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Neuroprotection by lowering cholesterol: A decrease in membrane cholesterol content reduces transporter-mediated glutamate release from brain nerve terminals

N. Krisanova, R. Sivko, L. Kasatkina, T. Borisova *

The Department of Neurochemistry, Palladin Institute of Biochemistry, NAS of Ukraine, 9 Leontovicha Street, Kiev 01601, Ukraine

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ABSTRACT

Background: In our earlier work, a reduction of cholesterol content increased the extracellular glutamate level in rat brain nerve terminals (synaptosomes) that was a result of the lack of transporter-mediated glutamate uptake. The aim of this study was to assess transporter-mediated release of glutamate from cholesterol-deficient synaptosomes. In stroke, cerebral hypoxia/ischemia, and traumatic brain injury, the development of neurotoxicity is provoked by enhanced extracellular glutamate, which is released from nerve cells mainly by glutamate transporter reversal — a distinctive feature of these pathological states. Methods: Laser scanning confocal microscopy, spectrofluorimetry, radiolabeled assay, and glutamate dehydrogenase assay. Results: Cholesterol acceptor methyl-β-cyclodextrin (15 mM) reduced the cholesterol content in the synaptosomes by one quarter. Transporter-mediated glutamate release from synaptosomes: 1) stimulated by depolarization of the plasma membrane; 2) by means of heteroexchange with competitive transportable inhibitor of glutamate transporters DL-threo-β-hydroxyaspartate; 3) in low [Na^+] medium; and 4) during dissipation of the proton gradient of synaptic vesicles by the protonophore cyanide-p-trifluoromethoxyphenyl-hydrazon (FCCP); was decreased under conditions of cholesterol deficiency by ~24, 28, 40, and 17%, respectively. Conclusions: A decrease in the level of membrane cholesterol attenuated transporter-mediated glutamate release from nerve terminals. Therefore, lowering cholesterol may be used in neuroprotection in stroke, ischemia, and traumatic brain injury which are associated with an increase in glutamate uptake reversal. This data may explain the neuroprotective effects of statins in these pathological states and provide one of the mechanisms of their neuroprotective action. However, beside these disorders, lowering cholesterol may cause harmful consequences by decreasing glutamate uptake in nerve terminals.

1. Introduction

Glutamate is not only a key excitatory neurotransmitter in the mammalian CNS, but also a potent neurotoxin. In stroke, cerebral hypoxia/ischemia, hypoglycemia, and traumatic brain injury, the development of neurotoxicity is provoked by an increase in the concentration of extracellular glutamate. Excessive extracellular glutamate overstimulates glutamate receptors initiating an excessive calcium entry through mainly N-methyl-D-aspartate ionotropic receptors, causing excitotoxicity, neuronal injury and death. In norm, extracellular glutamate between episodes of exocytotic release of the neurotransmitter is maintained at a low level, thereby preventing continual activation of glutamate receptors and protecting neurons from excitotoxic injury [1]. The enzymes for glutamate degradation have not been found in the synaptic cleft, so it is the only possibility to maintain a low extracellular glutamate concentration that is realized by high-affinity Na^+-dependent glutamate transporters through neurotransmitter uptake in neurons and glial cells. They use Na^+/K^+ electrochemical gradients across the plasma membrane as a driving force [2]. However, glutamate transporters can also act in the outward direction, so their function is reversible. Under conditions of energy deprivation and failure of the electrochemical gradient of the plasma membrane, glutamate transporters change the direction of their work and start to release the neurotransmitter into the extracellular space. A decrease in extracellular [Na^+] and/or intracellular [K^+] as well as an increase in extracellular [K^+] and/or intracellular [Na^+] and/or intracellular [Glu] in nerve terminals thermodynamically favor glutamate transport in the outward direction. It should be noted that transporter-mediated release is the main mechanism underlying the enhancement of the extracellular glutamate concentration under pathological conditions such as stroke, cerebral hypoxia/ischemia, hypoglycemia, and traumatic brain injury [3–5]. Kinetic data predicts that glutamate release through reverse transport can be dramatic because 1 μm^2 of neuronal cell membrane can release 140,000

Abbreviations: MβCD, methyl-β-cyclodextrin; GDH, glutamate dehydrogenase; DL-THA, DL-threo-β-hydroxyaspartate; DL-TBOA, DL-threo-β-benzyloxyaspartate; FCCP, cyanide-p-trifluoromethoxyphenyl-hydrazon

* Corresponding author. Tel.: +380 44 234 3254; fax: +380 44 279 6365.
E-mail address: tborisov@biochem.kiev.ua (T. Borisova).

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molecules of glutamate per second at an elevated extracellular [K^+] of 50 mM and reduced [Na^+] of 50 mM (Vm = −40 mV) [6]. Neuronal glutamate transporters are more reversible in comparison with the glial ones. While the neuronal glutamate transporters are functionally converted from an uptake-predominant to a release-predominant state by a reduction in [Na^+] from 145.2 mM to about 60 mM, uptake of glutamate by glial GLT-1 is maintained [7]. Therefore, transporter-mediated glutamate release from the neurons mainly contributes to an increase in ambient glutamate concentration under pathological conditions.

As glutamate transporters are integral membrane proteins, their function is tightly associated with the plasma membrane, and thus can be modulated by changes in its physical and chemical properties. Certain level of membrane cholesterol, which is an abundant constituent of eukaryotic membranes, is very important for normal functioning of a number of membrane proteins involved in synaptic transmission, such as ion channels, pumps, receptors and transporters [8–10]. Cholesterol depletion differently influences transporter-mediated glutamate uptake in mouse brain plasma membrane vesicles, rat brain nerve terminals, primary cortical cultures and in hippocampal astrocytes [8,11,12]. Exocytotic release of glutamate from nerve terminals is significantly decreased after depletion of membrane cholesterol [10,13,14].

So, the changes in glutamate uptake and exocytotic release of glutamate in nerve terminals as well as the functioning of ion channels under conditions of cholesterol deficiency are well documented in the literature. In contrast, non-exocytotic transporter-mediated glutamate release from nerve terminals, the main mechanism of glutamate release in stroke, cerebral hypoxia/ischemia, and traumatic brain injury, is not yet assessed. It is clear that a delay in elevation of ambient glutamate has a potential for preventing brain damage under these pathological states. The main question we ask is how reduced cholesterol content of neuronal membrane can modulate the pathogenic mechanisms underlying neurotoxicity? In addition, as cholesterol depletion decreases glutamate uptake, whether it also influences glutamate transporter reversal. In cholesterol-deficient nerve terminals, we assessed transporter-mediated release of glutamate: (*) stimulated by the depolarization of the plasma membrane; (**) by heteroexchange with transportable inhibitor of glutamate transporters DL-threo-β-hydroxyaspartate (DL-THA); (***) in low-Na^+ medium; (****) during dissipation of the proton gradient of synaptic vesicles; (*****) under conditions of energy deprivation.

This study is also of interest because of increasing evidence that statins (widely applicable cholesterol-reducing drugs that reduced the membrane cholesterol level in the brain [15,16]) have neuroprotective features under several pathological conditions, including stroke, cerebral ischemia, traumatic brain injury, and excitotoxic amino acid exposure [17–20]. The exact molecular mechanisms underlying these findings remain poorly understood.

2. Materials and methods

2.1. 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, Kiev. They were housed in a quiet, temperature-controlled room (22–23 °C) and were provided with water and dry food pellets ad libitum. All procedures conformed to the guidelines of the Palladin Institute of Biochemistry. 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 [21] with slight modifications. 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). The Ca^{2+}-free medium contained 1 mM EGTA (Sigma, U.S.A.) and no added Ca^{2+}. Protein concentration was measured as described by [22].

2.2. The treatment of nerve terminals with methyl-β-cyclodextrin (MβCD) and the determination of cholesterol concentration

The treatment of synaptosomes with 15 mM MβCD (Sigma, U.S.A.) (37 °C, 30 min) was carried out in standard oxygenated salt solution, then synaptosomal suspension was washed with 10 volumes of ice-cold standard salt solution, sedimented, and then the pellet was resuspended in this solution to a final concentration of 1 mg protein/ml and immediately used in the experiments. Control synaptosomes were simultaneously incubated without MβCD for 30 min at 37 °C, and then also subjected to washing procedure similarly with MβCD experiments. MβCD complexed with cholesterol (15 mM MβCD and 2.3 mM cholesterol; Sigma, U.S.A.) was prepared as described by [23]. The treatment of synaptosomes by MβCD/cholesterol complex was similar with the abovementioned.

The extraction of lipids from the aliquots of synaptosomal suspension of untreated (control), MβCD-treated and MβCD/cholesterol-treated samples was performed according to [24]. Quantitative determination of cholesterol level was carried out according to [25].

2.3. Confocal imaging of filipin-labeled nerve terminals

We used the fluorescent probe filipin, which binds to membrane cholesterol [26], to clarify the alterations in membrane cholesterol content of synaptosomes. The fluorescent dye filipin (50 μg/ml) (Sigma, U.S.A.) was administered to synaptosomal suspension (final protein concentration of 0.2 mg/ml). Filipin-labeled synaptosomes were evaluated under the confocal laser scanning microscope LSM 510 META, Carl Zeiss, objective Plan-Apochromat 100×/1.4 Oil DIC, 405 nm excitation and >505 nm emission. For confocal imaging, 1 μl of filipin-labeled synaptosomal suspension (0.2 mg/ml) was squashed and spread between two glass surfaces. Filipin-labeled synaptosomes were viewed in the absence of MβCD, then 1 μl of MβCD stock solution (30 mM) was added to the thin layer of synaptosomal suspension through the hole in the upper glass at 5 s time point after starting the time series and fluorescence images were captured with camera in each 5 s.

2.4. L-[^{14}C]Glutamate release experiments

Control, MβCD- or MβCD/cholesterol-treated synaptosomes were diluted in standard salt solution to 2 mg of protein/ml and after pre-incubation at 37 °C for 10 min were loaded with L-[^{14}C]glutamate (1 nmol/mg of protein, 238 mCi/mmol) (Amersham, UK) in Ca^{2+}-supplemented oxygenated standard salt solution at 37 °C for 10 min. After loading, the suspension was washed with 10 volumes of ice-cold oxygenated standard salt solution; the pellet was resuspended in this solution to a final concentration of 1 mg protein/ml and immediately used for release experiments. Release of L-[^{14}C]glutamate from synaptosomes was performed in Ca^{2+}-free incubation medium according to the following method: samples (125 μl of the suspension, 0.5 mg of protein/ml) were incubated for different time intervals within the range 0–6 min at 37 °C and rapidly sedimented in a microcentrifuge (20 s at 10,000×g). Release was measured in the aliquots of the supernatants (100 μl) and the pellets by liquid scintillation counting with scintillation cocktail ACS (1.5 ml)

(Amersham, UK). Total synaptosomal L-[^{14}C]glutamate content was equal to 200,000 ± 15,000 cpm/mg protein. Release of the neurotransmitter from synaptosomes incubated without stimulating agents was used for assay of tonic (basal) release. Stimulated release of the neurotransmitter was calculated by subtracting the basal value from the value of total release.

2.5. Glutamate dehydrogenase assay: the assessment of the extracellular level and release of endogenous glutamate from nerve terminals

The changes in the extracellular level of glutamate in the synaptosomes were detected using glutamate dehydrogenase assay [27,28]. 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 thermostated 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 the synaptosomes to the incubation media was detected as an increase in NADH fluorescence.

To analyze transporter-mediated glutamate release, synaptosomes were preloaded with cold glutamate (50 μM) at 37 °C for 10 min, then the procedures were similar with those in the experiments with L-[^{14}C]glutamate. The concentration of cold glutamate in the aliquots of the supernatants was determined based on the value of NADH fluorescence in each probe.

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

2.6. 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. Cholesterol concentration in nerve terminals after treatment with methyl-β-cyclodextrin (MβCD)

Cyclic oligosaccharide MβCD, which is composed of a lipophilic cavity and hydrophilic outer surface, effectively extracts cholesterol from the membranes of a variety of cells [8,12,14,29–31]. We used isolated brain nerve terminals (synaptosomes), which retain all features of intact nerve terminals, e.g., ability to maintain the membrane potential, exocytotic and transporter-mediated release as well as accomplish uptake of neurotransmitters. Quantitative assessment of cholesterol concentration (see Materials and methods) showed that the treatment of synaptosomes with 15 mM MβCD for half an hour reduced the cholesterol level from 0.095 ± 0.004 μmol of cholesterol/mg of protein in the control to 0.075 ± 0.002 μmol of cholesterol/mg of protein after the treatment with the acceptor ($P \leq 0.05$, Student's *t*-test, n = 4) (Fig. 1, A). Whereas, cholesterol content of synaptosomes treated with MβCD complexed with cholesterol (2.3 mM cholesterol in 15 mM MβCD) was not changed considerably, thereby making these synaptosomes appropriate for using as an additional control in the analysis of changes associated with cholesterol deficiency. We confirmed the above data using confocal laser scanning microscopy with the fluorescent probe filipin, which binds to membrane cholesterol [26]. The profiles of the fluorescence intensity of filipin represented in Fig. 1, B showed that MβCD quickly and effectively extracted cholesterol from the synaptosomes.

3.2. The extracellular level of glutamate in cholesterol-deficient nerve terminals

As it was mentioned in the Introduction, the certain level of ambient glutamate is very important for proper synaptic transmission, whereas an increase in this level causes neurotoxicity [1,32,33]. In our recent studies, we revealed that the extracellular level of preloaded L-[^{14}C]glutamate became one third higher after the treatment with 15 mM MβCD and consisted of 0.193 ± 0.013 nmol/mg protein in the control and 0.282 ± 0.013 nmol/mg protein in MβCD-treated synaptosomes ($P \leq 0.05$, Student's *t*-test, n = 8) [12]. The extracellular glutamate concentration is determined by a balance between glutamate uptake mediated by Na^+-dependent glutamate transporters and tonic release of glutamate. Tonic release of L-[^{14}C]glutamate (in the presence of DL-threo-β-benzyloxyaspartate (DL-TBOA) in the incubation media) was decreased from 0.075270 ± 0.005785 nmol/mg of protein in the control to 0.047285 ± 0.005785 nmol/mg of protein in MβCD-treated synaptosomes ($P \leq 0.05$, Student's *t*-test, n = 4). Glutamate dehydrogenase assay (see Materials and methods) was also used for the assessment of the extracellular level and tonic release of endogenous glutamate in cholesterol-deficient nerve terminals. As shown in Fig. 2, A, the ambient level of endogenous glutamate was ~35% higher in cholesterol-deficient nerve terminals in comparison with the control ones. Tonic release of endogenous glutamate from the synaptosomes was measured starting from 3 min time point when the most of ambient glutamate was converted to α-ketoglutarate by glutamate dehydrogenase. In cholesterol-deficient nerve terminals, tonic release of endogenous glutamate was decreased by ~25% at 1 min time point in comparison with the control (Fig. 2, B). For the analysis of the effect of MβCD per se irrespective to cholesterol accepting capacity, we used 15 mM MβCD complexed with cholesterol (2.3 mM). The application of this complex caused insignificant changes in the extracellular glutamate level and tonic release of glutamate from the synaptosomes as compared to the control. Thus, the results of the assessment of the extracellular level and tonic release of glutamate from cholesterol-deficient synaptosomes obtained with glutamate dehydrogenase assay were in accordance with our data on radiolabeled glutamate.

3.3. Stimulated by depolarization of the plasma membrane transporter-mediated release of glutamate from cholesterol-deficient nerve terminals

Depolarization of the plasma membrane of nerve terminals by high-KCl in Ca^{2+}-free medium causes reversal of glutamate transporters and release of glutamate from the cytosol. The value of transporter-mediated release of L-[^{14}C]glutamate from the synaptosomes measured at 3 min time point was decreased by ~24% after cholesterol extraction with 15 mM MβCD that equals to 0.101325 ± 0.006750 nmol/mg of protein in the control and 0.0772 ± 0.0048 nmol/mg of protein in cholesterol deficiency ($P \leq 0.05$, Student's *t*-test, n = 5) (Fig. 3, A). (However, at 6 min time point it was equal to 0.141855 ± 0.008865 nmol/mg of protein in the control and 0.136065 ± 0.008865 nmol/mg of protein in MβCD-treated synaptosomes). Similar to the previous section, the application of 15 mM MβCD complexed with cholesterol (2.3 mM) did not induce changes in transporter-mediated release of L-[^{14}C]glutamate from the synaptosomes as compared to the control. Thus, data on high-KCl-evoked transporter-mediated release of L-[^{14}C]glutamate from the synaptosomes showed that the initial velocity of this release was decreased under conditions of cholesterol deficiency.

To measure net transporter-mediated release of glutamate from cholesterol-depleted synaptosomes in these experiments, it was plausible to inhibit glutamate uptake in order to neglect its contribution. It is so because released glutamate is continuously removed from the extracellular medium by glutamate transporters

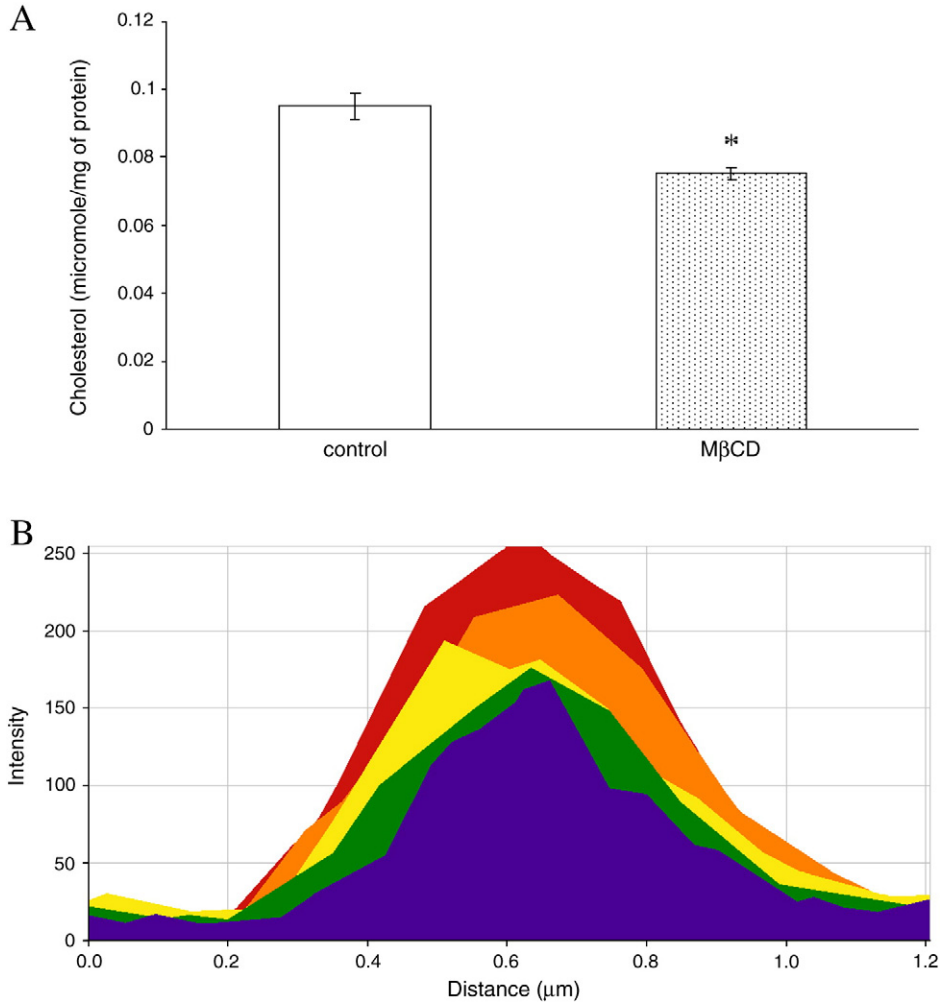

Fig. 1. (A) A decrease in the concentration of cholesterol in nerve terminals after treatment with MβCD (15 mM) at 37 °C for 30 min. Quantitative determination of cholesterol level was carried out according to Zlatkis et al. [25]. Data are means ± SEM of four independent experiments. *, $P \leq 0.05$ as compared to the control. (B) The profiles of the fluorescence intensity of cholesterol-sensitive fluorescent dye filipin recorded from the confocal images of filipin-labeled synaptosomes following the addition of MβCD.

with different effectiveness in the control and cholesterol deficiency. In this case, the application of glutamate transporter inhibitors is not possible because non-transportable one DL-TBOA attenuates as direct as reversed transport of glutamate, whereas transportable inhibitor DL-threo-beta-hydroxyaspartate (DL-THA) causes release of glutamate by means of heteroexchange. Weak uptake shown in cholesterol-depleted nerve terminals should increase the extracellular glutamate concentration, and thus the apparent release of L-[^{14}C]glutamate. Therefore, it was reasonable to analyze depolarization-evoked transporter-mediated release of endogenous glutamate from cholesterol-deficient synaptosomes using glutamate dehydrogenase assay. Unfortunately, we clarified that this release could not be registered in the synaptosomes after the treatment with MβCD (however, the rest of the tonic release was detected under these conditions, see the previous section). It is so because the treatment with MβCD for 30 min followed by washing procedure with 10 volumes of standard solution (see Materials and methods) led to the removal of the large amount of endogenous glutamate from the synaptosomes. This data is in accordance with our recent study that the addition of 15 mM MβCD caused dissipation of the proton gradient of synaptic vesicles and massive release of L-[^{14}C]glutamate from the synaptosomes [14].

So, we preloaded cold (non-radioactive) glutamate to MβCD-treated synaptosomes and measured depolarization-evoked transporter-mediated release of the neurotransmitter using glutamate dehydrogenase assay. As shown in Fig. 3, B, stimulated by high-KCl synaptosomal glutamate release in Ca^{2+}-free medium for 6 min was decreased after cholesterol depletion and consisted of 3.3333 ± 0.3400 nmol/mg of protein in the control and 1.5606 ± 0.1600 nmol/mg of protein in cholesterol deficiency ($P \leq 0.05$, Student's *t*-test, n = 5). This data was in accordance with the above results obtained with radioactive L-[^{14}C]glutamate (Fig. 3, A), where we demonstrated a significant decrease in the velocity of transporter-mediated L-[^{14}C]glutamate release at 1 and 3 min time points under conditions of cholesterol deficiency. However, the difference in this release between the control and cholesterol-deficient synaptosomes presented in Fig. 3, A (6 min point) was not so significant as in Fig. 3, B. It is so because of the existence of L-[^{14}C]glutamate uptake, which, in addition, is more effective in the control than in MβCD-treated synaptosomes [12,14]. In contrast, glutamate dehydrogenase experiments showed "pure" transporter-mediated release, when released glutamate was metabolized by the enzyme.

3.4. Release of glutamate from cholesterol-deficient nerve terminals by means of heteroexchange

In this set of the experiments, we evaluated the release of L-[^{14}C] glutamate by means of heteroexchange with transportable inhibitor of glutamate transporters DL-THA, which is a substrate for glutamate transporters that competitively inhibits glutamate uptake, but does

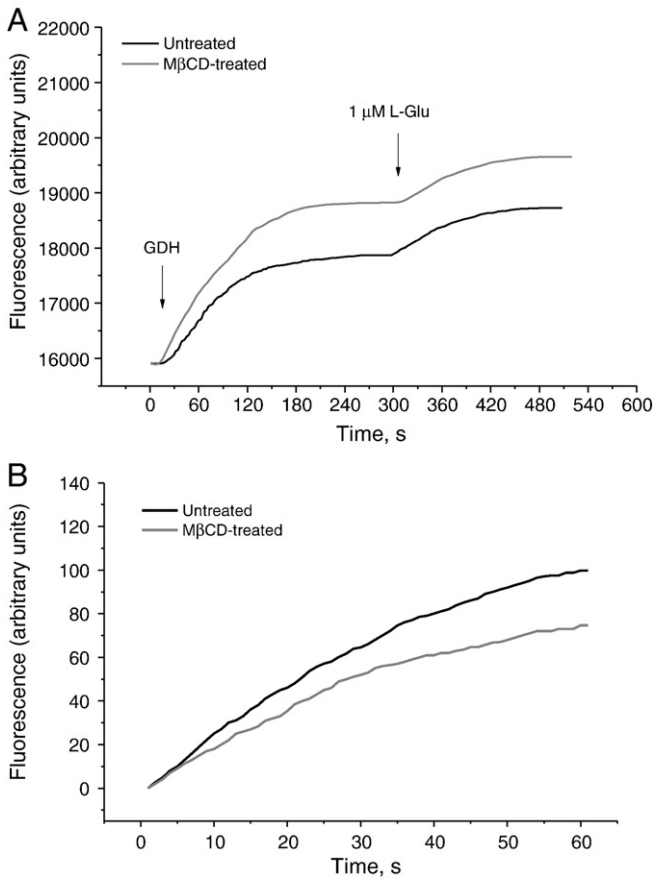

Fig. 2. (A) The extracellular level of endogenous glutamate in control (black line) and cholesterol-deficient (gray line) rat brain synaptosomes; (B) Tonic release of endogenous glutamate from control (black line) and cholesterol-deficient (gray line) rat brain synaptosomes assessed with glutamate dehydrogenase assay. Control and 15 mM MβCD-treated 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 were measured by the changes in NADH fluorescence (excitation and emission wavelengths of 340 and 460 nm, respectively). Trace is representative of three independent experiments.

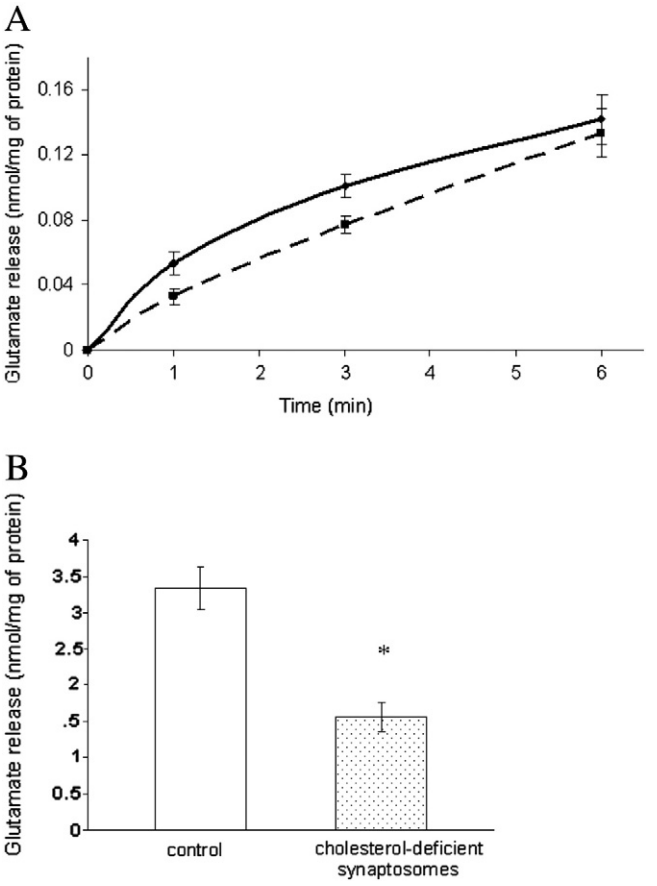

Fig. 3. Stimulated by high-KCl (35 mM) transporter-mediated release of preloaded L-[^{14}C]glutamate (A) and cold glutamate (glutamate dehydrogenase assay) (B) from control synaptosomes (solid line in A; empty column in B) and synaptosomes preliminary treated with 15 mM MβCD (dashed line in A; dotted column in B). Control and MβCD-treated synaptosomes were loaded with L-[^{14}C]glutamate (1 nmol/mg of protein, 238 mCi/mmol) (A) or 50 μM cold glutamate (B) as described in Materials and methods. After loading, samples (0.5 mg of protein/ml) were preincubated for 8 min at 37 °C, then at different time points the aliquots of the samples were centrifuged and L-[^{14}C]glutamate radioactivity was determined (A) or fluorescence intensity of NADH was measured at 6 min time point using glutamate dehydrogenase assay (B). Data are means ± SEM of five independent experiments, each performed in triplicate. *, $P \leq 0.05$ as compared to the control.

not prevent molecular transport mechanism. Heteroexchange and transporter-mediated release of glutamate have a common rate-limiting step in the transport process, and so heteroexchange may be used for the evaluation of transporter-mediated release of glutamate [34].

It was revealed that release of L-[^{14}C]glutamate by heteroexchange with 100 μM DL-THA was decreased (by ~28%) at 6 min time point from 0.137995 ± 0.009650 nmol/mg of protein to 0.099395 ± 0.008680 nmol/mg of protein as a result of the treatment of nerve terminals with 15 mM MβCD ($P \leq 0.05$, Student's *t*-test, n = 4) (Fig. 4). Using MβCD complexed with cholesterol (2.3 mM cholesterol in 15 mM MβCD), it was shown that the treatment with the complex did not change significantly heteroexchange of L-[^{14}C]glutamate with DL-THA in comparison with the control (Fig. 4). As release of glutamate by heteroexchange is justified for the evaluation of transporter-mediated glutamate release, we confirmed that the latest was decreased under conditions of cholesterol deficiency. This result is in accord with the above data on stimulated by depolarization transporter-mediated release of glutamate from cholesterol-depleted synaptosomes.

3.5. Glutamate release in low-Na^+ medium from cholesterol-deficient nerve terminals

As was mentioned earlier, Na^+/K^+ gradient is a driving force for glutamate uptake by transporters, and thus a reduction in the extracellular Na^+ concentration (up to 21 mM) is expected to inhibit uptake and facilitate the reversal of transporters resulting in the release of cytoplasmic glutamate into the extracellular space. Using monovalent organic cations N-methyl-D-glucamine (NMDG) to replace extracellular Na^+, we revealed that L-[^{14}C]glutamate release for 6 min was equal to 0.066585 ± 0.005790 nmol/mg of protein in the control and 0.039565 ± 0.005790 nmol/mg of protein in the cholesterol-depleted synaptosomes ($P \leq 0.05$, Student's *t*-test, n = 4) (Fig. 5). In synaptosomes treated with 15 mM MβCD complexed with cholesterol (2.3 mM), we did not find changes in L-[^{14}C]glutamate release in low-Na^+ medium as compared to the untreated control. Thus, it was demonstrated that the value of L-[^{14}C]glutamate release from cholesterol-deficient nerve terminals in low-Na^+ medium was lesser than that from the control.

3.6. Transporter-mediated glutamate release from cholesterol-deficient nerve terminals during dissipation of the proton gradient of synaptic vesicles

A principal assumption in the use of the protonophore carbonyl cyanide-p-trifluoromethoxyphenyl-hydrazon (FCCP) is the ability to

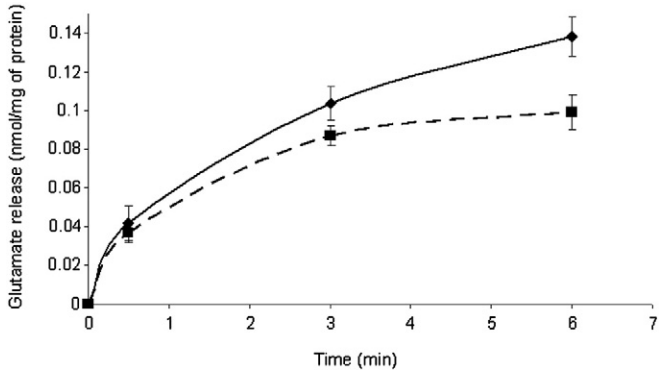

Fig. 4. Release of L-[^{14}C]glutamate by means of heteroexchange stimulated by transportable inhibitor of glutamate transporters DL-THA (100 μM) (Sigma, U.S.A.) from control synaptosomes (solid line) and cholesterol-deficient synaptosomes (dashed line). After loading of control and MβCD-treated synaptosomes with L-[^{14}C]glutamate (see Materials and methods), samples (0.5 mg of protein/ml) were preincubated for 8 min at 37 °C, at 1, 3 and 6 min time points, the aliquots of the samples were centrifuged. Data are means ± SEM of four independent experiments, each performed in triplicate.

dissipate the proton gradient and inhibit uptake of glutamate by synaptic vesicles [35–37]. These conditions should favor an augmentation of transporter-mediated (and also tonic) L-[^{14}C]glutamate release from nerve terminals. Recently, we have shown that FCCP-evoked L-[^{14}C]glutamate release from nerve terminals was significantly inhibited by DL-TBOA, so this release was mediated by glutamate transporters [38]. In this set of the experiments, we assessed L-[^{14}C]glutamate release from the control and cholesterol-depleted synaptosomes during application of FCCP and revealed that it was less in cholesterol deficient as compared to the control. As shown in Fig. 6 (the first pair of columns), FCCP-evoked release of L-[^{14}C]glutamate for 6 min was equal to 0.101711 ± 0.004300 nmol/mg of protein in the control and 0.084534 ± 0.004300 nmol/mg of protein in the cholesterol-deficient synaptosomes ($P \leq 0.05$, Student's *t*-test, n = 4). We applied high-KCl at 5 min time point after addition of FCCP to expand further synaptosomal transporter-mediated release of L-[^{14}C] glutamate. It was revealed that cholesterol deficiency decreased the latest from 0.127187 ± 0.004300 nmol/mg of protein in the control to 0.111458 ± 0.004300 nmol/mg of protein in MβCD-treated synaptosomes ($P \leq 0.05$, Student's *t*-test, n = 4) (Fig. 6, the second pair of columns).

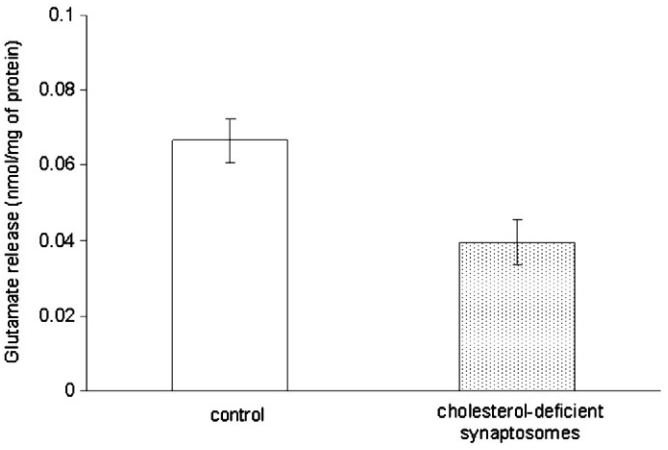

Fig. 5. Release of L-[^{14}C]glutamate from control (empty column) and cholesterol-depleted (dotted column) synaptosomes in low-Na^+ (21 mM Na^+, 105 mM NMDG; Sigma, U.S.A.), Ca^{2+}-free incubation media for 6 min. After loading of control and MβCD-treated synaptosomes with L-[^{14}C]glutamate (see Materials and methods), samples (0.5 mg of protein/ml) were preincubated for 8 min at 37 °C, then incubated for 6 min, rapidly sedimented in a microcentrifuge. Data are means ± S.E.M. of four independent experiments, each performed in triplicate. *, $P \leq 0.05$ as compared to the control.

3.7. Transporter-mediated glutamate release from cholesterol-deficient nerve terminals under conditions of energy deprivation

In energy deprivation experiments, we used iodoacetate (a potent inhibitor of G3P dehydrogenase) and rotenone (inhibitor of mitochondrial complex I)/oligomycin (inhibitor of ATP synthase). Similar to the previous section, we added high-KCl at 7 min time point after addition of iodoacetate or rotenone/oligomycin to expand transporter-mediated release of L-[^{14}C]glutamate and measure its initial velocity. As shown in Fig. 7 (the first and second columns), after application of iodoacetate (1 mM) and KCl (35 mM) to synaptosomes transporter-mediated release of L-[^{14}C]glutamate for 10 min was decreased from 0.14668 ± 0.01000 nmol/mg of protein in the control to 0.0801 ± 0.00800 nmol/mg of protein in the cholesterol-deficient synaptosomes (15 mM MβCD) ($P \leq 0.05$, Student's *t*-test, n = 3). The treatment of synaptosomes with rotenone/oligomycin (4 μM/4 μg/ml, respectively) and KCl (35 mM) resulted in a reduction of transporter-mediated release of L-[^{14}C]glutamate from 0.14282 ± 0.00500 nmol/mg of protein in the control to 0.11725 ± 0.00400 nmol/mg of protein in the cholesterol-deficient synaptosomes ($P \leq 0.05$, Student's *t*-test, n = 4) (Fig. 7, the third and fourth columns).

4. Discussion

In stroke, cerebral hypoxia/ischemia, traumatic brain injury and energy deprivation, glutamate is released from the nerve terminals into the extracellular space via glutamate transporters, thereby causing neurotoxicity, whereas beside these pathological states transporters predominantly operate in the inward direction. An increase in transporter-mediated release, and thus the extracellular level of the neurotransmitter may alter the functioning of NMDA receptors, which contributes to cognitive capacities and pathology [39]. Since the activity of glutamate transporters in the inward direction is decreased in cholesterol deficiency, we hypothesized that their reverse function could also be attenuated. Using cholesterol-deficient rat brain nerve terminals and applying radiolabeled technique and glutamate dehydrogenase assay (see Materials and methods), we have shown a decrease in transporter-mediated release of glutamate: (*) stimulated by the depolarization of the plasma membrane; (**) by means of heteroexchange with DL-THA; (***) in low-Na^+ medium; (****) during dissipation of the proton gradient of synaptic vesicles; (*****) under conditions of energy deprivation. As transporter-mediated release of glutamate from nerve cells determines the development of excitotoxicity in stroke, cerebral hypoxia/ischemia, and traumatic brain injury, we consider that the lowering of the level of membrane cholesterol, which attenuates the velocity of release, can have neuroprotective effect in these pathological states. It is clear that the lesser the transporter-mediated glutamate release is, the slower the development of neurotoxicity. The data of Abulrob et al. [40], who showed the neuroprotective activity of MβCD against oxygen-glucose deprivation in cortical neuronal cultures, may support our suggestion.

One of the main uncertainties of this study is the fact that in the experiments with radiolabeled glutamate, a decrease in transporter-mediated glutamate release from cholesterol-deficient nerve terminals has been registered against a background of enhanced extracellular glutamate concentration. Since transporter-mediated release of glutamate depends from the ratio glu_{int}/glu_{ex}, a decrease in the ratio per se may attenuate this release because of thermodynamic reasons. Also, cholesterol deficiency reduces the initial velocity of glutamate uptake [8,12] that may bring inaccuracy to the measurements. However, in the pathologies

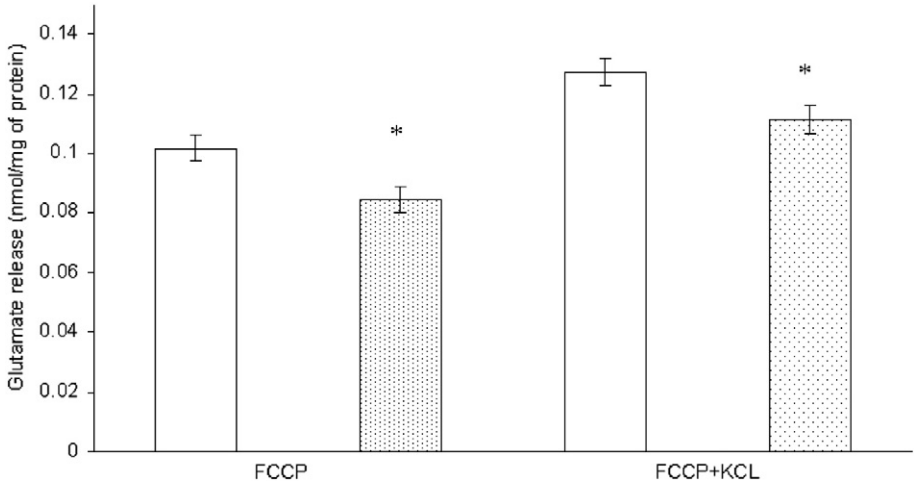

Fig. 6. Release of L-[^{14}C]glutamate in the presence of the protonophore FCCP (1 μM) (Sigma, U.S.A.) and FCCP + KCl (1 μM, 35 mM, respectively) from control (empty columns) and cholesterol-deficient (dotted columns) nerve terminals. After loading of the control and MβCD-treated synaptosomes with L-[^{14}C]glutamate (see Materials and methods), samples (0.5 mg of protein/ml) were preincubated for 8 min at 37 °C, then incubated for 6 min and rapidly sedimented in a microcentrifuge (FCCP-experiments). In FCCP + KCl experiments, KCl was added at 5 min time point after addition of FCCP, then synaptosomes were incubated for 6 min and rapidly sedimented. Data are means ± S.E.M. of four independent experiments, each performed in triplicate. *, $P \leq 0.05$ as compared to the control.

accompanied by glutamate uptake reversal, glutamate transporter activity in the inward direction can be negligible.

We suggest that the attenuation of transporter-mediated glutamate release from nerve terminals in cholesterol deficiency is a result of the reduction in the activity of glutamate transporters per se in response to the changes in the lipid surroundings. Butchbach et al. [8] reported that a loss of cholesterol led to the disturbance in cluster organization of glutamate transporters and changes in their trafficking in primary cortical cultures. Up- and down-regulations of glutamate transporter activity by exocytosis-like trafficking of transporter-containing vesicles [41] may be also affected in cholesterol deficiency because of the changes in the exocytotic process [10,13,14].

Our data may explain neuroprotective properties of widely applicable cholesterol-reducing drugs, statins, which are selective inhibitors of

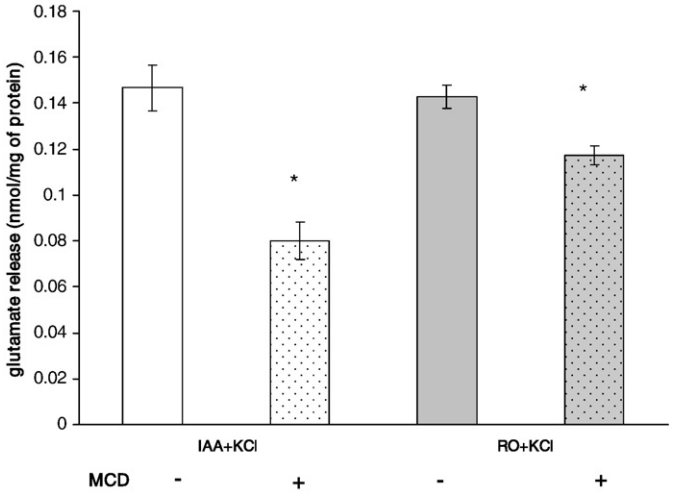

Fig. 7. Release of L-[^{14}C]glutamate in the presence of iodoacetate (IAA) and KCl (1 mM and 35 mM, respectively) (the first and second columns) and rotenone/oligomycin (RO) and KCl (4 μM/4 μg/ml and 35 mM, respectively) (the third and fourth columns) from control (clear columns) and cholesterol-deficient (dotted columns) nerve terminals. After loading of the control and MβCD-treated synaptosomes with L-[^{14}C]glutamate (see Materials and methods), samples (0.5 mg of protein/ml) were preincubated for 7 min at 37 °C with iodoacetate or rotenone/oligomycin, then after addition of KCl were incubated for 3 min and rapidly sedimented in a microcentrifuge. Data are means ± S.E.M. of three independent experiments, each performed in triplicate. *, $P \leq 0.05$ as compared to the control.

3-hydroxyl-3-methyl-glutaryl coenzyme A reductase, the rate-limiting enzyme of the mevalonate pathway for cholesterol biosynthesis. Recent data of the literature showed that the treatment with statins diminished the level of cholesterol in the brain [15]. Sierra et al. [16] found that monacolin J derivatives (natural and semi-synthetic statins) were the best candidates for the prevention of neurodegeneration due to their high capacity for brain–blood barrier penetration and cholesterol lowering effect on neurons. Increasing evidence indicates that statins, e.g. simvastatin, atorvastatin, and pravastatin, may be beneficial during acute stroke as well as post-ischemically in animal models and have neuroprotective features under conditions of cerebral ischemia, traumatic brain injury, and excitotoxic amino acid exposure [42–47]. Funck et al. [17] demonstrated that atorvastatin treatment had effects on pentylenetetrazol-induced seizures. Berger et al. [44] showed that relatively high dose of pravastatin administered repetitively after stroke onset improved neurological outcome. Sironi et al. [43] found that the treatment with statins after induction of focal ischemia in rats reduced the extent of brain damage. In summary, the data of the literature suggest that statins activate neuroprotective mechanisms, however its nature is far from being clear.

Several hypotheses on the possible mechanisms of the neuroprotective effect of statins are forwarded. The first ones consider that the neuroprotective action of statins is independent of cholesterol reduction. There are evidences that statins act on the nitric oxide synthase system [48] and inhibit release of potentially damaging cytokines such as IL-6 in the early phase of cerebral ischemia [44]. It may involve non-sterol mechanisms based on the effects on the endothelial cells, macrophages, platelets and smooth muscle cells [44,49]. The other point of view is that statins and MβCDs protect neurons from death changing excessive stimulation of NMDA receptors [18,39,50,51]. Ponce et al. [52] demonstrated that a decrease in cholesterol level by simvastatin in primary neuronal cultures protected from NMDA-induced neuronal damage probably by reducing the association of NMDA receptors to lipid rafts. Abulrob et al. [40] also suggested that cholesterol extraction from detergent-resistant microdomains affected NMDA receptor subunit distribution and signal propagation resulting in neuroprotection of cortical neuronal cultures against ischemic and excitotoxic insults. Modulation of NMDA receptors after simvastatin treatment could explain their anxiolytic-like activity and anti-inflammatory mechanisms in experimental model of Parkinson's disease [53]. Ramirez et al. [54] showed that simvastatin reduced the deleterious effects caused by kainate, including the

severity of seizures, excitotoxicity and oxidative damage in the hippocampus and other limbic structures of the brain cortex.

We propose the possible mechanism of the neuroprotective effect of statins based on our data that the reduction of the level of membrane cholesterol decreases transporter-mediated glutamate release from nerve terminals. This does not contradict NMDA-dependent and non-sterol mechanisms of statin action (and even may have additive or synergetic effect), but seems to be actual for the early phase of neuroprotection. The data of Berger et al. [44] may be considered in support of our hypothesis over the others. Using cerebral microdialysis in a temporary middle cerebral artery occlusion model in Wistar rats, the authors demonstrated that an increase in the extracellular level of striatal glutamate in the ischemic hemisphere was attenuated by pravastatin compared to placebo [44]. It may be suggested that in these experiments a decrease in cholesterol content most likely reduced transporter-mediated release of glutamate thereby causing a reduction in the extracellular glutamate concentration in the ischemic hemisphere.

Whether MβCD, which is used for the extraction of cholesterol in our experiments, may be employed for neuroprotection by direct application to injured brain regions. Recently, we showed that the addition of MβCD to nerve terminals and blood platelets caused dissipation of the proton gradient of their acidic compartments, i.e. synaptic vesicles and secretory granules, respectively [14,29]. In nerve terminals, this dissipation was accompanied with a dramatic increase in glutamate release, thus the presence of MβCD per se may cause an elevation of extracellular glutamate concentration. Intravenous administration of MβCD seems to be not effective because of its low permeability to brain–blood barrier [16,55,56] and its possible harmful influence on blood components. We demonstrated that MβCD decreasing the level of cholesterol in blood platelets caused a reduction of glutamate uptake [29]. Consequently, it may result in an increase in the glutamate concentration in the plasma, and then in the cerebrospinal fluid (because of glutamate balance between them [57]), thereby changing extracellular glutamate homeostasis in the brain.

Our experimental data showed that a decrease in the level of membrane cholesterol in nerve terminals reduced transporter-mediated glutamate release. The latest is the main mechanism of the enhancement of neurotoxic extracellular glutamate in stroke, cerebral hypoxia/ischemia, hypoglycemia, and traumatic brain injury. Therefore, a reduction of cholesterol content may be used for neuroprotection under these pathological conditions, i.e. “neuroprotection by lowering cholesterol”. Also, our data may explain the neuroprotective effect followed by the administration of statins in stroke, cerebral hypoxia/ischemia, seizures, excitotoxicity, oxidative damage and traumatic brain injury. However, beside these pathologies, the normal level of membrane cholesterol is very important for proper synaptic transmission and a decrease in membrane cholesterol content of nerve terminals may cause neurotoxic consequences because of weak glutamate uptake and the enlargement of the extracellular glutamate concentration.

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