ACTA NEUROBIOLOGIAE EXPERIMENTALIS

Research paper Acta Neurobiol Exp 2008, 68: 169–179

Preconditioning reduces hypoxia-evoked alterations in glutamatergic Ca^{2+} signaling in rat cortex

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The aims of this study were (1) to characterize calcium signaling in rat cortex induced by repeated *in vitro* application of the glutamatergic agonists L-glutamate, NMDA, AMPA and DHPG, (2) to analyze the influence of transient severe hypobaric hypoxia (180 Torr) administered *in vivo* on calcium responses to stimulation of glutamate receptors by their agonists, and (3) to evaluate the effects of preconditioning with intermittent mild hypobaric hypoxia (360 Torr), 24 h before the severe hypoxia, on these Ca^{2+} responses. Intracellular Ca^{2+} dynamics was studied using the fluorescent probes fura-2 and chlortetracycline to monitor free and bound calcium (Ca_i and Ca_b), respectively. In control cortical slices, application of L-glutamate, NMDA and AMPA induced concomitant increases in Ca_i and Ca_b, reflecting Ca^{2+} influx and its intracellular accumulation in neurons. DHPG, an agonist of group I mGlu receptors induced a decrease in Ca_b accompanied by a rise in Ca_i levels, indicating Ca^{2+} mobilization. In cortical slices collected 24 h after severe hypoxia, the responses of Ca_b to glutamate administration were increased, DHPG-induced shifts were reversed, the increase in Ca_b after the first application of AMPA was reduced, while after the second, Ca_b rises were potentiated, and the increases in Ca_b evoked by NMDA application were slightly suppressed. The alterations of responses in Ca_b to the selective agonists were completely prevented by preconditioning with mild hypoxia. Our results suggest that protection of normal glutamatergic calcium signaling contributes to tolerance to hypoxia induced by preconditioning.

Key words: brain, calcium, glutamate, hypobaric hypoxia, NMDA, AMPA, DHPG

INTRODUCTION

The brain is extremely sensitive to different forms of hypoxia. Even brief periods of global brain ischemia (ischemic hypoxia), hypoxemia or severe hypobaria may result in neuronal lesions in vulnerable brain regions, with accompanying neurological deficits and behavioral dysfunction (for recent reviews see Khot and Tirschwell 2006, Oechmichen and Meissner 2006, Perlman 2006). The results of numerous pathophysiological studies indicate the involvement of excessive stimulation of excitatory amino acid receptors, changes in the expression and function of these receptors and aberrant glutamate neurotransmission in the mechanisms of hypoxic/ischemic neuronal damage (Homola et al. 2006, Salinska et al. 2005, Quintana et al. 2006). It is also generally accepted that disturbances in calcium homeostasis can participate in the mechanism of nerve cell injury (Kristian 2004, Martinez-Sanchez et al. 2004, Yao and Haddad 2004). Several studies have demonstrated that hypoxia/ischemia induces calcium imbalance in brain neurons (Dubinsky and Rothman 1991, Bickler and Hansen 1994, 1996, Mitani et al., 1994). Our previous data indicated that exposure of rat cortical brain slices to severe anoxia *in vitro* destabilizes intracellular homeostasis of calcium with biphasic changes in the levels of both free and bound calcium. We also demonstrated the major role of NMDA receptors in this phenomenon (Semenov et al. 2000).

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Received 17 October 2007, accepted 24 April 2008

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Another of our previous studies showed that preconditioning of cortical slices *in vitro* with a short episode of anoxia reduces pathological calcium dynamics evoked by subsequent prolonged anoxia (Semenov et al. 2002).

It is well known that preconditioning of animals *in vivo* with episodes of mild hypoxia or ischemia induces tolerance to subsequent severe hypoxia/ischemia (for a review see Schaller and Graf 2002). This results in long-lasting morphological and behavioral neuroprotection (Duszczyk et al. 2005, 2006, Rybnikova et al. 2005a,b). In our studies we have used hypobaric hypoxia for inducing brain injury in rats as well as for preconditioning. This model corresponds to natural pathophysiological situations like decompression in civil or military aviation and adaptation to high altitudes (Mortazavi et al. 2003, Files et al. 2005). We have demonstrated that preconditioning of rats with repeated mild hypobaric hypoxia initiates the development of long-lasting tolerance of brain neurons to subsequent (no less than 24 h later) severe hypoxic insults applied either *in vitro* or *in vivo*. In particular, the calcium overload phenomenon induced by 10 min anoxia *in vitro* was shown to be significantly suppressed in brain slices obtained from rats preconditioned 24 h earlier (Semenov et al. 2004). In other studies, severe hypobaric hypoxia administered *in vivo* was shown to induce a dramatic decrease in brain NGFI-A expression, up-regulation of the expression of proapoptotic proteins and extensive apoptotic damage to hippocampal and neocortical neurons, while preconditioning of rats by repeated mild hypobaric hypoxia largely prevented these pathogenic events and increased the expression of antioxidative enzymes (Rybnikova et al. 2002, 2005a,b, 2006, Stroev et al. 2004, 2005). However, there is a lack of data describing how severe injurious hypoxic insult *in vivo* alters calcium-mediated glutamatergic signal transduction in the brain, and how preconditioning hypoxia modifies this effect.

The aims of the present study were to characterize, in rat cortical slices, pattern of calcium responses evoked by glutamate and selective glutamatergic agonists, to detect their persistent alterations 24 h after the animals were subjected to injurious severe hypobaric hypoxia and to identify the receptors that mediate these changes, and finally to evaluate the protective effects of preconditioning with mild hypobaric hypoxia.

METHODS

Animals

Eighty six adult male Wistar rats weighing 200–240 g used in this study were divided into 3 groups: (1) sham-treated controls (38 rats), (2) subjected to severe hypoxia (SH) (32 animals), and (3) preconditioned with mild hypoxia before exposure to severe hypoxia (MH + SH) (16 rats). Animal experiments were carried out in accordance with domestic regulations and the European Community Council Directive of 24 November 1986 (86/609/EEC). All efforts were made to minimize animal suffering and the number of animals required, and the experimental protocols were approved by local ethical committees.

Hypoxia

Hypoxia was induced by the exposure of rats to hypobaric conditions. A 50 l vacuum box (SPT-200, Horyzont, Warsaw, Poland) was used as a decompression chamber for the simultaneous hypobaric exposure of up to 6 rats. The pressure inside the chamber was reduced gradually at an average rate of 100 Torr/min, pausing for several minutes after every 100 Torr step. To avoid significant shifts in the pO_2, pCO_2, humidity and temperature inside the chamber during hypobaric exposure, the box was ventilated every 20 min by simultaneous pumping and opening of the inlet valve for 1 min while maintaining constant pressure. After hypobaric sessions lasting for 2 h at 360 Torr, equivalent to 5 000 m above sea level (mild hypoxia – MH), or 3 h at 180 Torr, equivalent to 11 000 m above sea level (severe hypoxia – SH), the chamber was restored to normal atmospheric pressure at a rate of about 40 Torr/min. For hypoxic preconditioning, MH was applied once a day for three days. SH an acute hypoxic test, was applied alone or 24 h after the MH. Control (sham-treated) animals were placed in the chamber for 3 h under normobaric conditions. Approximately 24 h after SH, all animals were anesthetized with chloral hydrate and decapitated. The brains were then rapidly removed for the preparation of cortical slices.

Preparation of cortical slices

The rat brains were chilled in ice-cold artificial cerebro-spinal fluid (ACSF) equilibrated with O_2. ACSF was composed of 124 mM NaCl, 5 mM KCl,

2.6 mM $CaCl_2$, 1.24 mM KH_2PO_4, 1.3 mM $MgSO_4$, 3 mM $NaHCO_3$, 10 mM glucose and 24 mM Tris-HCl (pH 7.4). Two tangential slices (400 μm) were then cut from the piriform cortex of both hemispheres using a tissue vibratome (752M Vibroslice, Campden Instruments, London, UK). The piriform cortex is demarcated by the lateral olfactory tract and rhinal fissure. Both slices from each animal were placed in 2 ml superfusion chambers and preincubated at 37°C for 2.5 h with a continuous flow of ACSF (1.2 ml/min).

Measurements of intracellular Ca^{2+} (free and bound)

Relative changes in the intracellular levels of free Ca^{2+} (Ca_i) and in Ca^{2+} bound to intracellular domains (Ca_b) were detected using the calcium-sensitive fluorescent probes fura-2 and chlortetracycline (CTC), respectively, as described in detail previously (Semenov et al. 2000, 2002). Briefly, to identify changes in Ca_i, one slice of each animal was preincubated for 90 min

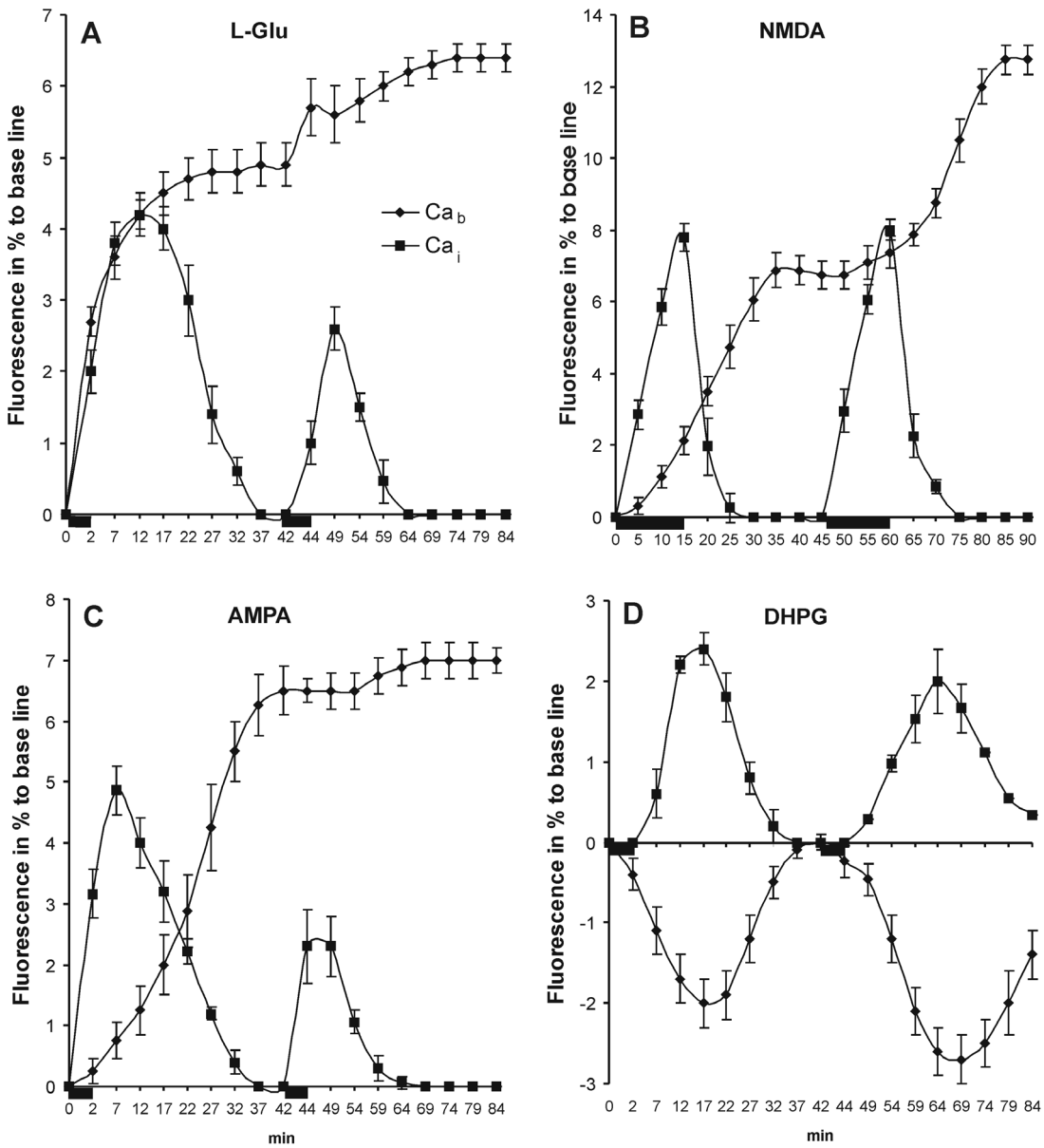

Fig. 1. Changes in intracellular free (Ca_i) and bound (Ca_b) calcium levels in cortical slices of control rats evoked by repeated application of glutamate receptor agonists: (A) 50 μM L-glutamate; (B) 100 μM NMDA; (C) 40 μM AMPA; (D) 100 μM DHPG. Black horizontal bars on abscissa of each panel mark the times of agonist application. Values represent means ± SEM. For each agonist, the number of slices used for Ca_b and Ca_i measurement were 8 and 6, respectively.

in ACSF containing 3.3 μM fura-2AM in 0.5% DMSO, while another (control) slice was incubated in ACSF with the same concentration of DMSO and used as an autofluorescence reference. Levels of fura-2 fluorescence intensity were then measured at 340 and 380 nm excitation and 510 nm emission wavelengths using a Hitachi F-2000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan) equipped with a xenon lamp. The measurements lasted a maximum of 10 s and were only repeated every 5 min to minimize photobleaching. The results of these measurements, corrected for autofluorescence and after further normalization, were used to calculate the ratio of fluorescence induced by excitation at 340 nm/380 nm. Changes in this fluorescence ratio, corresponding to changes in Ca_i levels were presented as a percentage of the baseline fura-2 fluorescence.

To measure the level of Ca^{2+} bound to hydrophobic intracellular domains (Ca_b), slices were prepared and preincubated as described above. Then both slices of each brain were incubated in ACSF containing 50 μM CTC for the entire superfusion experiment. Changes in fluorescence of Ca-CTC complexes were measured at 400 nm excitation and 522 nm emission wavelengths using an AvaSpec-2048 spectrometer (Avantes B.V. Eerbeek, Netherlands), equipped with an LED lamp (L2523UVC, Kingbright, Hong-Kong, China), mounted on a LUMAM-KF microscope (LOMO, St. Petersburg, Russia). The recorded fluorescence values were expressed as a percentage of the control steady-state level and used for estimation of Ca_b dynamics.

Glutamate receptor agonists

Glutamate receptor agonists L-glutamate (50 μM), AMPA (40 μM) and DHPG (100 μM) were bath-applied for two periods of 2 min separated by a 40 min interval. NMDA (100 μM) dissolved in ACSF without Mg^{2+} but containing 50 μM glycine, was applied in the perfusion medium twice for 15 min with a 30 min interval. Both slices of each animal were treated with only one agonist.

Statistics

The results are presented as means ± standard error of the mean (SEM) in two forms characterizing dynamics of Ca^{2+}-associated fluorescence (Figs 1 and 2) or integrated Ca^{2+} load (Fig. 3). The statistical significance of differences between means of the integrated values at $P<0.02$, was tested using analysis of variance (ANOVA) followed by Dunnett's test.

RESULTS

We initially examined the effects of different glutamate receptor agonists on intracellular levels of free and bound calcium (Ca_i and Ca_b, respectively) in slices of piriform cortex prepared from control (sham-treated) animals. Sham group was represented by 8–10 animals for each agonist tested. The dynamics of Ca_i and Ca_b are presented superposed in each panel of Fig. 1. The first application of L-glutamate induced a long-lasting increase in Ca_b that was accompanied by a significant increase in Ca_i (Fig. 1A). The increase in Ca_b persisted for at least 40 min, while that of Ca_i was transient, with the Ca_i level returning to control values after 37 min. A second application of the same dose of L-glutamate during 42–44 min of the experiment also induced increases in Ca_b and Ca_i; however, these effects were reduced compared to those of the first application. Slightly different calcium transients were observed after application of 100 μM NMDA (Fig. 1B). Both applications of NMDA (at 0–15 min and 45–60 min) induced equally pronounced rises in Ca_b and Ca_i. The first application of 40 μM AMPA also induced pronounced increases in Ca_b and Ca_i, while a reduced Ca_i response and only a negligible change in Ca_b was noticed after its second application (Fig. 1C), resembling the effects of L-glutamate. In general, application of the agonists of ionotropic glutamate receptors, NMDA and AMPA, induced increases in Ca_b and Ca_i reflecting Ca^{2+} influx into neurons and its accumulation in intracellular stores, presumably in mitochondria and endoplasmic reticulum (ER). Both applications of 100 μM DHPG, a selective agonist of group I metabotropic glutamate receptors, induced similar transient decreases in Ca_b, and these effects were accompanied by corresponding waves of increase in Ca_i (Fig. 1D). This result is consistent with mobilization of Ca^{2+} from intracellular stores.

In the experiments to determine the effects of severe hypobaric hypoxia (alone or preceded by preconditioning), we focused on changes in the level of Ca_b after the application of glutamate or selective glutamate receptor agonists. Severe hypoxia led to mortality of about 50%, whereas all the animals preconditioned with MH survived subsequent SH. SH-treated and surviving rats

as well as MH + SH-treated animals formed two equal groups of 16 animals each. Figures 2A and 3 show the effects of hypobaric hypoxia on glutamate-induced changes in Ca_b. In cortical slices prepared from the brains of rats submitted to SH 24 h earlier we noticed a significant potentiation of the increases in Ca_b evoked by L-glutamate. The response to the second application of this agonist was particularly amplified. In slices prepared from animals preconditioned with recurrent mild hypoxia before SH (MH + SH), the Ca_b responses to applications of glutamate were less potent than in non-preconditioned rats, but the response to the second application still greatly exceeded that observed in the control. These results indicate that 24 h after injurious brain hypoxia, responses of some or all types of glutamate receptors to activation by the agonist are enhanced,

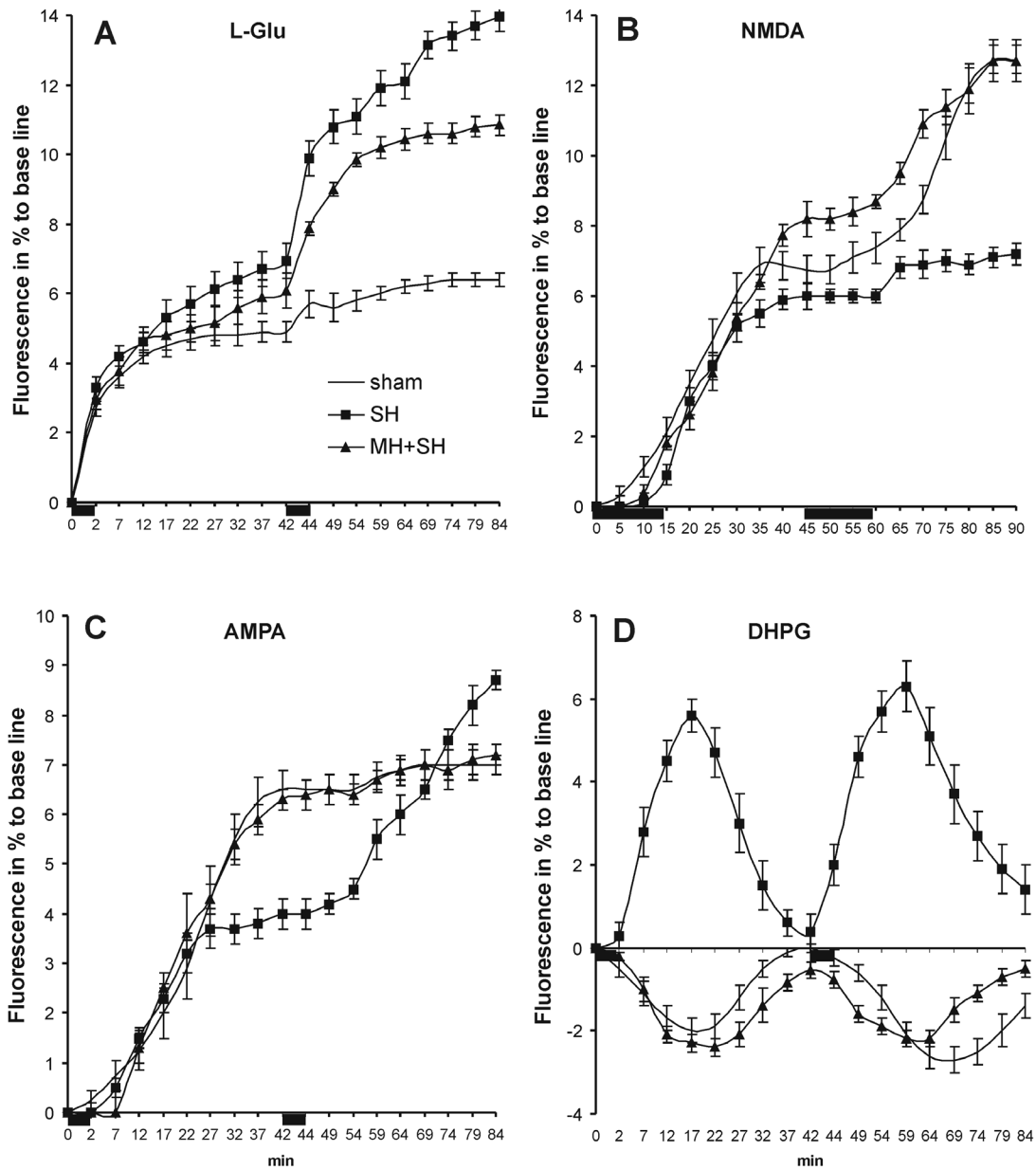

Fig. 2. Effects of severe hypoxia (SH) in non-preconditioned animals or rats preconditioned with intermittent mild hypoxia (MH + SH) on changes in intracellular bound calcium (Ca_b) in cortical slices evoked by repeated application of glutamate receptor agonists. Black horizontal bars on abscissa of each panel mark the times of agonist application. For each agonist each experimental variant represents mean value ± SEM obtained from eight slices (n=8). Conditions are as described in Fig. 1.

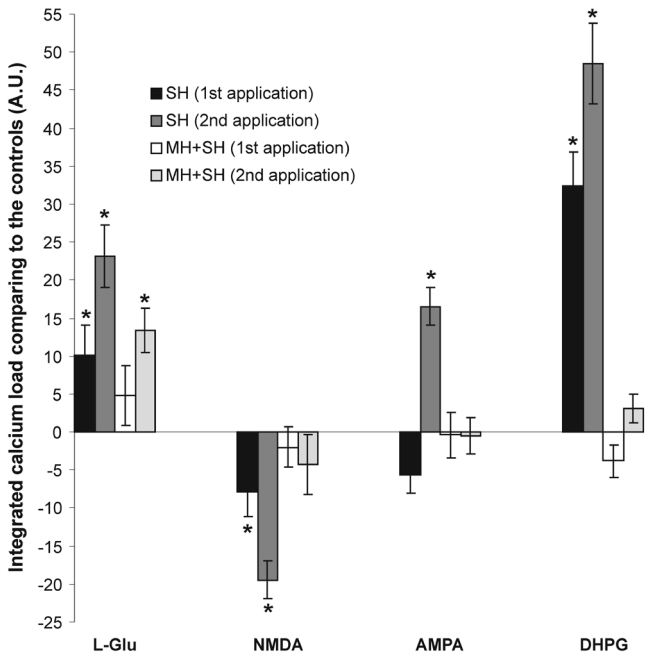

Fig. 3. Integrated values of Ca_b responses to glutamate receptor agonists in cortical slices of animals treated with SH or MH + SH, compared to control values. The data presented in Fig. 2 were integrated for equal time intervals for the first and second applications of the agonists in all experimental groups (for L-Glu, AMPA and DHPG, the respective time scales were 0–42 and 42–84 min; for NMDA, 0–45 and 45–90 min). The values obtained for SH and MH + SH groups were expressed as a percentage of the control values for the first and the second response, respectively, in Arbitrary Units (AU). The bars represent means ± SEM. **P*<0.02.

which may lead to calcium overload of brain cells. Moreover, our data demonstrate that preconditioning can reduce these potentially pathogenic changes.

To identify the glutamate receptors that may be involved in the mechanisms of post-hypoxic disturbances in glutamate-induced calcium transients and in the protection afforded by preconditioning, we studied post-hypoxic changes in Ca_b responses to the selective glutamate receptor agonists NMDA, AMPA and DHPG. As presented in Fig. 2B, the effects of NMDA application on Ca_b in cortical slices prepared from animals subjected to SH were suppressed. This effect was particularly noticeable after the second NMDA pulse (Fig. 3). Hypoxic preconditioning (MH + SH) prevented these changes (Figs 2B and 3). The Ca_b responses to AMPA were also clearly modified in cortical slices from animals subjected to SH (Fig. 2C). The increase in Ca_b after the first application of AMPA was suppressed compared to the control; on the contrary, the second response was significantly increased (Fig. 3). In slices prepared from animals preconditioned before SH, the responses of Ca_b to AMPA were close to the control (Figs 2C and 3). The responses of Ca_b to DHPG application in cortical slices from animals receiving SH were significantly different from the control (Figs 2D and 3). After both DHPG applications, instead of the characteristic decreases in Ca_b seen in the control, waves of reversible increases in Ca_b were observed. This reversal of Ca_b responses to DHPG was completely prevented by preconditioning (Figs 2D and 3).

DISCUSSION

The results of this study demonstrate that SH *in vivo* produces abnormal reactivity of glutamate receptors, particularly, suppressed deactivation of AMPA receptors and aberrant functional coupling of group I mGlu receptors with as yet unidentified plasmolemmal calcium channels. These changes point to sustained destabilization of glutamatergic calcium signaling in brain neurons after severe hypoxia that may lead to pathogenic neuronal calcium overload resulting in brain damage. Hypoxic preconditioning was found to prevent these changes.

In the present *in vivo* experiments we used a well-characterized model of hypobaric hypoxia for inducing injurious and preconditioning hypoxia (Miller et al. 2003, Kaur et al. 2005, Rybnikova et al. 2005 a,b). We found that SH induced high mortality during exposure (about 50%), and the surviving animals showed lesions to hippocampal neurons and behavioral impairment. Furthermore, preconditioning of rats with intermittent MH induced delayed and long lasting tolerance to subsequent SH. Our results confirmed that this protocol produces a substantial improvement in the rate of survival, decreases neuronal damage and reduces behavioral abnormalities (Rybnikova et al. 2005a,b). Recently, it was demonstrated in *in vivo* studies that enhanced expression of antioxidative and antiapoptotic gene products may participate in the mechanisms of tolerance to hypoxia induced by such preconditioning (Stroev et al. 2004, 2005, Rybnikova et al. 2006). Moreover, our previous experiments using the model of anoxic challenge of brain slices and their rapid *in vitro* preconditioning showed that acute prolonged anoxia (10 min) induces significant disturbances in

neuronal calcium metabolism, mainly mediated by NMDA receptors, and that short preconditioning anoxia (2 min) reduces this effect (Semenov et al. 2000, 2002). The present study used a combination of the *in vivo* model of SH alone or preceded by preconditioning with MH, with *in vitro* tests using cortical slices. The fluorimetric methods used for evaluating calcium dynamics in brain slices have been described in detail and discussed previously (Semenov et al. 2000, 2002). As has been repeatedly demonstrated in the control experiments, the results of calcium measurements using both setups utilized in this study do not differ.

The results of our control experiments confirmed some well-known properties of glutamate receptor calcium signaling and calcium homeostasis in neurons. Under standard experimental conditions, stimulation of brain slices with glutamate induced an increase in intracellular levels of Ca_i, resulting from the influx of extracellular Ca^{2+} into neurons and mobilization of intracellular stores. With such non-selective stimulation of both ionotropic and metabotropic glutamate receptors, the influx of calcium to neurons predominates, leading to enhanced accumulation of Ca^{2+} in the intracellular stores and to a rise in Ca_b. Our data also demonstrated a significant reduction in Ca_i and particularly Ca_b responses to the second application of L-glutamate, suggesting some deactivation of the receptors involved. Although the customary interpretation of this phenomenon suggests a neuronal origin of calcium responses to glutamate, it should be noted that glial cells are known to express both ionotropic and metabotropic glutamate receptors and respond to the application of agonists with increases in intracellular Ca^{2+} (Cai and Kimelberg 1997, Latour et al. 2001). So to some extent, the calcium responses recorded in this study can be attributed to glial cell signals.

As mentioned above, the response to L-glutamate, a universal and nonselective agonist of excitatory amino acid receptors, represents the sum of the responses of many types of glutamate receptor. In this study, the nature of calcium transients evoked by glutamate was examined using selective agonists of ionotropic and metabotropic glutamate receptors. Both NMDA and AMPA, like L-glutamate, induced increases not only in Ca_i but also in Ca_b. These effects may reflect the influx of extracellular Ca^{2+} into neurons and its accumulation in intracellular stores. It is noteworthy that the calcium responses evoked by the first and second application of NMDA were equal, whereas the second response to AMPA was significantly less pronounced than the first, suggesting deactivation of these receptors. These differences in the properties of two ionotropic glutamate receptors have been described previously in electrophysiological studies (for a review see MacDonald et al. 1998, Monaghan et al. 1998). NMDA receptors are well-characterized ligand-gated calcium channels that are highly permeable to calcium ions (McDermott et al. 1986, Mayer and Westbrook 1987), and therefore the occurrence of vivid calcium transients after the application of this agonist is not surprising. In contrast, AMPA channels with the standard heteromeric subunit composition, i.e. containing GluR2 subunits, are generally permeable to monovalent cations, and not to calcium (Geiger et al. 1995). The induction of Ca_b and Ca_i responses in rat cortical slices by AMPA may be partly explained by the presence of a proportion of AMPA receptors which are permeable to calcium. On the other hand, it was recently shown that the majority of cortical GABA-ergic interneurons, similarly to efferent pyramidal neurons, can express Ca^{2+}-permeable AMPA receptors (Fischer et al. 2002). This could explain the more pronounced Ca^{2+} response to the first pulse of AMPA, while the reduced response to its repeated application might be related to secondary GABA-ergic inhibition of neurons. Another generally accepted view is that under physiological conditions the majority of the Ca^{2+} influx that occurs in response to AMPA receptor stimulation may be due to voltage-sensitive calcium channels or reversed Na^+/Ca^{2+} exchangers (Mayer and Miller 1990, Czyz and Kiedrowski 2002). In either case, stimulation of the AMPA receptors is the primary event leading ultimately to calcium influx. In contrast to the effects of ionotropic glutamate receptor agonists, application of DHPG, an agonist of group I metabotropic glutamate receptors, resulted in transient decreases in Ca_b accompanied by symmetrical increases in Ca_i. The group I mGlu receptors are known to be coupled to phospholipase C and to IP3-mediated calcium release from the endoplasmic reticulum (Conn and Pin 1997). The observed pattern of calcium following DHPG application may be ascribed to mobilization of Ca^{2+} from intracellular stores. Contribution of the secondarily activated calcium influx *via* e.g. capacitative channels or NMDA receptors is also possible.

To study the effects of SH, with or without preconditioning, on calcium responses we focused on changes in Ca_b. This was because the initial control experi-

ments characterizing the interrelationship between Ca_i and Ca_b showed that changes in Ca_b more accurately reflect the calcium load of the cells, the major factor that determines calcium-mediated neuronal damage. In the present study we do not discriminate between different sub-pools of the bound calcium measured using CTC-Ca fluorescence. However changes in Ca_b could be interpreted as changes in the mitochondrial and ER bound calcium. Again, possible redistribution of calcium from mitochondria to ER and *vice versa* escapes our measurements. Our data demonstrated that the response of Ca_b to stimulation with L-glutamate was significantly potentiated in brain cortical slices prepared from rats 24 h after one episode of SH. The calcium response to the second application of this agonist was particularly increased. This facilitation of Ca_b responses seems to be quite a late posthypoxic event because shorter intervals after hypoxia (3 or 6 h) tested previously showed strongly suppressed responses to the same dose of exogenous L-glutamate (Miller et al. 2003). Preconditioning with three sessions of MH was found to significantly but incompletely reduce the alterations in the Ca_b response to glutamate induced by SH.

To consider the mechanism by which preconditioning acts to protect calcium homeostasis, we studied responses of Ca_b to NMDA, AMPA and DHPG in cortical slices prepared from animals 24 h after SH or MH + SH. The effects of SH on the response of Ca_b to repeated NMDA application was to reduce these responses, particularly to the second application of this agonist. Thus, modification of the responsiveness of NMDA receptors does not explain the enhanced increases in Ca_b in glutamate-treated cortical slices from rats surviving severe hypoxia. This post-hypoxic inhibition of calcium signals mediated by NMDA receptors was surprising. The NMDA receptor is generally thought of as the most prominent ligand-gated calcium channel in hypoxic or posthypoxic brain pathology induced by neuronal calcium overload (Choi 1988, Lee et al. 2000, Toescu 2004, Bano and Nicotera 2007). Previously, we found that 24 h after severe transient ischemia the NMDA-evoked currents in hippocampal pyramidal neurons were significantly reduced, but at the same time AMPA-evoked currents were facilitated (Tsubokawa et al. 1995). The observed suppression of NMDA receptors by SH was prevented by hypoxic preconditioning. At present it is difficult to speculate about the mechanisms of NMDA receptor inhibition after SH, and how preconditioning prevents this. One possible explanation may be interference, by NO-related species produced in the brain during and after severe hypoxia, with cysteine residues of the NMDA receptor subunits, resulting in decreased Ca^{2+} permeability of the NMDA channel (Lipton 1999). In contrast to the effect of NMDA application on Ca_b, a second pulse of AMPA induced a significant enhancement of the Ca_b responses, indicating that SH results in reduced deactivation of these receptors. Hypoxic preconditioning prevented these changes. The enhancement of glutamate receptor-mediated responses and particularly of AMPA-evoked currents has been described in hippocampal neurons of rats surviving severe forebrain ischemia. Moreover, the antagonism of these receptors prevented CA1 neuronal injury (Buchan et al. 1991, Li and Buchan 1993). This rather complex mechanism might be tentatively explained by inhibition of the deactivation of this receptor. Some role in these processes for oxidative stress, oxygen radicals and NO seems highly probable (Stroev and Samoilov 2006). Furthermore, it is known that ischemic insults modify the subunit composition of AMPA receptors, suppressing expression of the GluR2 subunit which regulates permeability of Ca^{2+}, while ischemic preconditioning prevents expression of AMPA receptors lacking GluR2 (Tanaka et al. 2002). To sum up, our data indicate that the increased responsiveness of AMPA receptors after SH may participate in enhancing calcium responses to glutamate, and these changes are prevented by preconditioning.

The most unexpected result of this study concerns the altered responses to stimulation of group I mGlu receptors by DHPG in cortical slices prepared from rats subjected to SH. Rather than the DHPG- induced decrease in Ca_b observed in control slices, we detected the reversal of this effect after hypoxia i.e. increases in Ca_b. These SH-evoked alterations were completely prevented by preconditioning. Similar responses were observed in cortical slices upon application of glutamate or agonists of ionotropic glutamate receptors, suggesting that they may reflect the influx of Ca^{2+} into neurons and its accumulation in intracellular stores, mainly in mitochondria and ER. This accumulation should be sufficiently large to override a decrease in Ca_b accompanying mobilization of calcium from intracellular stores. Thus, SH completely changed the pattern of calcium responses to activation of group I

mGlu receptors. Evidently, under these pathological conditions the classical functional unit observed in the control slices has been supplemented by activation of the plasmalemmal calcium channels. The interrelationship between group I mGlu receptors and ionotropic glutamate receptors or other receptors and channels has been demonstrated in several studies (Bandrowski et al. 2001, Pisani et al. 2001, Weber et al. 2001, Benquet et al. 2002, Blaabjerg 2003, Kotecha et al. 2003, Toescu 2004, Kelly et al. 2005). We propose that NMDA and AMPA receptors as well as TRP-channels and store-operated channels (SOCs/SMOCs) might respond to stimulation of group I mGlu receptors in brain cortical slices obtained from SH-treated animals. In the present study simultaneous measurements of Ca_b and Ca_i were performed only in the cortical slices of control rats (data presented on Fig. 1), while in experiments concerning the effects of SH or MH+SH on calcium homeostasis we focused exclusively on measuring changes in Ca_b. Our further experiments, especially concerning involvement of group I mGluRs will be aimed at changes in Ca_i as well, which should verify our explanation presented above. Moreover identification of the calcium stores (ER vs. mitochondrial) and of the channel(s) involved in Ca^{2+} influx into neurons upon activation of group I mGlu receptors are major goals of our future studies. The prevention of these SH-induced changes in calcium signaling by hypoxic preconditioning seems to be one of the mechanisms of induced tolerance to hypoxia. Hypoxic preconditioning is likely to involve the induction of antioxidative and antiapoptotic proteins (Rybnikova et al. 2006, Stroev and Samoilov 2006).

CONCLUSION

In this study we have identified hypoxia-induced abnormal reactivity of glutamatergic receptors in rat cortical slices, including suppressed deactivation of AMPA receptors and alterations in functional coupling of group I mGlu receptors with calcium channels. These changes may lead to potentially injurious excessive and sustained Ca^{2+} entry into neurons. Preconditioning of animals with mild hypoxia prevented these changes. We suggest that protection of normal glutamategic calcium signaling is among the mechanisms of tolerance to hypoxia induced by preconditioning.

Acknowledgement

This study was supported by RFBR grant (Russia) no. 07-04-00664, by the scientific network 26/E-40/SN-023/2006 and by statutory founds of the MCR (Warsaw).

REFERENCES

Bandrowski A, Aramakis V, Moore S, Ashe J (2001) Metabotropic glutamate receptors modify ionotropic glutamate responses in neocortical pyramidal cells and interneurons. Exp Brain Res 136: 25–40.

Bano D, Nicotera P (2007) Ca^{2+} signals and neuronal death in brain ischemia. Stroke 38: 674–676.

Benquet P, Gee C, Gerber U (2002) Two distinct signaling pathways upregulate NMDA receptor responses via two distinct metabotropic glutamate receptor subtypes. J Neurosci 22: 9679–9686.

Bickler P, Hansen B (1994) Causes of calcium accumulation in rat cortical brain slices during hypoxia and ischemia: Role of ion channels and membrane damage. Brain Res 665: 269–276.

Bickler P, Hansen B (1996) 2-Adrenergic agonists reduce glutamate release and glutamate receptor-mediated calcium cannels in hippocampal slices during hypoxia. Neuropharmacology 35: 679–687.

Blaabjerg M, Fang L, Zimmer J, Baskys A (2003) Neuroprotection against NMDA excitotoxicity by group I metabotropic glutamate receptors is associated with reduction of NMDA stimulated currents. Exp Neurol 183: 573–580.

Buchan A, Li H, Cho S, Pulsinelli W (1991) Blockade of the AMPA receptor prevents CA1 hippocampal injury following severe but transient forebrain ischemia in adult rats. Neurosci Lett 132: 255–258.

Cai Z, Kimelberg H (1997) Glutamate – receptor-mediated calcium responses in acutely isolated hippocampal astrocytes. Glia 21: 380–389.

Choi D (1988) Calcium-mediated neurotoxicity: relationship to specific channel types and role in ischemic damage. Trends Neurosci 11: 465–467.

Conn P, Pin J (1997) Pharmacology and function of metabotropic glutamate receptors. Annu Rev Pharmacol Toxicol 37: 205–237.

Czyz A, Kiedrowski L (2002) In depolarized and glucose-deprived neurons, Na^+ influx reverses plasolemmal K^+-dependent and K^+- independent Na^+/Ca^{2+} exchangers and contributes to NMDA excitotoxicity. J Neurochem 83: 1321–1328.

Dubinsky J, Rothman S (1991) Intracellular calcium concentration during "chemical hypoxia" and excitotixic neuronal injury. J Neurosci 11: 2545–2551.

Duszczyk M, Gadamski R, Ziembowicz A, Danysz W, Lazarewicz JW (2005) NMDA receptor antagonism does not inhibit induction of ischemic tolerance in gerbil brain in vivo. Neurotox Res 7: 283–292.

Duszczyk M, Ziembowicz A, Gadamski R, Lazarewicz JW (2006) Behavioral evaluation of ischemic damage of CA1 hippocampal neurons: effects of preconditioning. Acta Neurobiol Exp (Wars) 66: 311–319.

Files D, Webb J, Pilmanis A (2005) Depressurization in military aircraft: rates, rapidity, and health effects for 1055 incidents. Aviat Space Environ Med 76: 523–529.

Fischer W, Franke H, Scheibler P, Allgaier C, Illes P (2002) AMPA-induced Ca^{2+} influx in cultured cortical nonpyramidal neurons: pharmacological characterization using fura-2 microfluorimetry. Eur J Pharmacol 438: 53–62.

Geiger J, Melcher T, Koh D, Sakmann B, Seeburg P, Jonas P, Monyer H (1995) Relative abundance of subunit mRNAs determines gating and Ca^{2+} permeability of AMPA receptors in principal neurons and interneurons in rat CNS. Neuron 15: 193–204.

Homola A, Zoremba N, Slais K, Kuhlen R, Sykova E (2006) Changes in diffusion parameters, energy-related metabolites and glutamate in the rat cortex after transient hypoxia/ischemia. Neurosci Lett 404: 137–142.

Kaur C, Sivakumar V, Singh G, Singh J, Ling E (2005) Response of Purkinje neurons to hypobaric hypoxic exposure as shown by alteration in expression of glutamate receptors, nitric oxide synthases and calcium binding proteins. Neuroscience 135: 1217–1229.

Kelly P, Maccinnon R, Dietz R, Maher B, Wang J (2005) Postsynaptic IP3 receptor-mediated Ca^{2+} release modulates synaptic transmission in hippocampal neurons. Brain Res Mol Brain Res 135: 232–248.

Khot S, Tirschwell D (2006) Long-term neurological complications after hypoxic-ischemic encephalopathy. Semin Neurol 26: 422–431.

Kotecha S, Jackson M, Al-Mahrouki A, Roder J, Orser B, MacDonald J (2003) Co-stimulation of mGluR5 and N-methyl-D-aspartate receptors is required for potentiation of excitatory synaptic transmission in hippocampal neurons. J Biol Chem 278: 27742–27749.

Kristian T (2004) Metabolic stages, mitochondria and calcium in hypoxic/ischemic brain damage. Cell Calcium 36: 221–233.

Latour I, Gee Ch, Robitaille R, Lacaille J-C (2001) Differential mechanisms of Ca^{2+} responses in glial cells evoked by exogenous and endogenous glutamate in rat hippocampus. Hippocampus 11: 132–145.

Lee J, Grabb C, Zipfel G, Choi D (2000) Brain tissue responses to ischemia. J Clin Invest 106: 723–731.

Li H, Buchan A (1993) Treatment with an AMPA antagonist 12 hours following severe normothermic forebrain ischemia prevents CA1 neuronal injury. J Cereb Blood Flow Metab 13: 933–939.

Lipton S (1999) Neuronal protection and destruction by NO. Cell Death Differ 6: 943–951.

McDermott A, Mayer M, Westbrook G, Smith S, Barker J (1986) NMDA-receptor activation increases cytoplasmic calcium concentration in cultured spinal cord neurons. Nature 321: 519–522.

MacDonald J, Xiong X, Lu W, Raouf R, Orser B (1998) Modulation of NMDA receptors. Prog Brain Res 116: 191–208.

Martinez-Sanchez M, Striggow F, Schroder U, Kahlert S, Reymann K, Reiser G (2004) Na^{+} and Ca^{2+} homeostasis pathways, cell death and protection after oxygen-glucose-deprivation in organotypic hippocampal slice cultures. Neuroscience 128: 729–740.

Mayer M, Westbrook G (1987) Permeation and block of N-metyl-D-aspartic acid receptor channels by divalent cations in mouse cultured central neurons. J Physiol (London) 394: 500–527.

Mayer M, Miller R (1990) Excitatory amino acid receptors, second messengers and regulation of intracellular Ca2+ in mammalian neurons. Trends Pharmacol Sci 11: 254–260.

Miller O, Semenov D, Samoilov M (2003) Posthypoxic alteration of glutamatergic signal transduction in rat cortical neurons: the improving effect of preconditioning. Bull Exp Biol Med 135: 398–401.

Mitani A, Takeyasu S, Yanase H, Nakamura Y, Kataoka K (1994) Changes in intracellular Ca^{2+} and energy levels during in vitro ischemia in the gerbil hippocampal slice. J Neurochem 62: 626–634.

Monaghan D, Andaloro V, Skifter D (1998) Molecular determinants of NMDA receptor pharmacological diversity. Prog Brain Res 116: 171–190.

Mortazavi A, Eisenberg M, Langleben D, Ernst P, Schiff R (2003) Altitude-related hypoxia: risk assessment and management for passengers on commercial aircraft. Aviat Space Environ Med 74: 922–927.

Oechmichen M, Meissner C (2006) Cerebral hypoxia and ischemia: the forensic point of view: a review. J Forensic Sci 51: 880–887.

Perlman J (2006) Summary proceedings from the neurology group on hypoxic-ischemic encephalopathy. Pediatrics 117: S28–S33.

Pisani A, Gubellini P, Bonsi P, Conquet F, Picconi B, Centonze D, Bernardi G, Calabresi P (2001) Metabotropic glutamate receptor 5 mediates the potentiation of N-methyl-D-aspartate responses in medium spiny striatal neurons. Neuroscience 106: 579–587.

Quintana P, Alberi S, Hakkoum D, Muller D (2006) Glutamate receptor changes associated with transient anoxia/hypoglycaemia in hippocampal slice cultures. Eur J Neurosci 23: 975–983.

Rybnikova E, Tulkova E, Pelto-Huikko M, Samoilov M (2002) Mild preconditioning hypoxia modifies nerve growth factor-induced gene A messenger RNA expression in the rat brain induced by severe hypoxia. Neurosci Lett 329: 49–52.

Rybnikova E, Khozhai L, Tyul'kova E, Glushchenko T, Sitnik N, Pelto-Huikko M, Otellin V, Samoilov M (2005a) Expression of early gene proteins, structural changes in brain neurons in hypobaric hypoxia, and the correcting effects of preconditioning. Neurosci Behav Physiol 35: 383–388.

Rybnikova E, Vataeva L, Tyulkova E, Gluschenko T, Otellin V, Pelto-Huikko M, Samoilov M (2005b) Mild hypoxia preconditioning prevents impairment of passive avoidance learning and suppression of brain NGFI-A expression induced by severe hypoxia. Behav Brain Res 160: 107–114.

Rybnikova E, Sitnik N, Gluschenko T, Tjulkova E, Samoilov M (2006) The preconditioning modified neuronal expression of apoptosis-related proteins of Bcl-2 superfamily following severe hypobaric hypoxia in rats. Brain Res 1089: 195–202.

Salinska E, Danysz W, Lazarewicz JW (2005) The role of excitotoxicity in neurodegeneration. Folia Neuropathol 43: 322–339.

Schaller B, Graf R (2002) Cerebral ischemic preconditioning. An experimental phenomenon or a clinical important entity of stroke prevention? J Neurol 249: 1503–1511.

Semenov D, Samoilov M, Zielonka P, Lazarewicz JW (2000) Responses to reversible anoxia of intracellular free and bound Ca^{2+} in rat cortical slices. Resuscitation 44: 207–214.

Semenov D, Samoilov M, Lazarewicz JW (2002) Calcium transients in the model of rapidly induced anoxic tolerance in rat cortical slices: involvement of NMDA receptors. Neurosignals 11: 329–335.

Semenov D, Miller O, Samoilov M (2004) Influence of hypoxic preconditioning of rats in vivo to the dynamics of contents of intracellular calcium induced by long-lasting anoxia of the brain slices. Bull Exp Biol Med 138: 382–385.

Stroev S, Tjulkova E, Gluschenko T, Rybnikova E, Samoilov M, Pelto-Huikko M (2004) The augmentation of brain thioredoxin-1 expression after severe hypobaric hypoxia by the preconditioning in rats. Neurosci Lett 370: 224–229.

Stroev S, Gluschenko T, Tjulkova E, Spyrou E, Rybnikova E, Samoilov M, Pelto-Huikko M (2005) The effect of preconditioning on Cu, Zn-superoxide dismutase expression and enzyme activity in rat brain at the early period after severe hypobaric hypoxia. J Neurosci Res 53: 39–47.

Stroev S, Samoilov M (2006) Endogennye antioksidanty i gipoksicheskaja tolerantnost mozga. I.P. Pavlov Institute of Physiology of the RAS, St. Petersburg, 147 p.

Tanaka H, Calderone A, Jover T, Grooms S, Yokota H, Zukin R, Benett M (2002) Ischemic preconditioning acts upstream of GluR2 down-regulation to afford neuroprotection in the hippocampal CA1. Proc Natl Acad Sci U S A 99: 2362–2367.

Toescu E (2004) Hypoxia sensing and pathways of cytosolic Ca^{2+} increases. Cell Calcium 36: 187–199.

Tsubokawa H, Oguro K, Robinson H (1995) Single glutamate channels in CA1 pyramidal neurons after transient ischemia. Neuroreport 6: 527–531.

Weber J, Rzigalinski D, Ellis E (2001) Traumatic injury of cortical neurons causes changes in intracellular calcium stores and capacitative calcium influx. J Biol Chem 276: 1800–1807.

Yao H, Haddad GG (2004) Calcium and pH homeostasis in neurons during hypoxia and ischemia. Cell Calcium 36: 247–255.