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An amperometric glutamate biosensor for monitoring glutamate release from brain nerve terminals and in blood plasma

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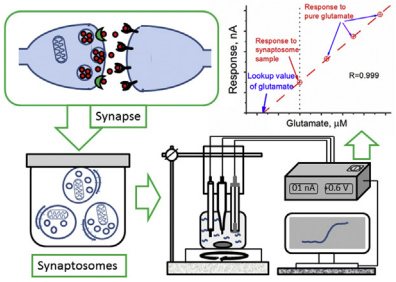
HIGHLIGHTS

- A biosensor-based approach for the analysis of glutamate transport was developed.
- Isolated rat brain nerve terminals (synaptosomes) were used for the studies.
- Tonic, exocytotic and transporter-mediated glutamate release rates were determined.
- The biosensor results were confirmed by traditional methods of glutamate analysis.

GRAPHICAL ABSTRACT



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ABSTRACT

An excess of the excitatory neurotransmitter, glutamate, in the synaptic cleft during hypoxia/ischemia provokes development of neurotoxicity and originates from the reversal of Na^+-dependent glutamate transporters located in the plasma membrane of presynaptic brain nerve terminals. Here, we have optimized an electrochemical glutamate biosensor using glutamate oxidase and developed a biosensor-based methodological approach for analysis of rates of tonic, exocytotic and transporter-mediated glutamate release from isolated rat brain nerve terminals (synaptosomes). Changes in the extracellular glutamate concentrations from 11.5 ± 0.9 to 11.7 ± 0.9 μM for 6 min reflected a low tonic release of endogenous glutamate from nerve terminals. Depolarization-induced exocytotic release of endogenous glutamate was equal to 7.5 ± 1.0 μM and transporter reversal was 8.0 ± 1.0 μM for 6 min. The biosensor data correlated well with the results obtained using radiolabelled L-[^{14}C]glutamate, spectrofluorimetric glutamate dehydrogenase and amino acid analyzer assays. The blood plasma glutamate concentration was also tested, and reliability of the biosensor measurements was confirmed by glutamate dehydrogenase assay. Therefore, the biosensor-based approach for accurate monitoring rates of tonic, exocytotic and transporter-mediated release of glutamate in nerve terminals was developed and its adequacy was confirmed by independent analytical methods. The biosensor measurements provided precise data on changes in the concentrations of endogenous glutamate in nerve terminals in response to stimulation. We consider that the glutamate biosensor-based approach can be applied in clinics for neuromonitoring glutamate-related parameters in brain samples, liquids and blood plasma in stroke, brain trauma,

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therapeutic hypothermia treatment, etc., and also in laboratory work to record glutamate release and uptake kinetics in nerve terminals.

1. Introduction

Glutamate is a key excitatory neurotransmitter in the central nervous system because of its involvement in almost all aspects of normal brain functioning. The main mechanism of glutamate release from presynaptic nerve terminals to the synaptic cleft is stimulated exocytosis. Neuronal injury and death in stroke, cerebral hypoxia/ischemia, hypoglycemia, traumatic brain injury, etc., are mainly provoked by an increase in the concentration of extracellular glutamate in the synaptic cleft that overstimulates the glutamate receptors and initiates an excessive calcium entry. Under these pathological conditions, excessive extracellular glutamate originates from the neuronal cytoplasm and is released through the membrane Na^+-dependent glutamate transporters operated in a reverse mode [1]. Beside the stimulated exocytotic release of glutamate and pathological glutamate transporter reversal, unstimulated tonic release from nerve terminals also deserves attention. This release occurs permanently via several mechanisms and is an important constituent that balances the ambient level of glutamate in the synaptic cleft between the episodes of exocytosis [2,3].

Recently, we have revealed that alterations in the extracellular glutamate level during therapeutic hypothermia can be unique for each patient [4]. Therefore, the test parameters and clinical criteria for continuous glutamate monitoring and evaluation of individual hypothermia-induced effects should be developed for personalized medicine practice.

Excessive extracellular glutamate can be removed from brain interstitial fluids to the blood plasma for the maintenance of proper extracellular glutamate homeostasis in the mammalian central nervous system [5–7]. The glutamate concentration in the blood plasma increases in case of ischemic stroke and other neurological disorders [8].

The traditional methods of glutamate determination include high performance liquid chromatography (HPLC), gas chromatography-mass spectrometry (GC-MS), and spectrophotometry. These techniques are sensitive and powerful, but they require very expensive and complex equipment that limits their application in the laboratory work and monitoring kinetics of neurotransmitter release/uptake in clinics [9]. The electrochemical biosensors for the glutamate determination are faster, more user-friendly and cheaper than the traditional methods [10]. Furthermore, the biosensors can be miniaturized for the detection of glutamate in living tissues that cannot be achieved by other methods [11]. Thus, the development and application of the glutamate-sensitive biosensors is a new perspective for simplifying analysis procedure and decreasing its price that can result in their wider involvement in the neurochemical research.

Currently, a number of glutamate-sensitive biosensors were developed. They are based on glutamate oxidase (GluOx) [12,13] or glutamate dehydrogenase (GLDH) [14,15]. Both enzymes oxidize glutamate to ketoglutarate, although the first enzyme also generates hydrogen peroxide, whereas the second one reduces a cofactor (NAD^+). Both biosensor types appeared to be efficient in the determination of glutamate concentration in biological and food samples. However, GLDH requires the addition of the factor to the working buffer or its co-immobilization with the enzyme. This fact makes the GLDH-based biosensor more complex in comparison with the GluOx-based one. Furthermore, the stability of the GluOx-based biosensor is much better [16–19].

In our previous study, we developed a biosensor-based method for monitoring the rate of glutamate uptake that takes into consideration the extracellular level of endogenous glutamate in the preparations of nerve terminals [20].

The aim of this study was to upgrade the recently constructed amperometric glutamate oxidase-based biosensor and develop a methodological algorithm for precise monitoring the rates of exocytotic glutamate release and the glutamate transporter reversal (pathological ischemia-related glutamate transport mechanism) in nerve terminals. The experimental data obtained with the glutamate biosensor were confirmed by independent analytical methods.

2. Materials and methods

2.1. Design of amperometric transducers

In this work, we used in-lab made platinum disc electrodes as amperometric transducers (Fig. 1A and B). The electrodes were produced according to the following algorithm. First, 3 mm long platinum wire of 0.4 mm in diameter was sealed in the terminal part of a glass capillary of 3.5–5 mm in outer diameter. An open end of the wire served as a working surface of the transducer. Then the platinum wire was connected by fusible Wood alloy to the conductor placed inside the capillary. A contact pad was attached to the other end of the conductor for connection with the measuring setup. The working electrode surface was obtained by grinding with alumina powder (particles of 0.1 μm and 0.05 μm) and treated with pure ethanol before the bioselective element immobilization. The electrode surface was periodically restored using the same grinding procedure. During the operation of the amperometric biosensor, we used a three-electrode scheme of the amperometric analysis (Fig. 1, C). The working amperometric electrodes, an auxiliary platinum electrode and an Ag/AgCl reference electrode were connected to the PalmSens potentiostat (Palm Instruments BV, The Netherlands).

2.2. Modification of amperometric transducer with phenylenediamine

The proposed biosensor operation is based on the measurement of the oxidation current flowing to the working electrode at applied potential (+0.6 V vs Ag/AgCl). The current is generated due to the decomposition of hydrogen peroxide, a product of the enzymatic reaction of glutamate oxidation, on the working electrode. Biological samples contain numerous substances (ascorbic acid, cysteine, dopamine, etc.) that can be also oxidized at the electrode resulting in the errors in glutamate measurements. To improve the selectivity of the biosensor, we placed a permselective membrane onto the electrode surface (below the enzyme layer). This membrane was based on poly(*m*-phenylenediamine) (PPD) and contained pores that allowed the access of hydrogen peroxide molecules to the electrode surface, but blocked the molecules of a larger size. The membrane was prepared by a method described in Ref. [21]. Briefly, a three-electrode system with a bare working electrode was

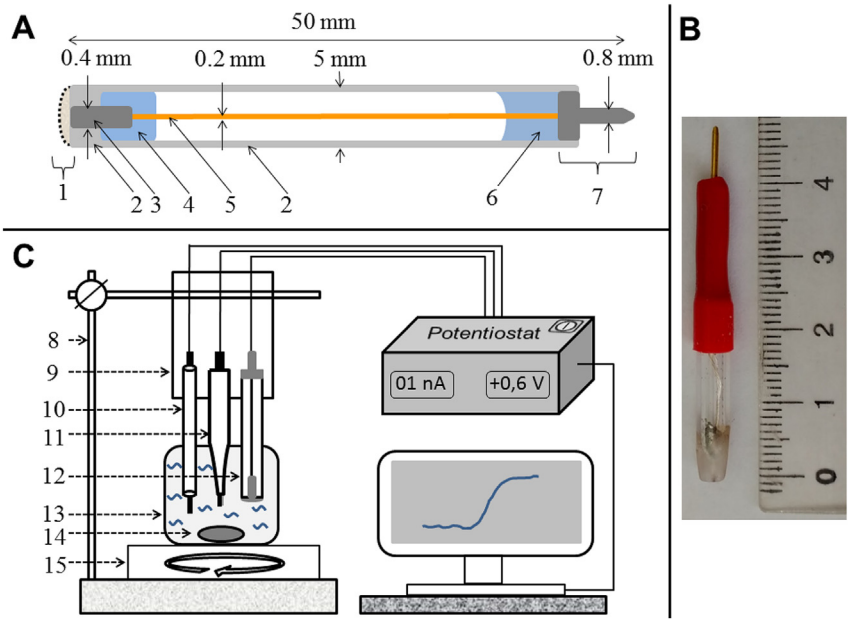

Fig. 1. Scheme (A) and photo (B) of glutamate biosensor and amperometric setup (C) (1-bioselective membrane; 2- glass capillary; 3-platinum wire; 4- fusible Wood alloy; 5 - Ag-conductor; 6- epoxy; 7- contact pad; 8- stand; 9-holder; 10- auxiliary platinum electrode; 11- Ag/AgCl reference electrode; 12- working amperometric electrode; 13- working cell; 14-magnet; 15- magnetic stirrer).

immersed in 5 mM solution of m-phenylenediamine (Sigma-Aldrich Chemie, Germany). Afterwards, we obtained 5–10 cyclic voltammograms and tested the effectiveness of PPD membrane. As was shown in our previous works with the same transducer, the membrane almost completely blocked the access of large electroactive substances (ascorbic acid, dopamine, cysteine, paracetamol, and uric acid) to the electrode surface [22,23]. Next, the enzyme was immobilized onto the PPD membrane surface.

2.3. Fabrication of bioselective element of the biosensor

The bioselective element of the biosensor was obtained by covalent immobilization of the enzyme and auxiliary substances on the surface of amperometric transducer. The initial solution contained 8% (hereinafter - mass fraction) of glutamate oxidase (EC 1.4.3.11, from *Streptomyces* sp. (recombinant) with an activity of 7 U mg^{-1}, Yamasa Corporation, Tokyo, Japan), 4% of bovine serum albumin (Sigma-Aldrich Chemie, Germany), 10% of glycerol (Sigma-Aldrich Chemie, Germany) in 100 mM phosphate buffer, pH 6.5. Glycerol was added to stabilize the enzyme during its immobilization, to prevent early drying and to improve the membrane adhesion to the transducer surface. This solution was mixed with 0.4% aqueous solution of glutaraldehyde (crosslinking agent) (Sigma-Aldrich Chemie, Germany) in the ratio 1:1. Once this mixture was deposited onto the transducer surface, it was dried for 40 min in air at room temperature. The unbound components of biomembrane and the excess of glutaraldehyde were washed out of the biosensor with the working buffer solution.

2.4. Measuring procedure

The measurements were carried out at room temperature in an open 2-mL measuring cell at constant stirring and at the constant potential of +0.6 V vs Ag/AgCl reference electrode ("amperometric detection" technique). As a working buffer served 25 mM HEPES (Sigma-Aldrich Chemie, Germany), pH 7.4.

The glutamate concentrations in the working cell were obtained by addition of the aliquots of stock solutions (50 mM–1 mM). All measurements were carried out in three replications.

2.5. Isolation of rat brain nerve terminals (synaptosomes)

Wistar rats (males 100–120 g body weight from the vivarium of M.D. Strazhesko Institute of Cardiology, National Medical Academy of Sciences of Ukraine) were maintained in accordance with the European Guidelines and International Laws and Policies. The animals were kept in the animal facilities of the Palladin Institute of Biochemistry, National Academy of Sciences of Ukraine, Kyiv. They were housed in a quiet, temperature-controlled room (22–23 °C) and were provided with water and dry food pellets *ad libitum*. Before brain removing, rats were decapitated. All procedures conformed to the guidelines of the Palladin Institute of Biochemistry. The total number of animals used in the study was 12. The cerebral hemispheres of decapitated animals were rapidly removed and homogenized in ice-cold solution containing 0.32 M sucrose, 5 mM HEPES-NaOH, pH 7.4 and 0.2 mM EDTA (Sigma, U.S.A.). The synaptosomes were prepared by differential and Ficoll-400 (Amersham, UK) density gradient centrifugation of rat brain homogenate according to the method of Cotman [24] with slight modifications [25,26]. All manipulations were performed at 4 °C. The synaptosomal suspensions were used in experiments during 2–4 h after isolation. The standard saline solution was oxygenated and contained (in mM): NaCl 126; KCl 5; $MgCl_2$ 2.0; NaH_2PO_4 1.0 (Sigma, U.S.A.); HEPES 20 (Sigma, U.S.A.); pH 7.4 and D-glucose 10 (Sigma, U.S.A.). The Ca^{2+}-supplemented medium contained 2 mM $CaCl_2$ (Sigma, U.S.A.). Protein concentration was measured as described by Larson [27].

2.6. Glutamate release experiments

Synaptosomes were diluted in the standard saline solution to the concentration of protein 2 mg mL^{-1} and after pre-incubation at 37 °C for 10 min were loaded with L-[^{14}C]glutamate (1 nmol mg^{-1} of protein, 238 mCi $mmol^{-1}$) in Ca^{2+}-supplemented oxygenated

standard saline solution at 37 °C for 10 min. After loading, the suspension was washed with 10 vol of the ice-cold oxygenated standard saline solution; the pellet was resuspended in this solution to a final concentration of protein 1 mg mL^{-1} [28]. The synaptosomal suspension (125 μl of the suspension, 0.4 mg mL^{-1} of protein) was pre-incubated at 37 °C for 8 min (typical experimental approach to restore ionic gradients). The release of L-[^{14}C]glutamate from synaptosomes was analyzed in the Ca^{2+}-containing and Ca^{2+}-free incubation media. To assess the release characteristics synaptosomes were incubated for different time intervals 0–30 min and rapidly sedimented in a microcentrifuge (20 s at 10,000 × *g*). The glutamate release was measured in aliquots of the supernatant (100 μl) and the pellets by liquid scintillation counting with scintillation cocktail ACS (1.5 mL). Total synaptosomal L-[^{14}C] glutamate content was equal to 200000 ± 15000 cpm mg^{-1} protein. The release of the neurotransmitter from the synaptosomes incubated without stimulating agents was used for assay of tonic release [29].

For the biosensor experiments, synaptosomes were diluted in the standard saline solution to the protein concentration of 0.4 mg mL^{-1}. The synaptosomal suspensions (1 mL) were pre-incubated at 37 °C for 8 min (typical experimental approach to restore the ionic gradients). The release of endogenous glutamate was carried out in Ca^{2+}-containing and Ca^{2+}-free incubation media. The samples for analysis of the release of endogenous glutamate from synaptosomes were prepared, as described above.

2.7. Glutamate dehydrogenase assay

The extracellular level, release and total concentration of endogenous glutamate in the preparations of synaptosomes and the glutamate concentration in the blood plasma were detected using glutamate dehydrogenase assay [30]. The measurements were carried out in the supernatant aliquots after rapid sedimentation of synaptosomes in a microcentrifuge (20 s at 10,000 × g). Appropriate controls were set for unspecific decrease/increase of the fluorescent signal.

2.8. Assay using amino acid analyzer

The extracellular level, release and total concentration of endogenous glutamate in the synaptosomal preparations were evaluated using Amino Acid Analyzer T 339. Synaptosomes were incubated at 37 °C for 15 min, then the release was started by the addition of 35 mM KCl in the Ca^{2+}-containing and Ca^{2+}-free incubation media and each preparation was immediately sedimented in a microcentrifuge (20 s at 10,000 × g). Two times diluted preparations (3% sulfosalicylic acid) were analyzed by Amino Acid Analyzer T 339 by the method of ion exchange chromatography. Final protein concentration in the synaptosomal preparations was 0.4 mg mL^{-1}.

2.9. Statistical analysis

The results were represented as mean ± S.E.M. of *n* independent experiments. The differences between two groups were compared by two-tailed Student's *t*-test. The differences were considered significant when $P \leq 0.05$.

3. Results

3.1. The glutamate biosensor engineering and characterization

At the beginning of development of the glutamate-sensitive biosensor, it was necessary to select a type of transduction and a type of bioselective element. In this work, the amperometric transducers were used, since the characteristics of amperometric biosensors depend weakly on the ionic strength and buffercapacity of an analyzed sample (in comparison with the potentiometric and conductometric biosensors), so these biosensors are well-suited for analysis of biological samples [31,32]. The two enzymes can be used for creation of a bioselective element of the biosensor (GluOx or GLDH), as was described in the Introduction section. Here we report the GluOx-based biosensor system chosen, firstly, because this enzyme shows better stability, and secondly, it does not require any external co-factor. The enzyme was immobilized on the sensitive surface of the amperometric transducer and catalyzed the following reaction (1):

$$\text{Glutamate} + O_2 \xrightarrow{\textit{Glutamate oxidase}} \alpha - \text{ketoglutarate} + NH_3 + H_2O_2 \quad (1)$$

A positive potential (+0.6 V vs Ag/AgCl) was applied to the transducer, and for this reason hydrogen peroxide generated by GluOx was decomposed in reaction (2), resulting in the formation of electrons directly registered by the amperometric transducer:

$$H_2O_2 \xrightarrow{+600\ mV} 2H^+ + O_2 + 2e^- \quad (2)$$

A current generated during H_2O_2 decomposition was directly proportional to the glutamate concentration.

At the first stage of research, we optimized the GluOx immobilization, since the immobilization procedure greatly affects the enzyme activity and the biosensor characteristics. The immobilization of the enzyme in the bioselective membrane on the transducer surface was performed by covalent cross-linking of GluOx and BSA by glutaraldehyde. We preferred this method of the enzyme immobilization because it is well studied and effective; numerous enzymes were successfully immobilized by glutaraldehyde during the biosensor development [33,34]. To optimize the immobilization conditions, we studied the dependence of the biosensor response on the concentration of GluOx in the bioselective element, the concentration of glutaraldehyde and duration of the immobilization. We checked also a limit of the glutamate detection (LOD) in all experiments, because the biosensor was designed for the measurements of low concentrations of glutamate.

The experiments with different GluOx concentrations in the biomembrane demonstrated that the biosensor responses were almost the same at GluOx concentration from 1% to 4% and the responses decreased at the concentrations below 1% (Fig. 2, A) because the amount of enzyme was insufficient for the most effective GluOx catalysis. An increase in the GluOx concentration up to 6% led to a slight decrease in the biosensors sensitivity to glutamate, probably because of a decrease in biomembrane permeability. Furthermore, LOD slightly decreased at an increase of the GluOx concentration, and the minimal LOD was observed at 4% GluOx concentration. Thus, 4% GluOx was selected for further experiments. The optimal concentration of glutaraldehyde was 0.4%. A lower concentration of glutaraldehyde was insufficient for maximum immobilization of the enzyme in the biomembrane. Higher concentrations of glutaraldehyde caused inactivation of GluOx in the biomembrane.

The duration of GluOx immobilization with 0.4% glutaraldehyde was studied in the range from 20 to 40 min (Fig. 2, B). The biosensors were operational in all cases, and there was no statistically significant difference in their LOD. The highest responses were obtained at 30 min, so this time was used in further experiments.

On the next stage of work, the stability and reproducibility of the biosensor response were studied as well as the changes of LOD in

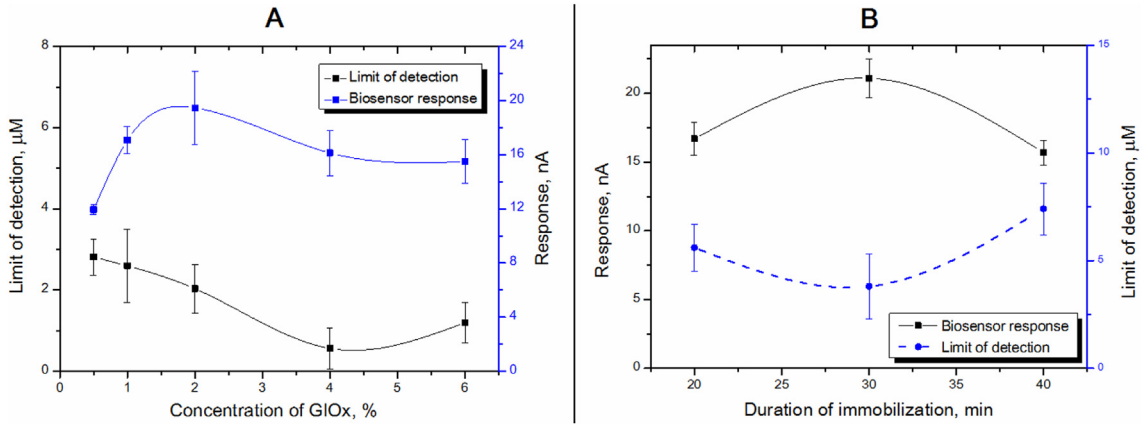

Fig. 2. Dependence of the biosensor responses and the limit of glutamate detection on the concentration of GluOx in the bioselective element of biosensor (A) and on the duration of enzyme immobilization (B). Glutamate concentration – 100 µM. Measurements were done in 25 mM HEPES buffer, pH 7.4, at a constant potential of +0.6 V vs Ag/AgCl. Points on the plots represent mean ± standard deviation of values obtained with 5 biosensors.

time. This experiment was necessary to demonstrate that the biosensor can be used for multiple determinations of glutamate in samples. To perform the experiment, the biosensor responses were obtained continuously in a span of 3 h. The biosensor measurement of the glutamate concentration took about 2 min, and after that the biosensor was washed with working buffer for 10 min. Typical results for a single biosensor of 5 studied are shown in Fig. 3. There was no significant decrease in the biosensor responses during this experiment; this indicated that GluOx was tightly immobilized on the transducer surface and was not washed out or lost the activity during measurements. A relative standard deviation of the biosensor responses was 1.9%–2.5% that is quite a good result (the usual deviation of the responses of enzyme-based biosensor lies between 2% and 4%). LOD also did not change significantly during the experiment.

The linear range of the glutamate determination was from 2 µM to 400 µM. The typical calibration curve of the biosensor for the glutamate determination is shown in Fig. 4, A. The range of high glutamate concentrations was not shown on the curve since glutamate does not reach high concentrations in the biological samples, hence we did not use this part of the biosensors dynamic range. To demonstrate the biosensor work in optimal conditions a typical response of the biosensor to glutamate is shown in Fig. 4, B. As seen, the response is quick, less than a minute, with a low noise -to- signal ratio. The limit of glutamate detection was 2 µM. It was measured as the glutamate concentration, the response to which is three times larger than the baseline noise (see a scheme in Fig. 4, B). Since the glutamate concentration in synaptosomal samples was expected to be 7–30 µM, the biosensor sensitivity was enough to perform the analysis even after some dilution of the samples in the working cell.

GluOx from *Streptomyces* sp. that was used in this work is highly selective for glutamate [35]. Thus, other amino acids might not interfere with the biosensor operation. According to our results, the biosensor was not sensitive to most amino acids. The biosensor response to the addition of glutamine, asparagine, and aspartic acid (1 mM) to the working cell was insignificant (<1 nA) and could not interfere with the glutamate detection.

Altogether the results demonstrate that the characteristics of the proposed biosensor are sufficient for the selective determination of glutamate in solution, and the next stage of the work was devoted to the detection of this neurotransmitter in the synaptosomal samples.

3.2. Monitoring tonic, exocytotic and transporter-mediated release of endogenous glutamate from nerve terminals using the glutamate biosensor

The nerve terminals (synaptosomes) isolated from rat cortex were used in the experiments. Synaptosomes retain all

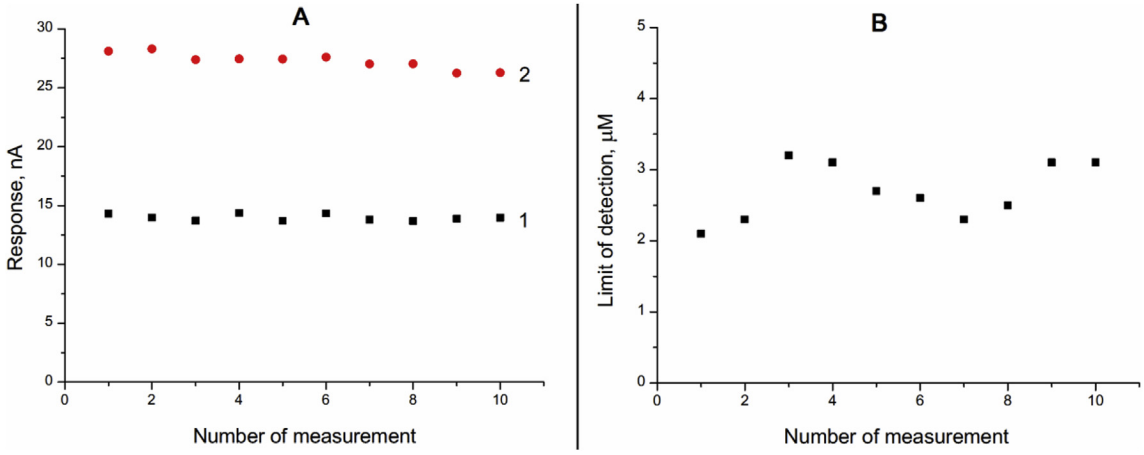

Fig. 3. Reproducibility of the biosensor responses on 50 µM (1) and 100 µM (2) glutamate (A) and the limit of glutamate detection (B) during 3 h. Measurements were done in 25 mM HEPES buffer, pH 7.4, at a constant potential of +0.6 V vs Ag/AgCl.

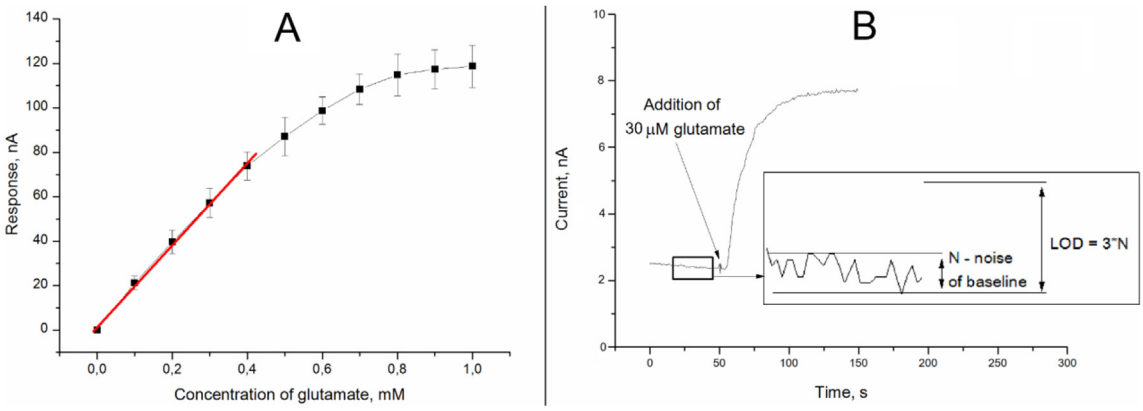

Fig. 4. Calibration curve of the biosensor for glutamate determination (A) and typical response of the biosensor (B). Measurements were done in 25 mM HEPES buffer, pH 7.4, at constant potential of +0.6 V vs Ag/AgCl. Points on the plot (A) represent mean ± standard deviation of values obtained with 5 independent biosensors.

characteristics of the intact nerve terminals, e.g., the ability to maintain a potential of the plasma membrane, to accomplish depolarization-evoked release and uptake of the neurotransmitters. Two types of experiments with synaptosomes were performed, i.e. the experiments (i) without stimulation to assess the changes in the extracellular glutamate concentrations, and (ii) with stimulation to evaluate the exocytotic and transporter-mediated glutamate release. For the biosensor measurement 100–200 μL aliquots of supernatant (see Materials and methods section) were added to the working cell and the responses were recorded. Then the aliquots of a standard glutamate solution were injected into the same cell and three more glutamate measurements were done. The glutamate concentration in the synaptosomal sample was calculated according to the proportional dependence of the response value on the glutamate concentration in the cell.

The time-course of changes in the extracellular glutamate concentrations in the samples of nerve terminals measured by the glutamate biosensor are shown in Fig. 5, A. The values were obtained using a standard addition approach for the glutamate biosensor measurements [20]. The extracellular glutamate concentration in the preparations varied between 7.9 ± 0.9 and 11.5 ± 0.9 μM depending on the incubation period and reflected a low and even negative tonic glutamate release at definite time intervals. Tonic glutamate release from nerve terminals was 0.2 μM for the first 6 min of monitoring. The data reflect the ability of synaptosomes to maintain the dynamic balance of tonic release vs. uptake of glutamate and confirms our recent suggestion on the existence of the dynamic gradient of glutamate across the plasma membrane. Long-term monitoring of the extracellular glutamate level in the synaptosomal preparations without stimulation is of value for clarification of synaptosomes` energetic status and functional state. It is clear from Fig. 5, A that the synaptosomal preparations were able to keep a definite level of extracellular glutamate and so were of appropriate quality. These data are in accordance with our recent data on the kinetic characteristics of glutamate uptake measured with similar biosensor [20], where we demonstrated the existence of a definite level of the basal glutamate signal (7.9 μM) in uptake experiments.

Exocytotic release measured with the biosensor was calculated as a difference between the glutamate release in Ca^{2+}-containing and Ca^{2+}-free media at a 6 min time point. As shown in Fig. 5B, exocytotic release of endogenous glutamate from nerve terminals (sample volume was 1 mL, protein concentration – 0.4 mg mL^{-1}) measured by the glutamate biosensor was equal to 7.5 ± 1.0 μM ($P \leq 0.05$, Student's *t*-test, n = 4, as compared to the basal extracellular level of endogenous glutamate). The rate of exocytotic glutamate release from nerve terminals stimulated by depolarization of their plasma membrane is an extremely important physiological parameter. It reflects the efficiency of exocytotic machinery at the presynaptic level.

The transporter-mediated release stimulated by depolarization of the plasma membrane in Ca^{2+}-free media was 8.0 ± 1.0 μM (a sample volume was 1 mL, protein concentration – 0.4 mg mL^{-1}) ($P \leq 0.05$, Student's *t*-test, n = 4, as compared to the basal extracellular level of endogenous glutamate) (Fig. 5, B). The transporter-mediated release is the main mechanism of glutamate release under the conditions of energy deprivation, hypoxia, ischemia that causes an increase in extracellular glutamate concentration thereby provoking neurotoxicity and cell death.

3.3. *Verification of glutamate biosensor measurements using L-[^{14}C] glutamate*

In the neuroscience area, the glutamate release kinetics in nerve terminals is measured primarily using radioactive labeled L-glutamate [36]. The release characteristics for other amino acid neurotransmitters are assessed in analogical way using radioactive labeling technique, where the aliquots of the synaptosomal suspension (or the culture cells) after the different manipulations are sedimented in a microcentrifuge. Radioactivity in the pellets and supernatants is determined by standard techniques using scintillation cocktail (see Materials and methods section).

In our experiments, the rate of L-[^{14}C]glutamate release from nerve terminals was determined based on a decrease in the amount of pellet's radioactivity and an increase in the supernatant's one. In the L-[^{14}C]glutamate release experiments, the extracellular glutamate concentration varied from 12 to 20% of total amount of radioactivity accumulated by nerve terminals. In the measurements with biosensor, the extracellular glutamate concentration in the preparation of nerve terminals varied between 7.9 and 11.5 μM (Fig. 5, A). The exocytotic component of L-[^{14}C]glutamate release was equal to 7 ± 1% of total amount of radioactivity accumulated by nerve terminals, and the transporter-mediated component was equal to 12.0 ± 1.5% of total amount of radioactivity accumulated by nerve terminals for 6 min.

3.4. *Difficulties in the expression of biosensor release results as a percentage of total concentration of endogenous glutamate in the preparations of nerve terminals*

As it was mentioned in the previous subsection, the data on L-[^{14}C]glutamate release were typically expressed as a percentage of

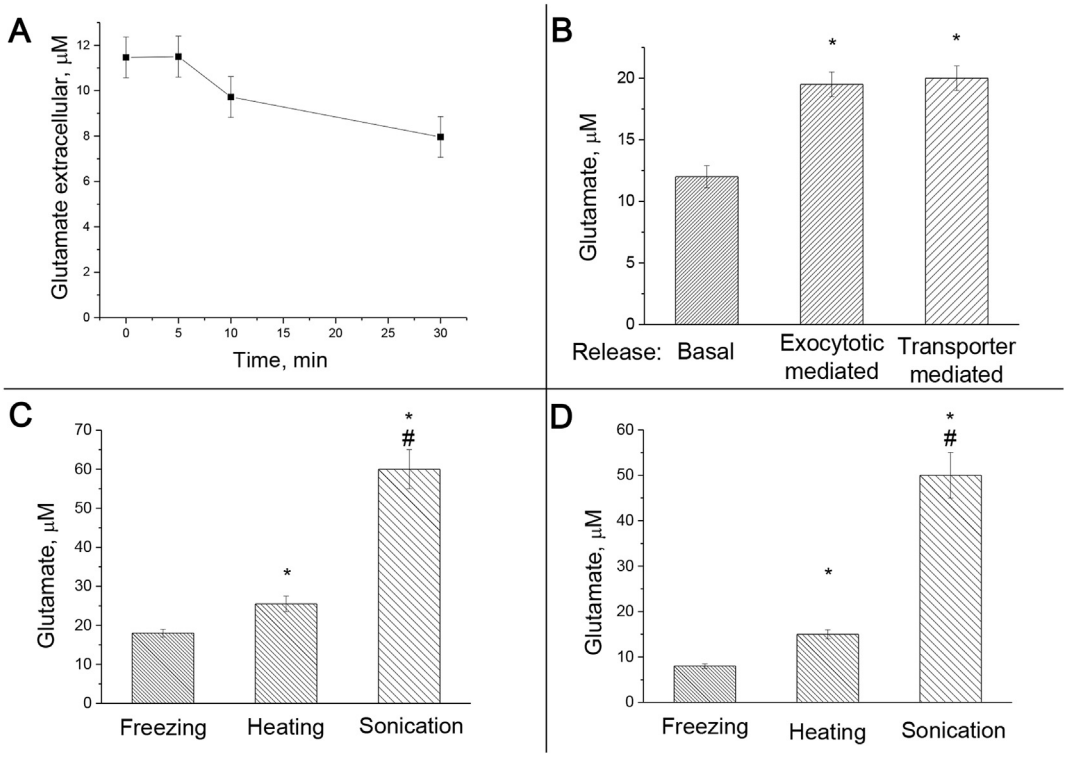

Fig. 5. A – The time course of changes in the extracellular glutamate concentration in the preparation of nerve terminals measured by the glutamate biosensor. The values were obtained using standard addition approach for glutamate biosensor measurements. B – Exocytotic (the second bar) and transporter-mediated (the third bar) release of endogenous glutamate from nerve terminals for 6 min measured by the glutamate biosensor. The values were obtained using standard addition approach for glutamate biosensor measurements. *, $P \leq 0.05$, Student's *t*-test, n = 4 as compared to the basal level of glutamate in the synaptosomal preparations. C – The total concentration of endogenous glutamate in the nerve terminal preparations measured by the glutamate biosensor. The membrane compartments of nerve terminals were disrupted by freezing (the first bar), heating (the second bar) and sonication (the third bar). The values were obtained using standard addition approach for glutamate biosensor measurements. *, $P \leq 0.05$, Student's *t*-test, n = 4 as compared to freezing approach; #, $P \leq 0.05$, Student's *t*-test, n = 4 as compared to heating approach. D – The concentration of endogenous glutamate inside of nerve terminals measured by the glutamate biosensor. The membrane compartments of nerve terminals were disrupted by freezing (the first bar), heating (the second bar) and sonication (the third bar). The values were obtained using standard addition approach for the glutamate biosensor measurements. *, $P \leq 0.05$, Student's *t*-test, n = 4 as compared to freezing approach; #, $P \leq 0.05$, Student's *t*-test, n = 4 as compared to heating approach.

total amount of radioactivity accumulated by nerve terminals during their preliminary loading with L-[^{14}C]glutamate. In this context, the question arises how to disrupt synaptosomes and synaptic vesicles inside of them to measure the total amount of endogenous glutamate by the biosensor. Usage of detergents for membrane solubilization and some other aggressive approaches [37–39] are not appropriate for the glutamate biosensor measurements.

To evaluate the total amount of glutamate in nerve terminals by the glutamate biosensor, we used several methodological approaches to disrupt nerve terminals and membrane compartments inside of them, i.e., freezing at - 20 °C for 2.5 h, heating at +70 °C for 1 h and repeated sonication (5 times) at 22 kHz for 1 min. As shown in Fig. 5C, the total amount of glutamate in the synaptosomal preparations measured by the glutamate biosensor (a sample volume was 1 mL, a protein concentration – 0.4 mg mL^{-1}) was 18.0 ± 1.0 μM after freezing; 25.5 ± 2.0 μM after heating ($P \leq 0.05$, Student's *t*-test, n = 4, as compared to freezing approach); and 60.0 ± 5.0 μM after repeated sonication ($P \leq 0.05$, Student's *t*-test, n = 4 as compared to freezing and heating approach). We also used maghemite γFe_2O_3 nanoparticles for better disruption of synaptosomes but this approach did not bring advantages in comparison with the sonication without nanoparticles. Therefore, the most effective methodological approach to disrupt the membrane compartments of nerve terminals is the repeated sonication.

As shown in Fig. 5 D, the concentration of endogenous glutamate inside of synaptosomes (a sample volume was 1 mL, and protein concentration – 0.4 mg mL^{-1}) at different disruption approaches was 8.0 ± 0.5 μM for freezing samples; 15.0 ± 1.0 μM for heating samples ($P \leq 0.05$, Student's *t*-test, n = 4); and 50.0 ± 5.0 μM for sonicated samples ($P \leq 0.05$, Student's *t*-test, n = 4). The value was calculated by subtraction of the ambient glutamate concentration from the total concentration of endogenous glutamate in the preparation of nerve terminals.

The extracellular glutamate concentration obtained with the biosensor was represented as a percentage of the total glutamate concentration in the preparations of nerve terminals. In the calculations, we used the concentration of total glutamate after the repeated sonication of nerve terminals (60.0 ± 5.0 μM). It was revealed that in the biosensor experiments the extracellular glutamate level was 16.0 ± 1.7% of the total amount of glutamate in nerve terminals. Taking into account the total glutamate concentration in the preparation of nerve terminals, depolarization-induced exocytotic release was calculated to be 12.5% of the total amount of glutamate in nerve terminals that is 1.8 times higher than that measured with L-[^{14}C]glutamate (7 ± 1% of the total amount of radioactivity accumulated by nerve terminals). Depolarization-induced transporter-mediated release was 13.0 ± 1.4% of the total amount of glutamate in nerve terminals for 6 min. For comparison, in the L-[^{14}C]glutamate experiments it was 12.0 ± 1.5% of the total amount of radioactivity accumulated by nerve terminals for 6 min.

Therefore, the values of the ambient level and transporter-mediated release of glutamate were consistent between the measurements using the biosensor or L-[^{14}C]glutamate when one took into consideration the total amount of glutamate in nerve

terminals, though no consensus (between the biosensor and L-[^{14}C] glutamate experiments) was revealed in the exocytotic parameter. Please, find explanation elsewhere in the Discussion section.

3.5. Verification of glutamate biosensor data using spectrofluorimetric glutamate dehydrogenase assay

Here, we used the spectrofluorimetric glutamate dehydrogenase assay to clarify the parameters that were inconsistent in the biosensor and L-[^{14}C]glutamate experiments. We demonstrated, using glutamate dehydrogenase, that the ambient level of endogenous glutamate in the synaptosomal preparations was 15 ± 1 μM (A), release of endogenous glutamate from synaptosomes in Ca^{2+}-containing media was 15.0 ± 0.9 μM ($P \leq 0.05$, Student's *t*-test, $n = 3$) (Fig. 6 A, B). The total concentration of synaptosomal endogenous glutamate determined using freezing approach was 30 ± 2 μM ($P \leq 0.05$, Student's *t*-test, $n = 3$) (Fig. 6, C) and using sonication was 60 ± 5 μM ($P \leq 0.05$, Student's *t*-test, $n = 3$) (Fig. 6, D). The glutamate dehydrogenase spectrofluorimetric measurements were carried out in 50 μl aliquots of the supernatant after sedimentation of synaptosomes (0.4 mg mL^{-1} of protein) added to 1 mL of the buffer solution in the cuvette. In this context, the values represented in Fig. 6, E were calculated in accordance with the protein concentration. Therefore, the data obtained with the glutamate dehydrogenase spectrofluorimetric assay were very similar to those obtained by the glutamate biosensor and also confirmed the effectiveness of sonication for disruption of synaptosomes to release vesicular glutamate.

3.6. Verification of glutamate biosensor data using amino acid analyzer

The ambient level, release and total concentration of endogenous glutamate in synaptosomes were measured using the amino

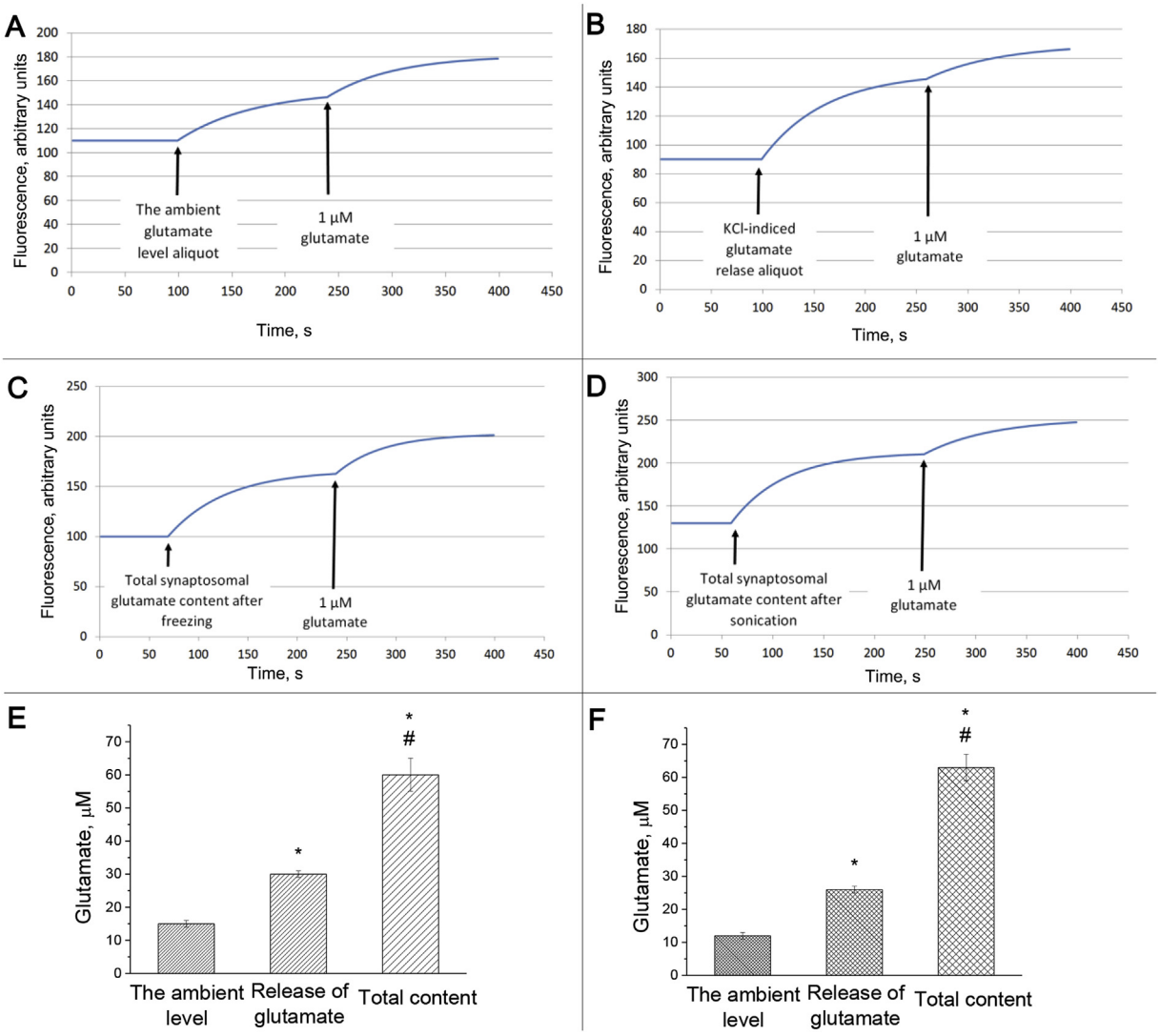

Fig. 6. The extracellular level (A), release in Ca^{2+}-supplemented media (B) and total concentration of endogenous glutamate after freezing (C) and sonication (D) in rat brain synaptosomes assessed with the glutamate dehydrogenase assay. E – Calculation of the glutamate dehydrogenase results presented in Figures A, B, D. *, $P \leq 0.05$, Student's t-test, $n = 3$ as compared to the ambient level of endogenous glutamate; #, $P \leq 0.05$, Student's t-test, $n = 3$ as compared to glutamate release value. The synaptosomal suspension (0.4 mg mL^{-1} of final protein concentration) was centrifuged and the aliquots (50 μL) were added to an enzymatic assay solution containing glutamate dehydrogenase. The concentration of endogenous glutamate in synaptosomes was measured by the changes in NADH fluorescence (excitation and emission wavelengths of 340 and 460 nm, respectively). The trace is representative of three independent experiments. F – The extracellular level (the first bar), release in Ca^{2+}-supplemented media (the second bar) and total concentration of endogenous glutamate (the third bar) in rat brain synaptosomes assessed by amino acid analyzer. *, $P \leq 0.05$, Student's t-test, $n = 3$ as compared to the ambient level of endogenous glutamate; #, $P \leq 0.05$, Student's t-test, $n = 3$ as compared to the glutamate release value.

acid analyzer (see Methods). According to the procedure of sample preparation for the amino acid analyzer, we diluted the synaptosomal preparations two times with 3% sulfosalicylic acid before the measurements. The ambient concentration of endogenous glutamate in the synaptosomal samples (the volume was 1 mL, final protein concentration – 0.4 mg mL^{-1}) was very close to its value in the glutamate biosensor measurements and corresponded to 12.0 ± 1.0 μM ($P \leq 0.05$, Student's *t*-test, n = 3), the total concentration of endogenous glutamate corresponded to 63.0 ± 4.0 μM ($P \leq 0.05$, Student's *t*-test, n = 3) (Fig. 6, F). Ca^{2+}-dependent release (the sum of exocytosis and glutamate transporter reversal) corresponded to 14.0 ± 1.0 μM ($P \leq 0.05$, Student's *t*-test, n = 3) (Fig. 6, F) and these values were also very close to the biosensor data.

3.7. Measurement of glutamate concentration in the blood plasma with the biosensor and the data verification by the spectrofluorimetric glutamate dehydrogenase assay

A balance between the glutamate concentration in the interstitial brain fluid and blood plasma has been recently demonstrated [5–8]. The glutamate concentration in the blood plasma can be considered as the extracellular one for the blood organelles which able to perform the release and transporter-mediated glutamate uptake, thereby contributing to the maintenance of the extracellular glutamate homeostasis in the brain. The glutamate concentration in the blood plasma of rats was determined using the glutamate biosensor and varied between 50 and 75 μM. To perform measurements, aliquots of the blood plasma were added to the working cell of the biosensor, after recording the response, the biosensor was washed rigorously with working buffer and used for the next analysis. The biosensor could analyze 6 to 8 samples of the blood plasma sequentially, after that the enzyme membrane of biosensor became contaminated with components of the blood plasma and its characteristics deteriorated. The plasma glutamate concentration was also measured using the glutamate dehydrogenase spectrofluorimetric approach and varied in the similar range. The amino acid analyzer cannot be used for the determination of the plasma glutamate concentration because of a high protein concentration in the samples. The addition of 3% sulfosalicylic acid during sample preparation resulted in pellet formation that brought inaccuracy in the measurements.

4. Discussion

In comparison to the classical analytical methods of glutamate determination (i.e. spectrophotometry, different types of chromatography and radiolabeled technique), the proposed glutamate biosensor-based approach for the analysis of glutamate release has several advantages. The biosensor does not require radiolabeled L-[^{14}C]glutamate preloading or other additional procedures, and thus the synaptosomal samples can be analyzed without pretreatment. The biosensor directly measures changes in the absolute values of endogenous glutamate concentrations in nerve terminals in response to different stimulation. Beside the experimental laboratory work, the glutamate biosensor-based approach can be applied in clinics for neuromonitoring the glutamate-related parameters and extracellular glutamate concentration in the brain samples, liquids and also in the blood plasma. The cost of overall measuring setup (including the biosensor, a potentiostat and auxiliary equipment) is quite small (about 4000 €) that is far lower than a cost of any classical method. The measuring setup is portative, and can be transported to another institution for on-site determination of the glutamate concentration in order to avoid storage and transportation of the synaptosomal samples. In medical practice during neuromonitoring, the transportability and portability of the biosensor is also an advantage. The biosensor analysis is very quick (several minutes for one sample). The biosensor is very specific to glutamate because the enzyme, GluOx, specifically catalyzes only glutamate oxidation, and the working electrode is covered with a semipermeable membrane that prevents oxidation of interfering compounds. This high selectivity was confirmed by comparison of the results with the traditional scintillation method (based on L-[^{14}C]glutamate), spectrofluorimetry (glutamate dehydrogenase-based) and amino acid analyzer in the present and our previous works [20].

In this study, for the first time: 1) the electrochemical glutamate oxidase-based biosensor was optimized for the determination of low micromolar glutamate concentrations; 2) the algorithm of the analysis of exocytotic and transporter–mediated glutamate release was developed; 3) the comparative analysis of the biosensor data with those obtained with the radiolabeled L-[^{14}C]glutamate technique, spectrofluorimetric glutamate dehydrogenase-based assay and amino acid analyzer was performed.

Since the experiments on glutamate determination in the synaptosomal samples had been planned, some important steps of the biosensor bioselective membrane preparation were optimized for the analysis of low glutamate concentrations. The optimal concentrations of the enzyme (4%) and glutaraldehyde (0.4%) for the biomembrane preparation were selected, as well as the immobilization time (30 min). It has been shown that the linear part of the biosensor calibration curve gives a possibility to determine the concentrations of glutamate presented in the synaptosomal samples (7–30 μM).

One of the advantages of the biosensor is ability to measure the absolute values of endogenous glutamate in nerve terminals. The absolute values of the following parameters, i.e. the extracellular glutamate concentration, Ca^{2+}-dependent glutamate release, and the total glutamate concentration in sonicated synaptosomes, measured using the biosensor, glutamate dehydrogenase assay and amino acid analyzer revealed comparability. The ambient glutamate level at 6 min time point was 11.5 μM (biosensor), 15 μM (glutamate dehydrogenase) and 12 μM (amino acid analyzer). Ca^{2+}-dependent synaptosomal glutamate release was 15.5; 15; 14 μM, respectively (Figs. 5 and 6). The total concentration of endogenous glutamate in nerve terminals after sonication determined by the biosensor, glutamate dehydrogenase and amino acid analyzer assays was almost similar (Figs. 5 and 6) and corresponded to 60, 60, 63 μM respectively. The values of the ambient glutamate level and different manners of glutamate release were close, but not similar, in the biosensor and L-[^{14}C]glutamate assays. The differences between from one side the L-[^{14}C]glutamate assay and from the other side the biosensor, glutamate dehydrogenase and amino acid analyzer measurements can be due to the difference in the experimental protocols. The radiolabeled technique required preliminary loading of L-[^{14}C]glutamate to nerve terminals (see Method section), whereas the other approaches allow measuring endogenous glutamate which was kept by nerve terminals during isolation, centrifugation and other experimental manipulations. The ratio $[Glu\ release]_{transporter\text{-}mediated}/[Glu]_{extracellular}$, $[Glu\ release]_{exocytotic}/[Glu]_{extracellular}$ and also $[Glu\ release]_{in\ Ca2+\text{-}supplemented\ media}/[Glu]_{extracellular}$ were characteristic parameters of the release efficiency and were near in all used methodological approaches. So, the adequacy of the developed glutamate biosensor-based methodological approach that allows measuring and calculating glutamate release efficacy in the synaptosomes has been proven.

Comparative analysis of the data obtained with the biosensor, glutamate dehydrogenase and amino acid analyzer assays from one side, and radiolabeled L-[^{14}C]glutamate from the other side revealed a difference, when the results were represented as a percentage of total concentration of glutamate in nerve terminals.

To have corresponded data on the extracellular level of glutamate, exocytotic and transporter-mediate release in the biosensor and L-[^{14}C]glutamate experiments, the putative total endogenous glutamate concentration in nerve terminals determined by the biosensor must be not 60 μM (Figs. 5 and 6) but higher (approximately 80–100 μM). We suggested that this discrepancy can be explained by two facts. The first one was an incomplete disruption of the intrinsic compartments of synaptosomes even after repeated sonication, and thus not all endogenous glutamate was released to the incubation media and measured by the biosensor, amino acid analyzer and glutamate dehydrogenate assays. The rest of glutamate was still kept by the synaptic vesicles. The use of detergents and other approaches for synaptosome disruption to measure the total amount of endogenous glutamate was restricted in the biosensor application [36–38]. The second factor was an uncontrolled release of endogenous cytoplasmic glutamate dehydrogenase during synaptosome disruption and therefore metabolization of existed glutamate that in turn can decrease the total glutamate content before starting the biosensor, glutamate dehydrogenase and amino acid measurements. In contrast, the radiolabeled L-[^{14}C]glutamate technique allowed measuring the total L-[^{14}C]glutamate concentration without preliminary synaptosome disruption.

It is an aspiring task to apply the glutamate biosensor for *in vivo* measurements in brain tissue. Due to a relatively large size of the electrode used in this work, the biosensor fits only *ex vivo* applications. However, it is possible to apply platinum microelectrodes 50–100 μm in diameter for the biosensor construction and implant it into the living tissue [40]. The procedure of the biosensor preparation (deposition of PPD membrane and immobilization of GluOx) should be only slightly modified as the electrode material and detection principle would be similar. The creation of such microbiosensor for detection of glutamate release in certain brain and spinal cord regions needs more investigations.

Our previous results demonstrated the principal necessity and perspectives of glutamate monitoring using the biosensor in practice. This statement is confirmed by the following facts: 1) the extracellular glutamate level is unique for each synapse [2,3,41]; 2) the alterations in extracellular glutamate in the nerve terminal preparations during therapeutic hypothermia are specific for each experimental animal, and they can be expected to be individual for a patient, and so the necessity of personal neuromonitoring in therapeutic hypothermia was demonstrated [4]; 3) the kinetics of glutamate uptake by nerve terminals can be measured using the glutamate biosensor [20]; and 4) the biosensor can be applied for glutamate release assessment in nerve terminals that was shown in this study.

Blood platelets are of special interest and can be considered as a potential peripheral marker for the analysis of disturbance in the glutamate transport in brain nerve terminals [2,3]. Platelets express plasma membrane glutamate transporters EAAT 1–3, secretory granule vesicular glutamate transporters VGLUT 1 & 2, NMDA, AMPA, kainate and mGlu receptors. Recently, we have demonstrated similarity of glutamate transport process in nerve terminals and platelets. The latter, however, have restriction in application for neuromonitoring because they cannot be used directly for the assessment of the pathological glutamate transporter reversal, since this manner of glutamate release in platelets is rather ambiguous [4]. Glutamate is released from platelets exclusively by means of exocytosis [3]. In perspective, we plan to develop panels of glutamate-related biomarkers for comprehensive neuromonitoring, e.g. the glutamate concentration in the blood and cerebrospinal liquids, and the glutamate transport characteristics of platelets, etc. These glutamate-related diagnostic, prognostic and predictive biomarkers are necessary for standard clinical decision-making algorithms that could help to select optimal individual temperature regime for patients with prescribed therapeutic hypothermia in ischemic stroke, brain trauma and in cardiac surgery of the aortic arch. In this context, the glutamate biosensor can be applied in medical practice.

5. Conclusions

We propose a biosensor-based methodological approach for the analysis of effectiveness of tonic, exocytotic and transporter-mediated glutamate release from nerve terminals verified by the radiolabeled L-[^{14}C]glutamate, spectrofluorimetric glutamate dehydrogenase and amino acid analyzer assays. Reliability of the biosensor measurements of the glutamate concentration in the blood plasma was also confirmed by the spectrofluorimetric glutamate dehydrogenase assay. The glutamate biosensor-based approach is suggested to be applied in clinics for neuromonitoring glutamate-related parameters in the brain samples, liquids and blood plasma in stroke, brain trauma, during therapeutic hypothermia treatment, etc., and also in laboratory work to record the glutamate release and uptake kinetics in nerve terminals.

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Ethical approval

Experimental protocols were approved by the Animal Care and Use Committee of the Palladin Institute of Biochemistry (Protocol from 19/09–2012).

Conflicts of interest

The authors declare no competing interests and no other relationships or activities that could appear to have influenced the submitted work.

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