1} of tissue in in the Glu II group, and 0.51 ± 0.09 ng·mg^{-1} of tissue in the Glu III group. After 60 minutes of the experiment, the values found in all experimental

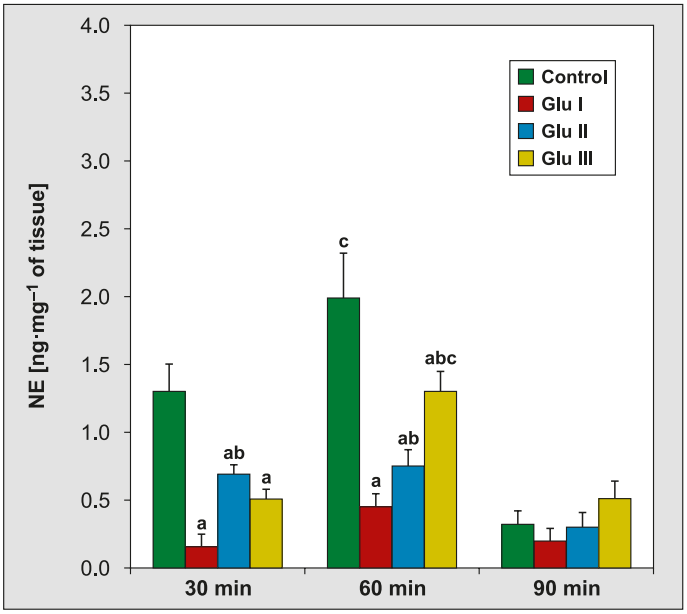

Fig. 2. Changes in the concentration of norepinephrine (NE) released from rabbit amygdala tissue after 30, 60, and 90 minutes of incubation in the presence of glutamate (L-Glu) at concentrations of 5 (Glu I), 50 (Glu II), and 200 (Glu III) µM (n = 12)

Explanations: a – significant differences (P ≤ 0.05-0.01) compared with the values in the control group; b – significant differences (P ≤ 0.05-0.01) between the Glu doses; c – significant differences (P ≤ 0.05-0.01) compared with the values observed after 30 minutes of incubation

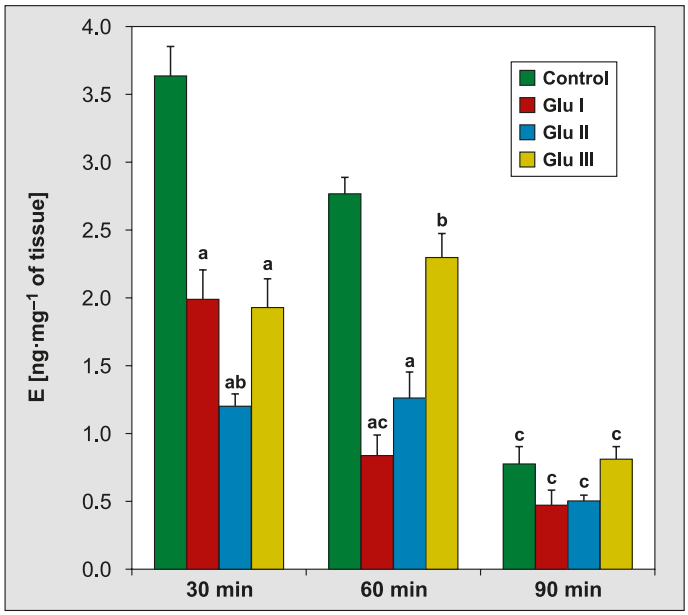

Fig. 3. Changes in the concentration of E released from rabbit amygdala tissue after 30, 60, and 90 minutes of incubation in the presence of glutamate (L-Glu) at concentrations of 5 (Glu I), 50 (Glu II), and 200 (Glu III) µM (n = 12)

Explanations: a – significant differences (P ≤ 0.05-0.01) compared with the values in the control group; b – significant differences (P ≤ 0.05-0.01) between the Glu doses; c – significant differences (P ≤ 0.05-0.01) compared with the values observed after 30 minutes of incubation

groups were still significantly lower than that found in the control group at that time. Although NE secretion increased to 0.51 ± 0.13 ng·mg^{-1} of tissue in the Glu III group, it was still significantly lower than that recorded at the same time in the control group ($P < 0.05$). After 90 minutes of incubation of the amygdala tissue, a significant reduction in the amount of NE secreted into the incubation medium was noted in all groups. NE concentrations determined at that time were significantly lower than those after 30 and 60 minutes of the experiment ($P < 0.01$).

Changes in the amount of E secreted from the amygdala into the incubation medium are shown in Figure 3. In the control group, it decreased gradually from 3.64 ± 0.62 ng·mg^{-1} of tissue after the first 30 minutes of the experiment to 0.77 ± 0.13 ng·mg^{-1} of tissue after 90 minutes ($P < 0.01$; Fig. 3). In all three experimental groups, the use of Glu for incubation of amygdala tissue resulted in a significant ($P < 0.01$) reduction in E secretion into the incubation medium after the first 30 min of the experiment. The strongest inhibition of E release was found in the Glu II group (1.20 ± 0.09 ng·mg^{-1} tissue). The values found in the other two groups, Glu I and Glu III, were very similar (1.99 ± 0.22 and 1.93 ± 0.21 ng·mg^{-1} of tissue). After another 30 minutes of the experiment, a further reduction in E secretion was observed in the Glu I group (0.94 ± 0.15 ng·mg^{-1} of tissue; $P < 0.01$), no changes were observed in the Glu II group (1.26 ± 0.19 ng·mg^{-1} tissue), and the change in the Glu III group was not significant (2.30 ± 0.18 ng·mg^{-1} tissue). After 90 min of incubation, a significant ($P < 0.01$) decrease in the secretion of this catecholamine into the incubation medium was observed both in the control group and in all experimental groups.

The results of our research showed a generally inhibitory effect of L-Glu on the release of catecholamines from rabbit amygdala slices (Fig. 1, 2, 3). As in our previous research, the three concentrations of Glu significantly inhibited *in vitro* CA release from the rabbit hypothalamus within 90 minutes of observation (50). Glu produces strong depolarizing effects on the neuronal terminals of various CNS structures, including adrenergic neurons. Thus, it can be assumed that L-Glu applied at concentrations 5, 50, and 200 times as high as physiologically normal levels, either damaged these terminals or stimulated the activity of enzymes transforming it into GABA by glutamate dehydrogenase (GAD) (50).

Another possibility is that L-Glu probably breaks them down in intracerebral CA pathway synthesis by inhibiting the activity of the DOPA-decarboxylase enzyme and transforming dihydroxyphenylalanine (DOPA) into 3-hydroxytryptamine (DA), which, in turn, was not transformed sufficiently into NE and subsequently into E.

Discussion of the results is challenging because there is no comparable information in the available

literature. Various neurons release both neuropeptides and other neurotransmitters or modulators into the structures of the nervous tissue of the amygdala, hippocampus, or hypothalamus. The obtained results of our own experiment did not take into account the possibility of additional effects of other biologically active substances present in amygdala, such as OXY, 5-HT, arginine vasopressin (AVP) or GABA, which could also influence the final results. Elucidation of this phenomenon would require further experimental investigations of interactions between L-Glu, CA, GABA, and OXY in the central structure of the HPA axis, which is the first to react when harmful factors, such as stressors, affect the organism.

Such a strong inhibition of CA release by the concentrations of L-Glu applied in this study would paradoxically not only cause adrenolysis of the amygdala but also intensify the stimulating effects of Glu on the neurons of the rabbit motivational structures investigated in this study (17, 50). Such hyperstimulation can lead to a weakening of neuronal function and to neuronal degeneration (apoptosis).

Glu, asparagine, and glutamine (Gln) are formed from their common precursor, aspartic acid (Asp). Glu acid itself is synthesized from its precursor, α-ketoglutarate, in the Krebs cycle. Glu is synthesized in neurons mainly as an excitatory transmitter, same as Asp, increasing the flow of positive ions (Na^+ and Ca^{2+}) by opening ion channels after binding to the appropriate specific receptors. Stimulation of these receptors is terminated by a Cl^--independent membrane transport system that is used only for reabsorption of Glu and Asp across the presynaptic membrane. Glu can is also reabsorbed (via reuptake) into neurons for later use. The excess Glu released at synapses is converted into Gln (a compound devoid of excitotoxic properties) by adjacent astrocytes (glial cells). Gln is safely transported *de novo* to neurons for conversion into Glu. Excessive accumulation of Glu in astrocytes is the cause of its neuroexcitotoxicity. Further absorption of the excess Glu is inhibited, which causes the continuous stimulation of neuronal terminals by depolarizing them in the subsynaptic space. This leads to the release of various transmitters, exhaustion of energy reserves, and finally to neurodegenerative changes and neuronal apoptosis (6, 48). Glu acts on specific N-Methyl-D-Aspartic acid receptors (iNMDAR) and non-NMDA, that is, metabotropic receptors (mGluR, group III). The NMDA receptor is an ion channel for Ca^{2+}, Na^+, and K^+ ions (18).

It appears that the results of this study are a consequence of the stimulating effect of Glu on group II and/or III receptors located presynaptically as inhibitory autoreceptors on the synapses of Glu-ergic amygdala neurons. When stimulated by Glu, they reduce the intracellular concentration of cAMP and cGMP by inhibiting the activity of adenylate and guanylate cyclase. Those receptors may also exert their actions through G proteins mediating Ca^{2+} channels. Stimulation of group I mGluR receptors on adrenergic synapses of the brain would cause depolarization of neuronal presynaptic terminals and an increased release of transmitters, including CA. This hypothesis would require experimental confirmation with the use of radiolabeled substances to diagnose the presence of specific mGluR receptors in an *in vitro* culture of rabbit neurons.

CA concentrations determined in the present study are similar to those found in our other studies under similar conditions (90 minutes of incubation) after applying the same concentrations of L-Glu to rabbit hypothalamus tissue (50). L-Glu, applied in concentrations 5, 50, and 200 times as high as physiologically levels, most strongly inhibited, about 92%, the release of CA, especially DA, after 30 minutes of incubation of the amygdala homogenate. These activities, along with a simultaneous reduction in the release of NE and E (by 40%), could indicate the inhibition of the enzymatic cascade in the synthesis of amygdala CA. This is supported by results reported by Humphries et al. (18) after the use of aspartame with 40% Asp in its composition. Asp is an analog of Glu saturating the same glutamatergic receptors in the brain. This type of aspartame caused signs (reduction in the release of NE and E) in rats that resulted from disturbances in CA distribution in various brain structures. CA are important transmitters governing the survival of individuals. DA, 5-HT, Glu, GABA, and acetylcholine (ACh) are especially important. Tyrosine hydroxylase activity is inhibited by high concentrations of DA due to the effect of this catecholamine on tetrahydrobiopterin (THB), which is a cofactor of tyrosine hydroxylase, through a feedback mechanism. This system is essential for preventing the excessive-synthesis of DA, an inhibitory transmitter in the brain. Therefore, if L-Glu reduces DA synthesis in the amygdala, which is favorable stronger stimulation of neurons by excitatory transmitters: Glu or ACh. Aspartame increases the synthesis of phenylalanine and tyrosine, and thus CA, including DA (7). According to Mehl-Madrona (http://www.healingarts.org/mehl-madrona/autism), a change in DA concentrations in the brain occurs after aspartame consumption by people suffering from Parkinson's disease. Bowen and Evangelista (http://www.wnho.net/aspartame_brain_damage.htm.) demonstrated that the intaking of aspartame resulted in higher plasma concentrations of phenylalanine and Asp, which penetrate the blood/brain barrier. On the other hand, a single oral administration of aspartame to mice resulted in opposite effects, that is, an increase in NE and DA (NE precursor) concentrations and a decrease in 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) concentrations in various regions of the brain. However, rats have a different mechanism of aspartame metabolism than humans (42).

Glu is the most abundant neurotransmitter in the brain. As previously noted, it is formed from α-ketoglutarate in the Krebs cycle. It is synthesized mainly in neurons as a neurotransmitter with the strongest stimulating effect on receptors. After saturating the appropriate type of specific receptors, it starts to intensify the flow of positive ions (Na^+ and Ca^{2+}) by stimulating these receptors and opening their ion channels. Glu is also reabsorbed by neurons for later use (stored). Astrocytes swollen with excess glutamate rupture and contribute to L-Glu hypercytotoxicity, as they are incapable of absorbing the entire excess pool of extracellular Glu (16). Continuous stimulation of presynaptic iNMDA and non-iNMDA receptors may damage these receptors.

Glu, as the strongest excitatory amino acid transmitter, and GABA, as a pre- and postsynaptic inhibitory amino acid transmitter, are closely related in the sense that GABA is synthesized by a simple modification of Glu (13). Glu is widely distributed in the forebrain, cerebellum, and other neurons, but becomes a neurotransmitter only when accumulated in synaptic vesicles at the axon terminals (23). Glu acts specifically on at least five of the eight receptor subtypes, especially NMDA receptors dominant in the not yet fully developed brain when transmission is weak and extremely plastic. It is these receptors that make the flow of Na^+ and Ca^{2+} ions possible after channel opening. NMDA receptors appear to be key players in LTP and synaptic plasticity because they support learning and lifelong memory storage (13).

Death of neurons can occur because of the action of Glu in two ways. First, the accumulation of Ca^{2+} ions in the cell leads to an increase in mitochondrial Ca^{2+} uptake and collapse. Second, ions of Ca^{2+} activate genes in cellular DNA which produce proteins that kill the cell by apoptosis (23).

In conclusion, the results of our *in vitro* experiment indicate an inhibitory effect of Glu on CA release from rabbit amygdala tissue. Our theoretical predictions regarding the results of the experiment indicated a different effect of Glu on the amygdala of rabbits in the secretion of all three catecholamines. Therefore, further studies on the possibility of Glu's receptor effect on target tissues are needed, especially since we initially demonstrated (unpublished data) that intraperitoneal (*i.p.*) injection of Glu at a concentration of 50 µM did not change the concentration of Glu in this tissue, while the use of antagonists and agonists of selected groups of glutamatergic metabotropic receptors contributed to an increase in Glu concentration in the amygdala tissue.

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