Talanta 135 (2015) 67–74

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Monitoring of the velocity of high-affinity glutamate uptake by isolated brain nerve terminals using amperometric glutamate biosensor

O. Soldatkin [a,*], A. Nazarova [b], N. Krisanova [b], A. Borysov [b], D. Kucherenko [c], I. Kucherenko [a], N. Pozdnyakova [b], A. Soldatkin [a,c], T. Borisova [b]

[a] Laboratory of Biomolecular Electronics, Department of Translation Mechanisms of Genetic Information, Institute of Molecular Biology and Genetics, NAS of Ukraine, 150 Zabolotnogo Str., Kyiv 03680, Ukraine
[b] The Department of Neurochemistry, Palladin Institute of Biochemistry, NAS of Ukraine, 9 Leontovicha Street, Kyiv 01601, Ukraine
[c] Institute of High Technologies, Taras Shevchenko National University of Kyiv, 64, Volodymyrska Str., Kyiv 01003, Ukraine

ARTICLE INFO

Article history:
Received 1 September 2014
Received in revised form
17 December 2014
Accepted 21 December 2014
Available online 29 December 2014

Keywords:
Biosensor
Amperometry
Glutamate
Synaptosomes
Nerve terminals

ABSTRACT

Glutamate is the major excitatory neurotransmitter in the central nervous system, which is involved in the main aspects of normal brain functioning. High-affinity Na^+-dependent glutamate transporters is key proteins, which transport extracellular glutamate to the cytoplasm of nerve cells, thereby preventing continuous activation of glutamate receptors, and thus the development of neurotoxicity. Disturbance in glutamate uptake is involved in the pathogenesis of major neurological disorders. Amperometric biosensors are the most promising and successful among electrochemical biosensors. In this study, we developed (1) amperometric glutamate biosensor, (2) methodological approach for the analysis of glutamate uptake in liquid samples of isolated rat brain nerve terminals (synaptosomes). The basal level of glutamate, the initial velocity of glutamate uptake and time-dependent accumulation of glutamate by synaptosomes were determined using developed glutamate biosensor. Comparative analysis of the data with those obtained by radioactive analysis, spectrofluorimetry and ion exchange chromatography was performed. Therefore, the methodological approach for monitoring of the velocity of glutamate uptake, which takes into consideration the definite level of endogenous glutamate in nerve terminals, was developed using glutamate biosensor.

1. Introduction

Glutamate is not only a key excitatory neurotransmitter in the central nervous system because of its involvement in many aspects of normal brain functioning, but also a potent neurotoxin. Ambient glutamate concentration is maintained at a low level between episodes of exocytotic release under normal physiological conditions, thereby preventing continual activation of glutamate receptors and protecting neurons from excitotoxic injury. In stroke, cerebral hypoxia/ischemia, hypoglycemia, traumatic brain injury, etc, the development of neurotoxicity is provoked by an increase in the concentration of ambient glutamate. Excessive extracellular glutamate overstimulates glutamate receptors initiating an excessive calcium entry through mainly *N*-metyl-D-aspartate ionotropic receptors, causes excitotoxicity, neuronal injury and death. There are no enzymes in the synaptic cleft that metabolize glutamate. The only way by which glutamate can be removed from the synaptic cleft is its uptake accomplished by high-affinity Na^+-dependent glutamate transporters, plasma membrane proteins of neurons and glial cells with eight putative transmembrane domains, that use Na^+/K^+ electrochemical gradients across the plasma membrane as a driving force [1]. Disturbance in glutamate uptake is involved in the pathogenesis of major neurological and neurodegenerative disorders and diseases.

A number of techniques are available for measuring glutamate concentration, including optical methods [2], patch clamp [3], and microdialysis [4]. However, mainly all of these techniques suffer from disadvantages, including: invasive sampling, temporal limitations, low signal-to-noise ratio, lack of economical feasibility, and/or ability to detect changes in glutamate transport under physiological conditions. These disadvantages limit the applicability of results regarding neurotransmitter kinetics [5].

The use of enzymatic biosensors with performance enhancing materials (e.g., carbon nanotubes) has been a major focus for many of these advances. However, these techniques are not used as main neuroscience research tools, due to relatively low sensitivity, excessive drift/noise, low signal-to-noise ratio, and inability to quantify rapid neurochemical kinetics during synaptic transmission [5]. Electrochemical methods are considered as one of the most potential approach, because of their simplicity, rapidity, high

* Corresponding author. Tel.: +380 442000328; fax: +380 445264397.
E-mail address: alex_sold@yahoo.com (O. Soldatkin).

http://dx.doi.org/10.1016/j.talanta.2014.12.031
0039-9140/

sensitivity and specificity. Amperometric biosensors are considered as the most promising and successful among electrochemical biosensors. They are used most often to determine glutamate concentration [6–9]. Using modified graphite electrode with stabilizing additives allows making glutamate biosensor with long term storage properties [10].

The analysis of the glutamate level in brain fluids, cell culture and serum as well as glutamate release from nerve cells with glutamate biosensor is documented in the literature. The importance of the initial velocity of high-affinity Na^+-dependent uptake and accumulation of glutamate by nerve terminals is underscored by the fact that defective glutamate uptake underlay negative neurological consequence in ischemia, amyotrophic lateral sclerosis, Alzheimer's disease, epilepsy, intoxications, AIDS, hepatic encephalopathy and traumatic brain injury as well as in some other conditions [1]. Glutamate uptake also plays important roles in peripheral organs and tissues [11].

The aim of this study was the development of amperometric biosensor based on the measurements of activity of glutamate oxidase and methodological approach adapted to this biosensor for adequate monitoring of the velocity of high-affinity glutamate uptake and glutamate accumulation by nerve terminals. Development of methodological algorithm was based on the comparative analysis of experimental data of glutamate biosensor with those obtained by radioactive analysis, spectrofluorimetry and ion exchange chromatography.

2. Materials and methods

2.1. Design of amperometric transducers

In this work, we used platinum disc electrodes of own production as amperometric transducers (Fig. 1). The electrodes were produced according to the following algorithm. First, 3 mm long platinum wire 0.4 mm in diameter was sealed in the end part of a glass capillary 3.5 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 alcohol before the bioselective element immobilization. The electrode surface was periodically restored using the same grinding procedure.

2.2. Modification of amperometric transducer with phenylenediamine

The work of amperometric biosensors is based on measurement of the electric current flowing through the working electrode at certain applied potential. In our work, the current is generated due to decomposition of hydrogen peroxide, a product of the enzymatic reaction of glutamate oxidation, on the working electrode. Since the solution tested may contain other electroactive compounds, which could be also oxidized at the electrode resulting in errors in measurements, the selectivity of amperometric electrode can be improved by coating the electrode with an additional polymer membrane restricting the diffusion of interfering substances to the electrode surface. Among a wide class of aromatic substances, which are capable of electropolymerization, phenylenediamine isomers are known to be used in biosensors most commonly [12–15]. The results of a series of comparative studies on the properties of poly(phenylenediamine) (PPD) based polymeric membranes, obtained from different monomers, demonstrated that the transducers modified with the *meta*-phenylenediamine-based polymer film had the best selectivity. This is why in our study we used this monomer for the formation of an additional membrane on the platinum electrode surface according to the method described in [15]. To prepare a PPD membrane, we immersed a three-electrode system with a bare working electrode in 5 mM solution of *m*-phenylenediamine (Sigma-Aldrich Chemie, Germany). Afterwards, we obtained 4–5 cyclic voltammograms and tested the effectiveness of PPD membrane [16]. Next, the bioselective elements were immobilized onto the PPD membrane surface.

Fig. 1. Scheme of the platinum disc electrodes. 1—bioselective membrane, 2—platinum wire, 3—internal conductor, 4—Wood alloy, 5—epoxy resin, 6—contact pad.

2.3. Fabrication of bioselective elements of the biosensor

Bioselective elements of biosensors were obtained by covalent immobilization of enzymes and auxiliary substances on the surface of amperometric transducer. 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, Yamasa Corporation, Tokyo, Japan), 4% bovine serum albumine (Sigma-Aldrich Chemie, Germany), 10% glycerol (Sigma-Aldrich Chemie, Germany) in 100 mM phosphate buffer, pH 6.5. Glycerol was added to stabilize enzymes during their 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 is deposited onto the surfaces of transducers, they were dried for 40 min in air at room temperature. After immobilization the biosensors were washed in the working buffer solution from unbound components of biomembranes and excess of glutaraldehyde.

2.4. Measuring procedure

We used three-electrode scheme of amperometric analysis. Working amperometric electrodes, an auxiliary platinum electrode and an Ag/AgCl reference electrode were connected to the PalmSens potentiostat (Palm Instruments BV, The Netherlands). The 8-channel device (CH-8 multiplexer) from the same manufacturer connected to the potentiostat allowed receiving signals from 8 working electrodes at a time, but usually only 2–3 working electrodes were in operation. In the course of measurement, the distance between the auxiliary platinum and all working electrodes was the same, about 5 mm. The measurements were carried out at room temperature in an open 3.5-ml measuring cell at constant stirring and at a constant potential of +0.6 V vs Ag/AgCl reference electrode. As a working buffer served 25 mM HEPES (Sigma-Aldrich Chemie, Germany), pH 7.4, with addition of 2 mM $Mg(NO_3)_2$ (Sigma-Aldrich Chemie, Germany). The glutamate concentrations in the working cell were obtained by addition of the aliquots of stock solutions (50–1 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, Medical Academy of Sciences of Ukraine) were maintained in accordance with the European Guidelines and International Laws and Policies. 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 removing the brain, 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 23, i.e. the development of liquid sample method for measurements of glutamate uptake—6 animals; the assessment of glutamate uptake and the extracellular glutamate level using L-[^{14}C]glutamate and parallel experiments with glutamate biosensor—6 animals; GABA uptake—4 animals; fluorescent measurements—3 animals; amino acid analysis—3 animals. The cerebral hemispheres of decapitated animals were rapidly removed and homogenized in ice-cold 0.32 M sucrose, 5 mM HEPES-NaOH, pH 7.4 and 0.2 mM EDTA (Sigma, U.S.A.). Synaptosomes were prepared by differential and Ficoll-400 (Amersham, UK) density gradient centrifugation of rat brain homogenate according to the method of Cotman [17] with slight modifications [18,19]. All manipulations were performed at 4 °C. The synaptosomal suspensions were used in experiments during 2–4 h after isolation. The standard salt solution was oxygenated and contained (in mM): NaCl 126; KCl 5; $MgCl_2$ 2.0; NaH_2PO_4 1.0 (all salts were from Reachim, Ukraine); 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 $CaCl_2$ (Reachim, Ukraine). Protein concentration was measured as described by Larson [20].

2.6. Uptake experiments

Uptake of L-[^{14}C]glutamate by synaptosomes was measured as follows: samples (250 μl of the suspension, 0.250 mg of protein/ml) were pre-incubated in standard salt solution at 37 °C for 10 min. Uptake was initiated by the addition of 10 μM L-glutamate supplemented with 420 nM L-[^{14}C]glutamate (0.1 μCi/ml), incubated during different time intervals (1, 10 min) at 37 °C and then aliquots (250 μl) were rapidly filtering through a Whatman GF/C filters. After twice washing with 4 ml ice-cold standard saline, filters were dried, then were suspended in a scintillation cocktail OSC and counted in a Delta 300 (Tracor Analytic, USA) scintillation counter. Non-specific binding of the neurotransmitter was evaluated in cooling samples sedimented immediately after the addition of radiolabeled glutamate. One animal was used to obtain one synaptosomal preparation, and each measurement was performed in triplicate.

2.7. Glutamate dehydrogenase assay: The assessment of the extracellular level of endogenous glutamate in nerve terminals

The extracellular level of glutamate in synaptosomes was detected using glutamate dehydrogenase assay. In the presence of glutamate, glutamate dehydrogenase reduced β-nicotinamide adenine dinucleotide (NAD^+) to NADH, a product that fluoresces, when excited with UV light. Synaptosomes (0.5 mg/ml of final protein concentration) were added to an enzymatic assay solution, which was composed of the standard salt saline, glutamate dehydrogenase (20 U/ml) (Sigma, U.S.A.) and NAD^+ (1 mM) (Sigma, U.S.A.) and preincubated at 37 °C for 10 min. Fluorescence intensity of NADH was measured in a stirred thermostatted cuvette (37 °C) at Hitachi MPF-4 spectrofluorimeter at excitation and emission wavelengths of 340 and 460 nm, respectively (slit bands were of 5 nm). Endogenous glutamate released from synaptosomes to the incubation media was detected as an increase in NADH fluorescence.

In all experiments, glutamate was added to synaptosomes at the end of the measurements to calibrate the activity of glutamate dehydrogenase.

2.8. Assessment of the ambient level of glutamate by amino acid analyzer

Synaptosomes (15 ml of suspension, 2 mg of protein/ml) were incubated for 30 min at 37 °C in standard oxygenated salt solution, and then samples were washed with 10 V of ice-cold standard salt solution and sedimented. The pellets were resuspended in 5 ml of standard oxygenated salt solution. For the evaluation of the ambient level of the neuromediator, synaptosomes (5 ml of suspension, 6 mg of protein/ml) were incubated at 37 °C for 15 min, then samples was rapidly sedimented in a microcentrifuge (20 s at 10,000 × *g*). 4 ml of the supernatants were concentrated in rotor evaporator up to 1.5 ml. Two times diluted preparations (3% sulfosalicylic acid) were analyzed by Amino Acid Analyzer T 339 by the method of ion exchange chromatography.

2.9. Statistical analysis

Results were expressed as mean ± S.E.M. of *n* independent experiments. Difference between two groups was compared by two-tailed Student's *t*-test. Differences were considered significant, when $P \leq 0.05$.

3. Results

3.1. Development of the glutamate biosensor and analysis of its main characteristics

The operation of amperometric biosensor for glutamate determination is based on the enzymatic reaction (1) in a bioselective membrane resulting in the glutamate oxidation and the formation of electrochemically active hydrogen peroxide. When applying positive potential, the reaction of hydrogen peroxide decomposition occurs on the electrode (2) resulting in the formation of electrons, which are directly registered by the amperometric transducer:

$$\text{Glutamate} + O_2 \xrightarrow[+600\ \text{mV}]{\text{Glutamate oxidase}} \alpha - \text{ketoglutarate} + NH_3 + H_2O_2 \quad (1)$$

$$H_2O_2 \rightarrow 2H^+ + O_2 + 2e^- \quad (2)$$

Poly(phenylenediamine) membrane covered the surface of the working electrode and prevented oxidation of electroactive molecules that are larger than hydrogen peroxide. Biosensor responses to some of electroactive substances with and without the poly (phenylenediamine) membrane indicate that the membrane indeed increased the selectivity of the biosensor (Fig. 2).

Response reproducibility is one of the key characteristics that show reliability of biosensor operation. For accurate measurement of glutamate concentration in solution the biosensor responses should be almost identical during time of operation.

Therefore, we investigated the response reproducibility during several hours of continuous operation. It took 3–5 min to carry out one measurement. An interval between measurements was about 10 min, during which we washed biosensors from the substrate with working buffer renewing it several times. The study was carried out at glutamate concentrations 0.05 mM, 0.1 mM, 0.5 mM, 1 mM. There was no notable decrease in the response values for 10 measurements; the relative standard deviation of responses was 5% on average.

The possibility of long-term usage of the biosensors is quite important. However, often there is a partial washout of the components of biological membranes from the transducers surface as a result of stirring of the buffer solution in the working cell. Besides, the enzyme activity somewhat decreases during storage. These factors can cause a certain drop in biosensor responses in the course of exploitation.

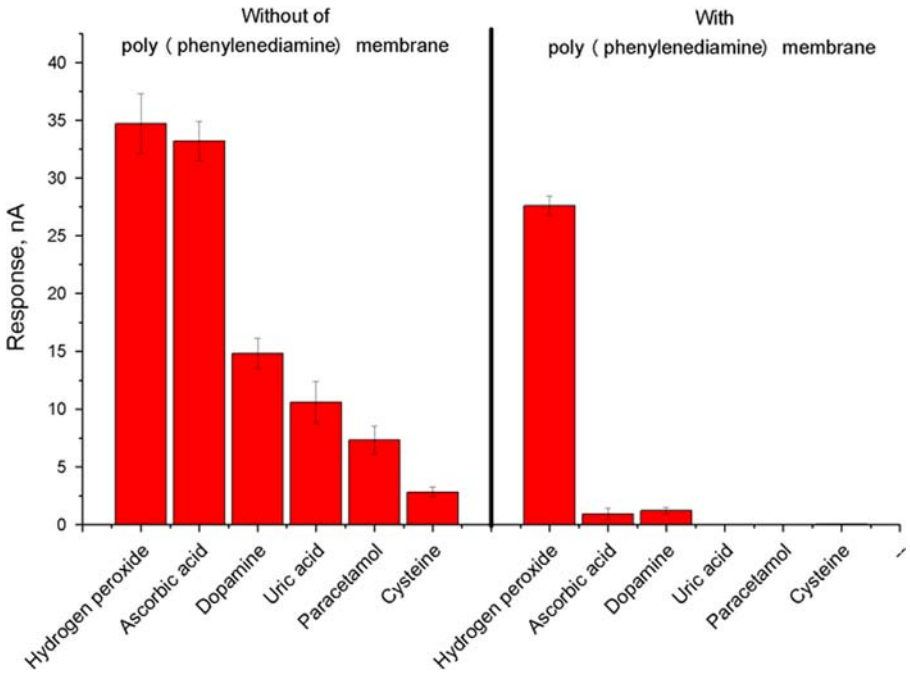

Fig. 2. Selectivity of amperometric transducer before and after deposition of poly(phenylenediamine) film. Electroactive substances were added in the following concentrations: hydrogen peroxide—0.05 mM, ascorbic acid—0.5 mM, dopamine—0.02 mM, uric acid—0.1 mM, paracetamol—0.1 mM, cysteine—0.1 mM.

The next stage of work was therefore aimed at testing the operational stability of the biosensor. During the day we received 8–12 responses to four different glutamate concentrations (ranging from 0.1 mM to 1 mM), and then the biosensor was stored dry at 4 °C until the next use. A few days later, we again measured the biosensor responses to glutamate of the same concentrations. Total duration of biosensor storage was 11 days. The responses to low glutamate concentrations (0.1 mM) remained stable throughout the period of measurement. On the other hand, responses to high glutamate concentrations (0.5 mM and 1 mM) slightly decreased. This evidences to some washout of the enzyme from the bioselective element. Although it had no effect on measuring low glutamate concentrations, the remained amount of enzyme could be deficient for complete reaction with glutamate of high concentrations, which led to a decrease in responses. However, the decrease was insignificant, which indicates the possibility of effective use of biosensors after storage.

Long-term storage of the biosensor was also investigated. The biosensors were stored dry at +4 °C for two months. During this time the biosensors lost 25% of activity.

The biosensor had following analytical characteristics. The detection limit of glutamate was 0.5–4 μM. It was measured as the glutamate concentration, the response to which is three times larger than the baseline noise. The detection limit slightly differed for every particular biosensor and increased during the biosensor operation. The linear working range was from 2–5 μM to 600–800 μM (depending on the particular biosensor); time of steady state response was 15–20 s; sensitivity to glutamate was 250–300 nA/mM. The typical calibration curve of the biosensor for glutamate determination is shown in Fig. 3. The linear part of this calibration curve is described by the equation $I = 315 \times C$ ($R^2 = 0.996$), where I—current (nA), C—glutamate concentration (mM).

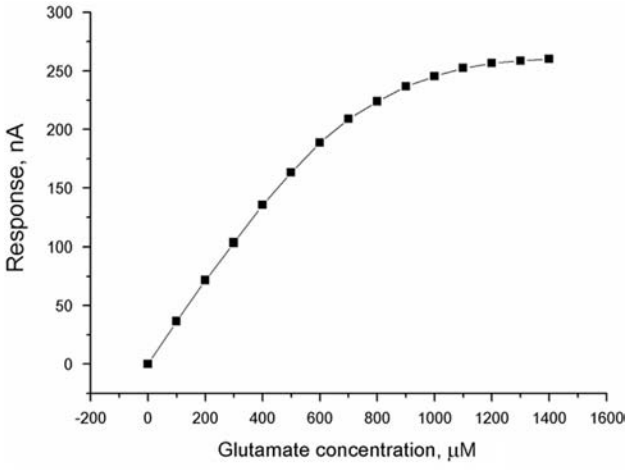

Fig. 3. Calibration curve of the biosensor for glutamate determination. Measurements in 25 mM HEPES buffer, pH 7.4, at a constant potential of +0.6 V vs Ag/AgCl reference electrode.

3.2. Development of liquid sample method for analysis of the initial velocity of high-affinity Na^+-dependent glutamate uptake and accumulation of glutamate in isolated brain nerve terminals

Velocity of high-affinity Na^+-dependent uptake and accumulation of glutamate by nerve terminals are extremely important physiological parameters and their registration is an actual task of medicine and neurotechnology. Assessment of these parameters allows evaluating activity of glutamate transporters and their ability to maintain low extracellular level of glutamate. We used isolated nerve terminals (synaptosomes) purified from rat cortex, which retain all features of intact nerve terminals, e.g., ability to maintain the membrane potential, exocytotic release as well as accomplish uptake of the neurotransmitters. Synaptosomes promises to be one of the best systems to explore the relationship between the structure of a protein, its biochemical and cell-biological properties, and physiological role [21].

In neuroscience area, the measurements of kinetic characteristics of glutamate uptake in synaptosomes are carried out presumably using radioactively labeled L-[^{14}C]glutamate [22–24]. Uptake of other amino acid neurotransmitters is measured similarly using radioactive labeled technique, where aliquots of synaptosomal suspension after different manipulation are filtered through fiberglass filters Whatman GF/C, which then are washed

with the buffer. Radioactivity remaining on the filter is determined by standard techniques using scintillation cocktail (see Section 2). In this context, the accumulation of L-[^{14}C]glutamate by nerve terminals is determined by an increase in the amount of radioactivity of the pellets collected on the filters. It is clear that the synaptosomal samples for the analysis of the kinetic characteristics of glutamate uptake using glutamate biosensor should be liquid. It should be noted that glutamate provides fast synaptic neurotransmission and synaptosomes accumulate approximately one-third of added radiolabeled glutamate for 1 min, whereas the measurements with glutamate biosensor take 3–5 min. Therefore, it is not correct to monitor a decrease in glutamate concentration directly in synaptosomal suspension because during the biosensor measurements synaptosomes continue to accumulate glutamate, and simultaneously glutamate is utilized by glutamate oxidase from the biosensor.

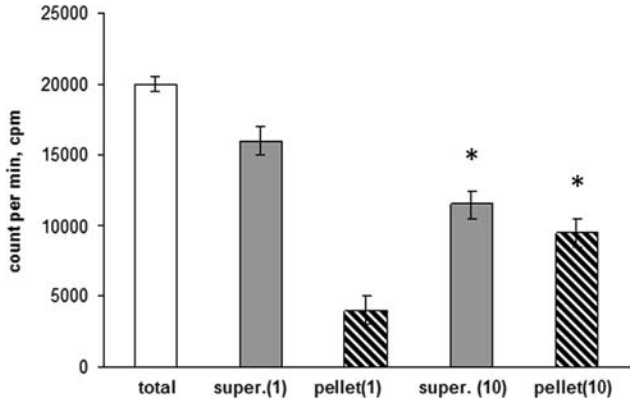

Fig. 4. Distribution of radioactivity in the supernatants (the second and fourth columns) and pellets (the third and fifth columns) in comparison with total radioactivity (the first column) during the measurement of the initial velocity of uptake (at 1 min time point) and accumulation of L-[^{14}C]glutamate by synaptosomes for 10 min. * $P \leq 0.05$, as compared to radioactivity in supernatant/pellets at 1 min time point, Student's t-test, $n=6$.

To avoid above difficulties, a new methodological approach for the analysis of the velocity of glutamate uptake in liquid samples was developed, the adequacy of which has been proven in parallel experiments using L-[^{14}C]glutamate. In this methodological approach, L-[^{14}C]glutamate uptake was registered not by filtering of synaptosomal suspension and analysis of radioactivity in the pellets, but in the supernatants after centrifugation of synaptosomal aliquots at a microcentrifuge. In this approach, L-[^{14}C]glutamate uptake by synaptosomes at definite time intervals was calculated as a reduction of radioactivity in the supernatant. Uptake of L-[^{14}C]glutamate by synaptosomes was measured as follows: samples (125 μl of the suspension, 0.2 mg of protein/ml) were pre-incubated in standard salt solution at 37 °C for 10 min. Uptake was initiated by the addition of 10 μM L-glutamate supplemented with 420 nM L-[^{14}C]glutamate (0.1 μCi/ml), incubated during different time intervals (1, 10 min) at 37 °C and then rapidly sedimented in a microcentrifuge (15 s at 10,000 × g). L-[^{14}C]Glutamate uptake was measured as a decrease in radioactivity of aliquots of the supernatant (100 μl) by liquid scintillation counting with scintillation cocktail ACS (1.5 ml). Nonspecific binding of the neurotransmitter was evaluated in cooling samples sedimented immediately after the addition of radiolabeled glutamate.

As shown in Fig. 4, the supernatant contained 80.0 ± 5.0% of total radioactivity added to synaptosomes ($n=6$), and the pellets (with the rest of supernatants)—20.0 ± 5.0% ($n=6$), respectively, when measured the initial velocity of L-[^{14}C]glutamate uptake by synaptosomes at 1 min time point. Measurements of L-[^{14}C]glutamate accumulation

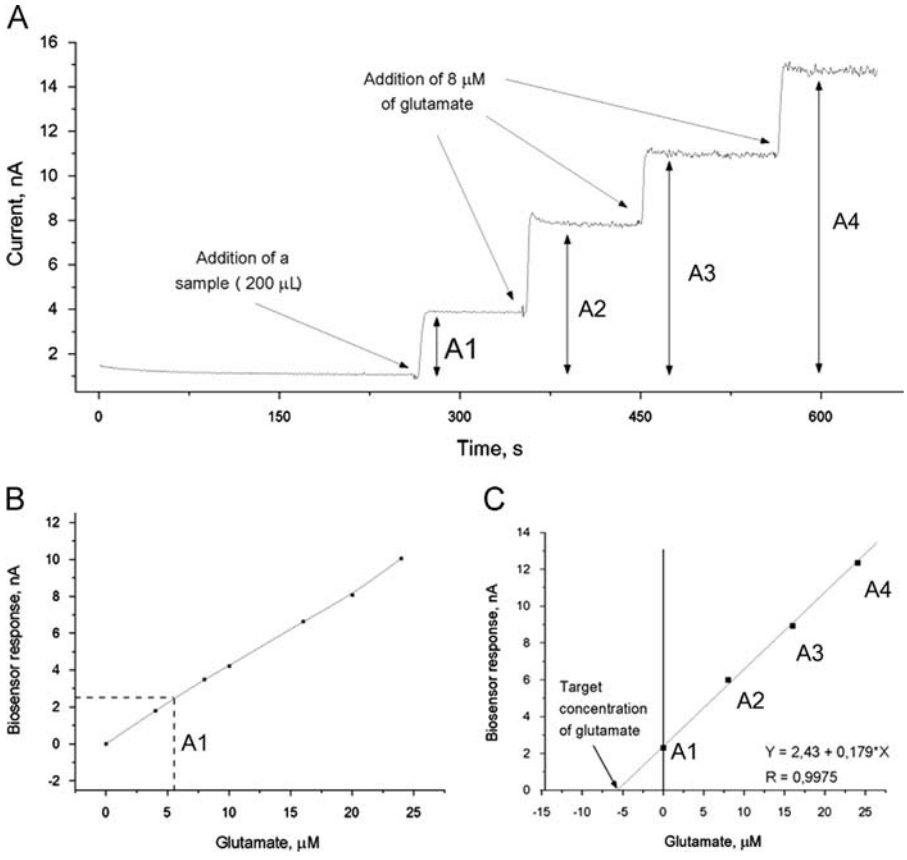

Fig. 5. An example of real experiment of glutamate determination via the biosensor. (A) Biosensor responses to addition of synaptosomal sample (A1) and three subsequent additions of pure glutamate (A2–A4). (B) Calibration plot for glutamate determination. (C) Plot for determination of glutamate via standard addition method.

by synaptosomes for 10 min revealed that the supernatant contained 58.0 ± 5.0% of total radioactivity added to synaptosomes ($P \leq 0.05$, as compared to radioactivity in supernatant at 1 min time point, Student's *t*-test, $n=6$), and the pellets (with the rest of supernatants)—42.0 ± 5.0%, respectively ($P \leq 0.05$, as compared to radioactivity in pellets at 1 min time point, Student's *t*-test, $n=6$).

The similar method was also appropriate for the analysis of the initial velocity of [^{3}H]GABA uptake by synaptosomes. The supernatant contained 72.0 ± 3.0% of total radioactivity added to synaptosomes ($n=4$), the pellets—28.0 ± 3.0% ($n=4$), respectively. When the inhibitor of [^{3}H]GABA uptake was applied, the supernatant become 79.6 ± 3.0% of total radioactivity added to synaptosomes ($n=4$), the pellets—20.3 ± 3.0% ($n=4$), respectively. Using classical method of filtering through fiberglass filters Whatman GF/C, the same inhibitor decreased the amount of radioactivity of the filter from 4.53 ± 0.2% of total radioactivity accumulated by synaptosomes ($n=4$) to 3.5 ± 0.48% ($n=4$), respectively.

This methodological approach has several advantages over pellet-based one. At first, more samples can be analyzed during the same time unit that is important for measurements of fast kinetics, and second, this method requires less amount of protein per sample. However, it should be noted that ^{14}C-radiloabeled neuromediators revealed the more accurate data in comparison with ^{3}H-labeled ones.

3.3. Measurements of the basal signal of glutamate biosensor in synaptosomal preparations

At first, it was demonstrated that standard salt solution (see Section 2) did not evoke biosensor response. Before starting uptake experiments, biosensor response was also measured in supernatant of synaptosomal preparation at zero time point, that is, just after preliminary incubation of cold synaptosomes at 37 °C for 10 min (see Section 2). This response is a very important characteristic of synaptosomes that reflects the basal level of the signal, which should be obviously taken into consideration in calculation of the initial velocity and accumulation of glutamate by nerve terminals. It should be noted that commonly used method of the registration of the velocity of glutamate uptake started by the addition of L-[^{14}C]glutamate/L-glutamate to synaptosomal suspension does not take into consideration the basal level of endogenous glutamate. Evaluation of this basal response of biosensor (as well as other measurements of synaptosomal samples) was carried out using two methods —the calibration plot and standard addition (Fig. 5). In calibration plot, glutamate concentration was determined by comparing biosensor response to sample addition with preliminary obtained calibration plot (Fig. 5B). In case of standard additions, biosensor responses to a sample and to 3 subsequent additions of pure glutamate were obtained (Fig. 5A). Next, values of the responses were plotted as shown on Fig. 5C and glutamate concentration was obtained on the intersection with abscissa. It was shown that the average basal level of glutamate in synaptosomes determined by two approaches consisted of 7.9 ± 0.6 μM ($n=6$) at a synaptosomal protein concentration of 0.4 mg/ml. As the addition of glutamate oxidase follow by the measurement of the basal signal, it may be concluded that the obtained value related to glutamate oxidase substrates only but not to the presence of electroactive substances.

The basal level of extracellular glutamate in synaptosomes was detected in fluorimetric experiments using glutamate dehydrogenase [25,26]. In the presence of glutamate, glutamate dehydrogenase reduced β-nicotinamide adenine dinucleotide (NAD^+) to NADH, a product that fluoresces, when excited with UV light. As shown Fig. 6 glutamate concentration detected with glutamate dehydrogenase assay consisted of 2.3 ± 0.3 μM ($n=3$) at a protein concentration of 0.5 mg/ml. Basal level of glutamate varies from

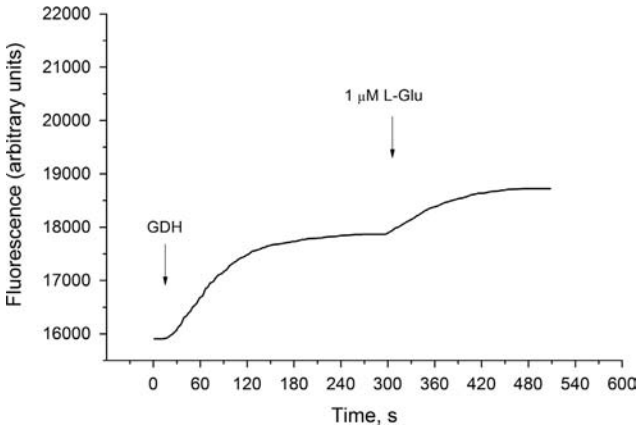

Fig. 6. The extracellular level of endogenous glutamate in rat brain synaptosomes assessed with glutamate dehydrogenase assay. Synaptosomal suspension (0.5 mg/ml of final protein concentration) was added to an enzymatic assay solution containing glutamate dehydrogenase (GDH). The extracellular level and tonic release (starting from 3 min time point) of endogenous glutamate in synaptosomes was measured by the changes in NADH fluorescence (excitation and emission wavelengths of 340 and 460 nm, respectively). Trace is representative of three independent experiments.

preparation to preparation and depends from functional status of the nerve terminals.

The ambient level of glutamate in synaptosomes was assessed with amino acid analyzer (see Section 2). The value of ambient glutamate consisted of 2.8 ± 0.3 μM ($n=3$) at a synaptosomal protein concentration of 2 mg/ml. This data is in accordance with data obtained with glutamate dehydrogenase assay but is slightly lower than those obtained with glutamate biosensor. Glutamine can be also a substrate for glutamate oxidases from different sources. The concentration of ambient glutamine determined with amino acid analyzer was approximately seven times lower in comparison with ambient glutamate and was 0.4 ± 0.03 μM ($n=3$) at a synaptosomal protein concentration of 2 mg per ml.

Basal signal of glutamate biosensor in synaptosomes per se is very important characteristic of glutamatergic neurotransmission because it reflects their ability to maintain the proper extracellular level of glutamate.

3.4. Analysis of the initial velocity of Na^+-dependent uptake and accumulation of glutamate in nerve terminals using glutamate biosensor

Comparative experiments determining the initial velocity of glutamate uptake by nerve terminals and accumulation of glutamate for 10 min were conducted using both L-[^{14}C]glutamate and glutamate biosensor. In calculations, the basal signal of biosensor in the aliquots of supernatants of synaptosomal suspension immediately after the initiation of uptake by the addition of cold glutamate (10 μM) was set as 100%. In experiments with L-[^{14}C]glutamate, radioactivity in the aliquots of supernatants of synaptosomal preparation after starting uptake with L-[^{14}C]glutamate/L-glutamate (10 μM) was set as 100%. In 1 min, the initial velocity of glutamate uptake by synaptosomes was measured in two ways with biosensor and radiolabeled method that consisted of 79 ± 5% ($n=6$) of the initial value (calibration plot) and 78 ± 5% ($n=6$) of the initial value (standard addition method) in biosensor experiments and 75 ± 5% ($n=6$) of the initial value using L-[^{14}C]glutamate. Fig. 7A shows a decrease in the glutamate concentration in the supernatant at 1 min time point after the initiation of uptake process by 10 μM of cold glutamate (the first and second columns) or L-[^{14}C]glutamate/L-glutamate (the third column).

The similar measurements were carried out at the time intervals that used to calculate the accumulation of glutamate by synaptosomes. It is shown that accumulation of glutamate by synaptosomes for 10 min consisted of 58 ± 4% (n=6) of the initial value (calibration plot) and 56 ± 4% (n=6) of the initial value (standard addition) in biosensor experiments and 53 ± 4% (n=6) of the initial value using L-[^{14}C]glutamate. Fig. 7B shows a decrease in the glutamate concentration in the synaptosomal supernatant for 10 min after the initiation of uptake process by 10 μM of cold glutamate or L-[^{14}C]glutamate/L-glutamate.

Therefore, it was shown that the values of the initial velocity of glutamate uptake and accumulation of glutamate by nerve terminals measured using glutamate biosensor and L-[^{14}C]glutamate were almost similar, if the above methodological approach and calculation algorithm, which take into account the level of the basal glutamate signal, were applied (Table 1).

4. Discussion

Analytical methods such as high performance liquid chromatography and gas chromatography/mass spectrometry [27] and coupled techniques (e.g., microdialysis) are capable of quantifying glutamate concentrations under physiological conditions [28], but cannot measure dynamic glutamate flux, and are temporally constrained (due to bulk sampling) [5,29]. Quantification of neurotransmitter transport dynamics is hindered by a lack of sufficient tools to directly monitor bioactive flux under physiological conditions [5]. Thus, real-time autonomous techniques, which can prove non-invasive quantification of glutamate transport dynamics in neuronal cultures, in particular, techniques which have the ability to quantify magnitude and direction of glutamate transport in the neural environment are desired [5]. Electrochemical microelectrodes resolve some disadvantages, and can

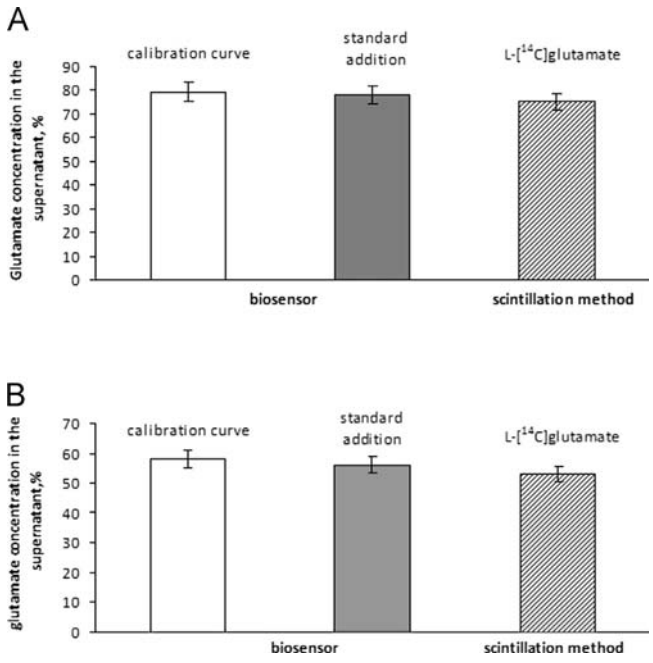

Fig. 7. A decrease in concentrations of cold glutamate (biosensor experiments) and L-[^{14}C]glutamate/L-glutamate (scintillation method) in the aliquots of supernatants of synaptosomal suspension in 1 min (A) and 10 min (B) after the initiation of glutamate uptake. The values were obtained with glutamate biosensor using calibration plot (the first column) and standard addition method (the second column) and scintillation technique (the third column). The basal signal of biosensor immediately after the initiation of uptake by the addition of cold glutamate (10 μM) was set as 100% (the first and second columns). Radioactivity in the aliquots of synaptosomal supernatant after starting uptake with L-[^{14}C] glutamate/L-glutamate (10 μM) was set as 100% (the third columns).

Table 1
Summarizing data on comparative analysis of the initial velocity and accumulation of glutamate by nerve terminals using glutamate biosensor and L-[^{14}C]glutamate.

Key characteristics of glutamate transport in nerve terminals	Biosensor measurements, % of the initial value	L-[^{14}C]Glutamate measurements, % of the initial value
The initial velocity of glutamate uptake	78.5	75
The accumulation of glutamate	57	53

successfully measure glutamate concentration in cultured cells, hippocampal slices, and in vivo in rats [29–32]. Amperometric transduction techniques are most commonly used for electrochemical detection of neurotransmitters (e.g., glutamate, serotonin and nitric oxide) [33,34]. The use of carbon nanotubes and nanopatterned metals such as platinum has been shown to increase electron transfer (and therefore sensitivity) in microelectrodes [35]. However, these techniques have relatively low sensitivity, excessive drift/noise, low signal-to-noise ratio that make difficult to quantify rapid neurochemical kinetics during synaptic transmission [5].

In this study: (1) amperometric glutamate biosensor and (2) methodological approach for the analysis of glutamate (and GABA) uptake in liquid synaptosomal samples were developed; and also (3) the basal level of glutamate, the initial velocity of glutamate uptake and accumulation of glutamate in nerve terminals were determined by glutamate biosensor; and (4) comparative analysis of this data with those obtained by radiolabeled technique, spectrofluorimetry and ion exchange chromatography were performed. The necessity of the development of a new methodological approach for the analysis of glutamate (and GABA) uptake is underscored by the fact that the samples for glutamate biosensor should be liquid, whereas the most common approach in the neuroscience area for the measurements of glutamate uptake by synaptosomes is application of radioactively labeled L-[^{14}C]glutamate and evaluation of its amount in synaptosomal pellets after different manipulations [36,37]. The adequacy of the developed approach was proven using L-[^{14}C]glutamate in parallel experiments. The registration of L-[^{14}C] glutamate uptake was performed not by filtering of synaptosomal suspension and analysis of radioactivity in the pellet, but in the aliquots of the supernatant after centrifugation in a microfuge. L-[^{14}C] Glutamate uptake by synaptosomes at definite time intervals can be calculated as a reduction of radioactivity in the supernatant.

The ambient level of glutamate is a balance between glutamate uptake and unstimulated glutamate release from nerve terminals and is very important for tonic activation of post-/pre-synaptic glutamate receptors [38]. In our experiments, the ambient level of glutamate in synaptosomes was 7.9 μM. It should be underlined that during measurements of the kinetic characteristics of glutamate uptake with radiolabeled L-[^{14}C]glutamate (it is actual also for other amino acid neurotransmitters), the ambient level of endogenous glutamate (or other neurotransmitters) is usually not taken into consideration. Developed methodological approach using glutamate biosensor allows measuring and calculating the kinetic characteristics taking into account the level of the basal glutamate signal.

Recently, McLamore et al. [5] developed biosensor that was used in self-referencing (oscillating) mode to measure endogenous glutamate release and uptake in neural cells during electrical stimulation. Using differentiated P19 cells, McLamore et al. [5] measured the basal level and release of glutamate stimulated by high-KCl, and also glutamate release stimulated by high-KCl after the treatment of the cells with the inhibitor of glutamate transporter DL-TBOA. It was shown that the basal extracellular concentration of glutamate in cell culture was 5.8 ± 0.6 μM that was similar to reported in vivo values measured using microdialysis 1–5 μM

[39] and GluOx biosensors 1.4–1.8 μM [29]. It should be noted that our data on the extracellular level of glutamate in synaptosomes is very similar with those measured in the cell culture.

As future perspective, we plan to develop algorithm for calculation of the velocity of stimulated by depolarization of the plasma membrane transporter-mediated release, Ca^{2+}-dependent release, and unstimulated release of glutamate from nerve terminals using glutamate biosensor and compare this data with those obtained with radiolabeled and spectrofluorimetric assay.

Also, glutamate biosensor is very perspective for the measurements of glutamate transport in blood platelets, which are suggested to serve a peripheral model of presynaptic nerve terminals [11,40,41]. In platelet experiments, it should be taken into consideration that there is a significant difference in the ambient glutamate level in nerve terminals and platelets, because the latest are surrounding with blood plasma, where a glutamate concentration consists of approximately 30 μM.

In summary, using developed amperometric glutamate biosensor, we devise a new method for the analysis of glutamate uptake in liquid samples that allow measuring the initial velocity of uptake and accumulation of glutamate by nerve terminals taking into account the basal glutamate level.

Funding

This work was support by the Program of NAS of Ukraine “Sensor systems for medico-ecological and industrial-technological requirement: metrological support and experimental operation”, Space Research Program, and STCU project # 6055.

Ethical approval

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

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

Acknowledgements

We would like to thank Dr. L. Kasatkina and M. Myasnikova for spectrofluorimetry and ion exchange chromatography.

References

[1] N.C. Danbolt, Prog. Neurobiol. 65 (2001) 1–105.
[2] J.L. Rickus, A.J. Tobin, J.I. Zink, B. Dunn, Mater. Res. Soc. Symp. Proc. 662 (2001) 155–161.
[3] R.I. Hume, L.W. Role, G.D. Fischbach, Nature 305 (1983) 632–634.
[4] A. Chen, C.E. Lunte, J. Chromatogr. A 691 (1995) 29–35.
[5] E.S. McLamore, S. Mohanty, J. Shi, J. Claussen, S.S. Jedlicka, J.L. Rickus, D.M. Porterfield, J. Neurosci. Methods 189 (2010) 14–22.
[6] R.L. Villarta, D.D. Cunnigham, G.G. Guilbault, Talanta 38 (1991) 49–55.
[7] Q. Li, S. Zhang, J. Yu, Appl. Biochem. Biotechnol. 59 (1996) 53–61.
[8] N.F. Almeida, A.K. Mulchandani, Anal. Chim. Acta 282 (1993) 353–361.
[9] S. Ghobadi, E. Csoregi, G. Marko-Varga, L. Gorton, Curr. Sepr. 14 (1996) 94–102.
[10] T.D. Gibson, J.N. Hulbert, S.N. Parker, et al., Biosens. Bioelectron. 10 (1992) 701–708.
[11] L. Kastkina, T. Borisova, Int. J. Biochem. Cell Biol. 45 (2013) 2585–2595.
[12] Q. Yang, P. Atanasov, E. Wilkins, Sens. Actuators, B 46 (1998) 249–256.
[13] O.M. Schuvailo, O.O. Soldatkin, A. Lefebvre, R. Cespuglio, A.P. Soldatkin, Anal. Chim. Acta 573 (2006) 110–116.
[14] S.C. Kelly, P.J. O'Connell, C.K. O'Sullivan, G.G. Guilbault, Anal. Chim. Acta 412 (2000) 111–119.
[15] S.J. Killoran, R.D. O'Neill, Electrochim. Acta 53 (2008) 7303–7312.
[16] O.O. Soldatkin, O.M. Schuvailo, S. Marinesco, R. Cespuglio, A.P. Soldatkin, Talanta 78 (2009) 1023–1028.
[17] C.W. Cotman, Methods Ensemol. 31 (1974) 445–452.
[18] T. Borisova, N. Krisanova, Adv. Space Res. 42 (2008) 1971–1979.
[19] N. Krisanova, I. Trikash, T. Borisova, Neurochem. Int. 55 (2009) 724–731.
[20] E. Larson, B. Howlett, A. Jagendorf, Anal. Biochem. 155 (1986) 243–248.
[21] C. Sudhof, Annu. Rev. Neurosci. 27 (2004) 509–547.
[22] T. Borisova, R. Sivko, A. Borysov, N. Krisanova, Cell Mol. Neurobiol. 30 (2010) 1013–1023.
[23] T. Borisova, N. Krisanova, R. Sivko, L. Kasatkina, A. Borysov, S. Griffin, et al., Neurochem. Int. 59 (2011) 272–279.
[24] N. Krisanova, L. Kasatkina, R. Sivko, A. Borysov, A. Nazarova, K. Slenzka, et al., Astrobiology 13 (2013) 679–692.
[25] N. Krisanova, R. Sivko, L. Kasatkina, T. Borisova, Biochim. Biophys. Acta, Mol. Basic Dis 1822 (2012) 1553–1561.
[26] N. Krisanova, R. Sivko, L. Kasatkina, A. Borysov, T. Borisova, Mol. Cell. Neurosci. 58 (2014) 95–104.
[27] S.S. Jedlicka, K.M. Little, D.E. Nivens, D. Zemlyanovdf, J.L. Rickus, J. Mater. Chem. 17 (2007) 5058–5067.
[28] C.J. Watson, B.J. Venton, R.T. Kennedy, Anal. Chem. 78 (2006) 1391–1399.
[29] B.K. Day, F. Pomerleau, J.J. Burmeister, P. Huett, G.A. Gerhardt, J. Neurochem. 96 (2006) 1626–1635.
[30] O. Niwa, T. Horiuchi, K. Torimitsu, Biosens. Bioelectron. 12 (1997) 311–319.
[31] W.H. Oldenziel, M. van der Zeyden, G. Dijkstra, W.E.J.M. Ghijsen, H. Karst, T.I.H.F. Cremers, et al., J. Neurosci. Methods 160 (2007) 37–44.
[32] J.J. Burmeister, G.A. Gerhardt, Anal. Chem. 73 (2001) 1037–1042.
[33] N. Dale, S. Hatz, F. Tian, E. Llaudet, Trends Biotechnol. 23 (2005) 420–428.
[34] G.S. Wilson, R. Gifford, Biosens. Bioelectron. 20 (2005) 2388–2403.
[35] S. Hrapovic, Y. Liu, K.B. Male, J.H.T. Luong, Anal. Chem. 76 (2004) 1083–1088.
[36] T. Borisova, N. Himmelreich, Adv. Space Res. 36 (2005) 1340–1345.
[37] T. Borisova, N. Krisanova, N. Himmelreich, Adv. Space Res 33 (2004) 1362–1367.
[38] T. Borisova, Cholesterol and Presynaptic Glutamate Transport in the Brain, Springer, New York, NY, 2013.
[39] D.A. Baker, Z. Xi, H. Shen, C.J. Swanson, P.W. Kalivas, J. Neurosci. 22 (2002) 9134–9141.
[40] L. Kasatkina, T. Borisova, Neurochem. Int. 56 (2010) 711–719.
[41] T. Borisova, L. Kasatkina, L. Ostapchenko, Neurochem. Int. 59 (2011) 965–975.