



Adaptation to intermittent hypoxia restricts nitric oxide overproduction and prevents beta-amyloid toxicity in rat brain

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ABSTRACT

This study tested the hypothesis that adaptation to intermittent hypoxia (AIH) can prevent overproduction of nitric oxide (NO) in brain and neurodegeneration induced by beta-amyloid (A β) toxicity. Rats were injected with a A β protein fragment (25–35) into the *nucleus basalis magnocellularis*. AIH (simulated altitude of 4000 m, 14 days, 4 h daily) was produced prior to the A β injection. A passive, shock-avoidance, conditioned response test was used to evaluate memory function. Degenerating neurons were visualized in stained cortical sections. NO production was evaluated in brain tissue by the content of nitrite and nitrate. Expression of nNOS, iNOS, and eNOS was measured in the cortex and the hippocampus using Western blot analysis. 3-Nitrotyrosine formation, a marker of protein nitration, was quantified by slot blot analysis. A β injection impaired memory of rats; AIH significantly alleviated this disorder. Histological examination confirmed the protective effect of AIH. Degenerating neurons, which were numerous in the cortex of A β -injected, unadapted rats, were essentially absent in the brain of hypoxia-adapted rats. Injections of A β resulted in significant increases in NOx and in expression of all NOS isoforms in brain; AIH blunted these increases. NO overproduction was associated with increased amounts of 3-nitrotyrosine in the cortex and hippocampus. AIH alone did not significantly influence tissue 3-nitrotyrosine, but significantly restricted its increase after the A β injection. Therefore, AIH affords significant protection against experimental Alzheimer's disease, and this protection correlates with restricted NO overproduction.

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Introduction

Multiple studies have demonstrated that adaptation to intermittent hypoxia (AIH) exerts protective effects on the cardiovascular system that reduce ischemic and reperfusion injury [1,2], hypertension [3] and myocardial infarction [4]. These protective effects have been attributed to stimulation of self-defense systems in the body, including those of antioxidants, heat shock proteins, prostaglandins and other so-called stress-limiting systems [5]. Adaptation to hypoxia also renders protection to nerve cells of brain. Prior studies have shown that adaptation to AIH in an altitude chamber prevented experimental epilepsy [6,7], and adaptation to high-altitude hypoxia in mountains improved formation and retention of a conditioned reflex in mice [8–10]. Adaptation to hypobaric hypoxia was successfully used clinically for treatment

of patients with schizophrenia and alcohol abuse [9]. In rats, AIH during chronic ethanol consumption attenuated oxidative damage to the brain and mitigated behavioral abnormalities during subsequent ethanol withdrawal [11]. AIH was effective against ischemic brain injury [12–14] and stroke [15] in mice and rats. Finally, AIH prevented the neurodegenerative process in rat brain during the development of experimental Parkinson disease [16].

Several classes of drugs are used in the therapy of Alzheimer's disease (AD). These include acetylcholine esterase inhibitors, drugs influencing NMDA and glutamate receptors, drugs affecting the synthesis of beta-amyloid (A β) and the formation of neurofibrillary tangles, and anti-inflammatory agents [17]. However, the efficacy of these drugs is limited, since each drug influences only a single step of the pathogenesis in AD, and these drugs have an effect on both damaged and normal cells. Thus, major attention is now paid to non-pharmacological approaches that may mobilize self-defense systems. Dietary restriction, physical and mental activity [18,19], or mild stress exposure, such as exposure to light or transcutaneous nerve stimulation [20] has been reported to reduce both the incidence and severity of neurodegenerative disorders in humans.

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Recently we demonstrated for the first time that adaptation to intermittent normobaric hypoxia reduced the loss of memory in rats with experimental AD induced by intracerebral injections of beta-amyloid (A β) [21,22]. Mechanisms of this protection remain unknown, although limited data available from Russian-language publications suggest that underlying protective mechanisms may include reduction of toxic reactive oxygen and nitrogen species [21,23]. The current study tested the hypothesis that adaptation to intermittent hypoxia can, indeed, prevent NO overproduction and neurodegenerative damage induced by A β toxicity in rat brain. We also sought to identify specific NO synthases responsible for the A β -induced NO overproduction and to determine the effects of AIH on these NO synthases.

Experimental procedures

Chemicals

Reagents were purchased from Assay Designs (Ann Arbor, MI) or Bio-Rad (Hercules, CA).

Animals

Male Wistar rats weighing 220 ± 50 g at the beginning of the study were maintained at controlled temperature (22–25 °C) and humidity (55%). A 12:12 h light–dark cycle was maintained with lights on between 7:00 AM and 7:00 PM. All animal procedures were performed in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals. Protocols were approved by the Animal Care and Use Committee of the Institute of General Pathology and Pathophysiology.

Adaptation to hypoxia

Adaptation to hypobaric hypoxia was produced in an altitude chamber at a simulated altitude of 4000 m above sea level. The total duration of adaptation was 14 days. On the 1st day, rats were “elevated” to 1000 m and the session lasted for 30 min; on the 2nd day – 2000 m for 1 h; on the 3rd day – 3000 m for 1.5 h; on the 4th day – 4000 m for 2 h; on the 5th day – 4,000 m for 3 h; and on the 6th and subsequent days – 4000 m for 4 h. The rate of “elevation” to the simulated altitude did not exceed 15 m/s. The last session was performed 24 h prior to the A β injection.

A β injections

A β (25–35) (Sigma–Aldrich, St. Louis, MO) was dissolved in physiological saline. Rats were anesthetized intraperitoneally with chloral hydrate (375 mg/kg body weight; Merck, Germany), and their heads were fixed in a stereotaxic apparatus. To model AD [24], animals were injected with 2 μ l of solution containing 0.4 nmol A β protein fragment (25–35), which was injected slowly into the *nucleus basalis magnocellularis* at both sides of the brain (AP–2.0 mm, lat. \pm 3.5 mm, vent. 5.6 mm) [25] with a 10 μ l Hamilton microsyringe. Untreated, control rats received no surgical treatment, while sham-operated rats were anesthetized and received bilateral injections of 2 μ l saline. Behavioral tests were performed 14 days after the A β injection. All animals were killed by decapitation 30 days after the A β or saline vehicle injections, and biochemical investigations and histopathological analyses were performed.

Behavioral testing

The passive, shock-avoidance, conditioned response test was used to evaluate memory function. For this test, a two-compartment,

one-way, step-through apparatus was used [26]. In the training trial, the rats were placed in the illuminated compartment and allowed to explore the compartments. The time that elapsed before the rat stepped into the dark compartment was recorded (pre-shock latent period 1, LP1). If the rat did not enter the dark compartment by 3 min, the rat was withdrawn from the experiment. Upon entry into the dark compartment, a mild, unavoidable foot-shock (1 mA, for 3 s) was delivered through the grid floor, and the rats were returned to their cages. After 24 h the test was repeated. The rats were placed again in the illuminated compartment, and the latency to step into the dark compartment was recorded (post-shock latent period, LP2). If the rat did not enter the dark compartment within 3 min, LP2 was set as 3 min.

Histopathological analysis

Five rats of each group were anesthetized intraperitoneally with chloral hydrate (375 mg/kg body weight; Merck, Germany) and then perfused intracardially with a mixture of 4% formaldehyde, glacial acetic acid and 95% ethanol (2:1:7 v/v/v). After this perfusion, the rats were decapitated, and their heads placed in a refrigerator at 4 °C for 20 h. To visualize degenerating neurons, 10 μ m sections of parietotemporal cortex were Nissl-stained with haematoxylin–eosin, acid vanadium fuchsin and cresyl violet. The number of degenerating neurons within the cortical layer was counted using a computer-compatible light microscope (BX51, Olympus, Japan) at x400 magnification and expressed as the number of degenerating neurons per a field of view (0.126 mm²). The software, Cell A, Version 2.4, was used to photograph neurons. The software, Infinity Analysis, Version 4.0, was used to count neurons. The mean number of degenerating neurons per field of view was calculated for at least 20 sections per each treatment group. The sections were taken from random parts of the parietotemporal cortex. All assessments of histological sections were performed blindly.

Measurement of nitrite and nitrate in brain tissue

Brain cortex was homogenized in liquid nitrogen, and deionized water was added to the samples at 1:1.5 ratio. The samples were frozen at –80 °C and unfrozen briefly and refrozen three times to destroy cell membranes. Then the samples were deproteinized by adding 1/20th volume of ZnSO₄ (300 g/L) and centrifuged at 3000 rpm for 6 min, warmed to 60 °C, and centrifuged again at 13,000 rpm for 30 min. Nitrate was reduced to nitrite in the supernatant using a cadmium reactor in the presence of 0.5 M NH₄OH, pH 9.0, as a buffer (plasma to buffer ratio 9:1, v/v). A sample aliquot was mixed with an equal volume of Griess reagent (Sigma–Aldrich, St. Louis, MO) and incubated for 10 min for color development at room temperature. Each sample was assayed in duplicate. The total content of nitrite and nitrate (NO_x) was measured in the supernatant. Calibration curves were made with sodium nitrite (5–50 μ M) in distilled water. Absorbance was measured at 540 nm by a spectrophotometer. The results were expressed as μ mol nitrite/mg tissue.

Western immunoblot analysis

Frozen cortex and hippocampus tissues from each animal were homogenized in six volumes (wt/vol) of lysis buffer (pH 7.4) composed of 10 mM HEPES, 0.1 mM EDTA, 0.32 mM sucrose, and protease cocktail, 10 μ l cocktail per 1 mL of lysis buffer. The protease cocktail contained 2.5 μ g/ml antipain, 2.5 μ g/ml leupeptin, 1.74 μ g/ml pepstatin A and 0.95 μ g/ml aprotinin (Sigma–Aldrich, St. Louis, MO). The homogenates were incubated for 15 min at 4–8 °C and centrifuged at 4 °C for 25 min at 13,000 rpm. The protein concentration was determined spectrophotometrically using the

Bradford reagent (Bioquant Protein, Merck, Germany). Crude homogenate protein (100 µg) from three animals of each group was heated for 7 min at 96 °C and then loaded on gradient (4–12%) sodium dodecyl sulfate-tris(hydroxymethyl)aminomethane glycine polyacrylamide gels. Proteins were transferred electrophoretically to polyvinylidene difluoride membranes, blocked with 5% nonfat dry milk, and incubated overnight at 4 °C with polyclonal anti-iNOS, anti-eNOS, and anti-nNOS antibodies (Assay Designs, Ann Arbor, MI). HeLa cell lysate was used as a positive control for iNOS and eNOS proteins, and mouse brain tissue extract was used as a positive control for nNOS protein (Assay Designs, Ann Arbor, MI). Specific proteins were detected using horseradish peroxidase-conjugated anti-rabbit secondary antibodies (Assay Designs, Ann Arbor, MI) and ECL reagents (Amersham, UK). The blots were scanned with a Bio-Rad (Hercules, CA) imaging densitometer, and optical densities of the protein bands were quantified with Photoshop software.

Nitrotyrosine slot blotting

Slot blotting was used to measure 3-nitrotyrosine (3-NT) formation. Crude homogenates of brain cortex and hippocampus (100 µg, see above) were mixed with equal volumes of sample buffer, boiled for 5 min, and loaded on nitrocellulose membranes using a Bio-Rad slot-blotting apparatus. Membranes were then blocked with 5% nonfat dry milk and probed overnight with primary polyclonal antinitrotyrosine antibody (1 µg/ml) and horseradish peroxidase-conjugated anti-rabbit secondary antibodies (Upstate Biotechnology, Charlottesville, VA). Nitrotyrosine molecular weight standard (Upstate Biotechnology) was used as positive control. Specific proteins were detected with the ECL kit, and the intensity of the slots was evaluated by densitometry, as mentioned above.

Statistical analyses

All measurements were performed in duplicate or triplicate, and mean values were computed for each rat. Data from each rat were averaged, and these values are presented as means ± SEM.

Student's *T*-test was used to identify selected group differences among plasma and brain tissue NOx concentrations. The non-parametric Wilcoxon paired *T*-test was used to compare pre- and post-shock latencies (LP1 and LP2) and to calculate ΔLP for each animal in a group. The non-parametric Wilcoxon–Mann–Whitney *U*-test was used to determine significance of intergroup differences in the passive shock-avoidance conditioned response test. The exact Fisher test was used to determine significance of differences in memory retention. Differences were considered significant at $p < 0.05$.

Results

Changes in memory retention

Previously Harkany et al. demonstrated [27] that Aβ exerted marked neurotoxic effects and induced cell death in rat brain after a 14 day survival period. Thus, in these experiments, we choose to investigate alterations of retention mechanisms two weeks post-surgery, using a step-through, passive shock avoidance test. 89% and 87% of naïve control and sham-operated control rats remembered the foot-shock, which was evident as increased post-shock latencies. After the Aβ injection, the percentage of rats remembering the foot-shock decreased to 75% ($p < 0.05$). AIH did not change the percentage of control and sham-operated rats that remembered the foot-shock, but AIH significantly increased the proportion of Aβ treated rats that remembered the foot-shock to 87%.

Fig. 1 shows that the Aβ injection significantly decreased the latency 24 h after the delivery of a mild foot-shock (LP2), compared to both sham-operated and control animals (68 ± 8 s for Aβ vs. 144 ± 17 or 149 ± 16 s for naïve and sham-operated control rats, respectively; $p < 0.05$ for both comparisons). This indicates short-term impairment of cortical retention mechanisms by Aβ injection. The performance of control and saline vehicle-injected rats did not significantly differ from each other. AIH alone did not change the memory retention (133 ± 17 s for adaptation to hypoxia vs. 144 ± 17 or 130 ± 11 s for naïve rats and sham-operated control rats, respectively, $p > 0.05$ for both comparisons). However, adaptation to hypoxia significantly alleviated the loss of memory in Aβ-injected rats; ΔLP (LP2–LP1) was greater in AIH rats injected with Aβ (93 ± 10 s) than in non-adapted rats injected with Aβ (68 ± 8 s, $p < 0.05$).

Histopathological findings

Histological examination confirmed the protective effect of adaptation to hypoxia (Fig. 2). The total number of visible neurons (degenerating plus normal) per field of view was slightly less in the brain of Aβ-injected rat but this difference was not statistically significant. Degenerating neurons were not found in rats which did not receive Aβ-injections (Figs. 2A, 2C, 2B). Multiple shrunk, hyperchromic degenerating neurons were present in the parietotemporal cortex of rats injected with Aβ (38 ± 6.2 in the field of view) (Fig. 2D). Neurons with these pathomorphological signs were essentially absent in the brain of rats injected with Aβ after adaptation to hypoxia (3 ± 2 degenerating neurons in the field of view, $p < 0.05$; Fig. 2E). Therefore, AIH prevented Aβ toxicity-induced neurodegeneration in the brain.

Measurement of NOx in brain tissue

Fig. 3 shows that injections of Aβ resulted in a significant increase in brain tissue NOx (26.0 ± 2.0 and 29.1 ± 3.9 µmol nitrite/mg tissue for naïve control and sham-operated rats, respectively, vs. 44.0 ± 3.3 µmol nitrite/mg tissue for Aβ, $p < 0.05$), which indicated NO overproduction. AIH did not induce any significant changes in NOx of naïve or sham-operated rats, but completely prevented NO overproduction in rats injected with Aβ (44.0 ± 3.3 for Aβ vs. 23.3 ± 4.0 µmol nitrite/mg tissue for adapted rats injected with Aβ, $p < 0.05$).

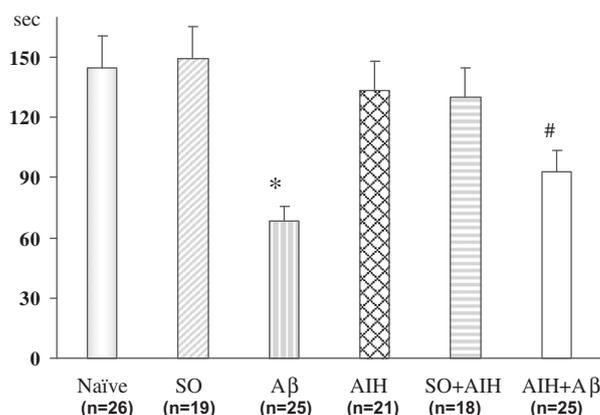


Fig. 1. Changes in post-shock latencies (ΔLP; LP2–LP1) in the 24 h memory retention trial of the passive avoidance test after Aβ injections in the nucleus basalis magnocellularis. The results are presented as M ± SEM. AIH = adaptation to hypoxia; SO = sham operation; *less than all other groups, $p < 0.05$; #greater than Aβ and less than non-Aβ rats, $p < 0.05$.

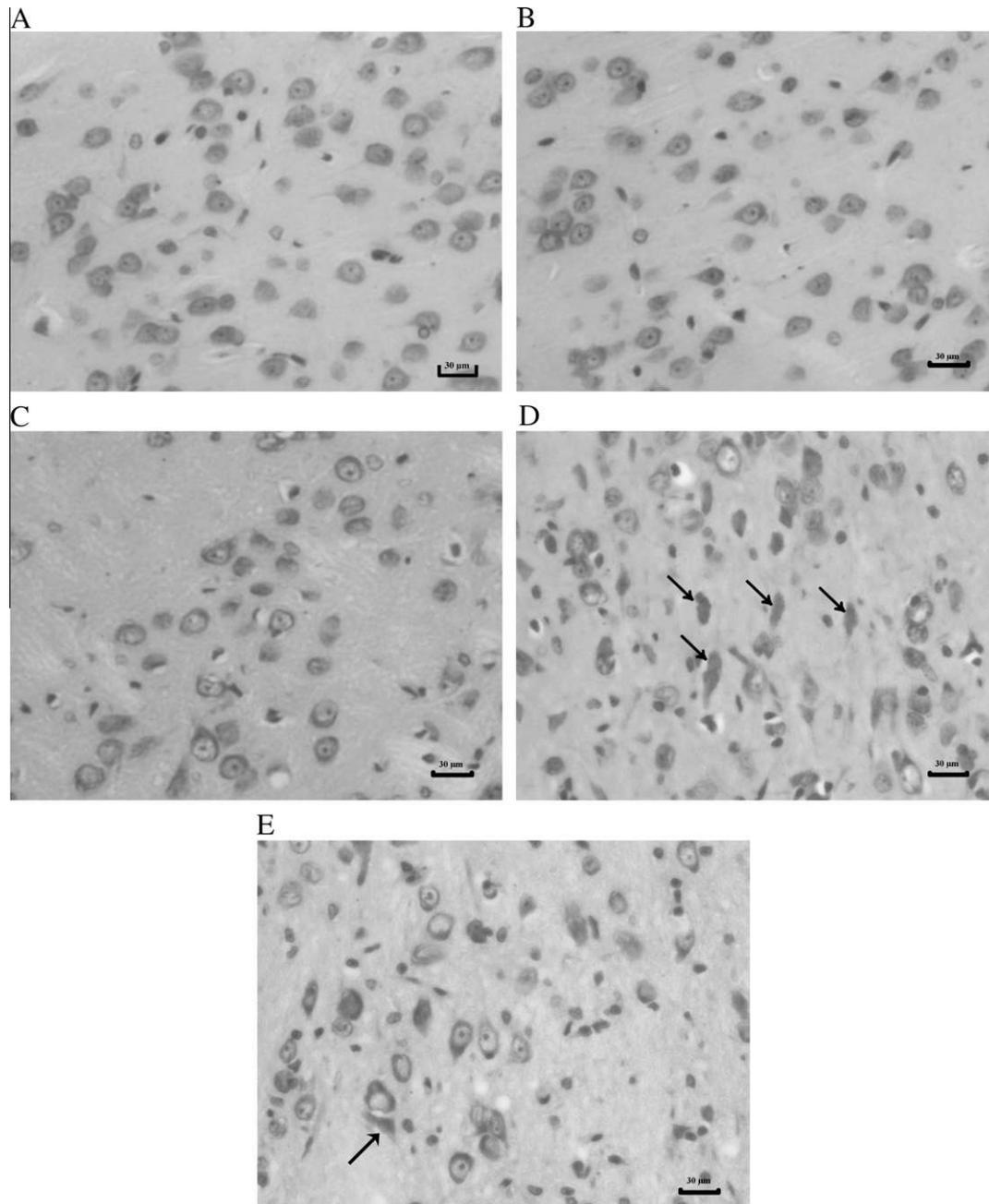


Fig. 2. Representative sections of Nissl-stained rat parietotemporal cortex ($\times 400$). (A) = naïve control; (B) = sham operation; (C) = adaptation to hypoxia; (D) = effect of A β (arrows indicate shrunken and hyperchromic, degenerating neurons); (E) = adaptation to hypoxia + A β .

Expression of NOS isoforms

Fig. 4 shows changes in nNOS (Fig. 4A), iNOS (Fig. 4B), and eNOS expression (Fig. 4C) in rat brain cortex and hippocampus after AIH and injection of A β . Sham operation did not induce any detectable changes in expression of any of the NOS isoforms. Injections of A β resulted in large increases in all three NOS isoforms in both cortex and hippocampus. The iNOS expression increased to a lesser extent in the cortex than in the hippocampus of A β -injected rats. AIH did not significantly influence eNOS, nNOS, and iNOS expression in the cortex. The expression of all three NOS isoforms was increased in the hippocampus of AIH rats, although these increases were considerably smaller than the increases in NOS isoform expression induced by A β . The most important result of these experiments was that adaptation to hypoxia significantly blunted the increases in

NOS isoform expression induced by A β in both cortex and hippocampus. This protection was most pronounced with respect to nNOS in the hippocampus and to eNOS in the cortex and hippocampus. This protection was most pronounced with respect to nNOS in the hippocampus and to eNOS in the cortex and hippocampus.

3-Nitrotyrosine content

Formation of 3-NT was evaluated in the cortex and hippocampus. Fig. 5 shows that A β injections were associated with increased 3-NT in cortex ($96 \pm 18\%$, $p < 0.05$; Fig. 5A) and in hippocampus ($120 \pm 26\%$, $p < 0.05$; Fig. 5B). Sham operation also resulted in statistically significant, but milder, increases in 3-NT in cortex ($38 \pm 12\%$, $p < 0.05$; Fig. 5A) and in hippocampus ($25 \pm 10\%$,

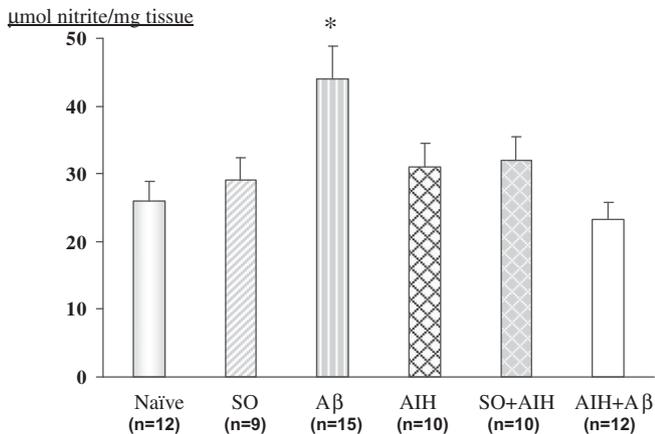


Fig. 3. Effect of adaptation to hypoxia on the nitrite + nitrate (NO_x) concentration (μmol nitrite/mg tissue) in brain tissue of rats with experimental Alzheimer's disease. The results are presented as M ± SEM. AIH = adaptation to hypoxia; SO = sham operation; *greater than all other groups.

Fig. 5B). AIH did not significantly influence tissue 3-NT in the hippocampus, but moderately increased 3-NT in the cortex by 42% compared to the naïve control ($p < 0.05$). However, AIH significantly restricted the increases in 3-NT in the cortex and hippocampus of rats injected with A β .

Discussion

The main findings of this study are that adaptation of rats with A β toxicity to intermittent hypobaric hypoxia (AIH) attenuated (1) NO overproduction by excessively expressed NOS isoforms and the resulting protein nitration in brain; (2) neurodegenerative processes resulting in loss of cortical neurons; and (3) impairment of memory retention. Taken together, these data show that AIH affords significant protection against experimental AD. This protection may be mediated by reducing NO overproduction and resulting protein nitration, one of AD's important pathogenetic mechanisms.

AD is characterized by formation of amyloid plaques, intracellular neurofibrillary tangles and pronounced cell death in the brain, resulting in progressive loss of memory and cognitive ability [28]. Early steps in the pathogenesis of AD include excessive production of reactive oxygen species and reactive nitrogen species [29].

Direct neurotoxic effects of excessive NO are mediated by mitochondrial dysfunction and ATP depletion, which lead to the collapse of mitochondrial membrane potential and the release of mitochondrial proteins. These proteins, including cytochrome *c* and Smac, in turn, activate the caspase cascade causing apoptotic death of neurons [30]. In addition, NO reacts with superoxide anion (O₂⁻) to produce peroxynitrite (ONOO⁻), an extremely strong oxidant that damages lipids, DNA, carbohydrates, and proteins. Both peroxynitrite decomposition and NO oxidation produce nitrite which is subsequently oxidized to nitrogen dioxide (NO₂) by reactions catalyzed by peroxidases [31–33]. Nitrogen dioxide is a potent nitrating species [34], and it readily nitrates tyrosine to form 3-NT [35]. Nitration of tyrosine affects many important enzymes and structural proteins, such as, manganese superoxide dismutase, neurofilament L, actin, and tyrosine hydroxylase [36]. This pathological nitration process is associated with development and progression of several neurodegenerative diseases, including AD, and 3-NT is found in large amounts in affected regions of the brains of patients with AD [37–39].

Nitrative damage occurs early in the progression of AD and may be important for transition from mild cognitive impairment to the

severe impairment characteristic of AD [39]. In the present study, we observed an increase in total protein nitration of both hippocampus and cortex of rats with experimental AD. The increase in 3-NT was greater in hippocampus than in cortex following injection of A β , consistent with the suggestion that the hippocampus is the region more vulnerable to AD [39,40].

Our measurements of the stable NO metabolites, nitrite and nitrate, showed that A β induced pronounced NO overproduction resulting from excessive expression of all three NOS isoforms. Interestingly, we found stronger induction of eNOS than of iNOS, although a prior report indicated that the iNOS isoform, which is induced in astrocytes associated with amyloid plaques, is a major source of NO in AD [41]. More recent evidence, however, has demonstrated also a significant role for eNOS [42,43]. While eNOS is found in endothelial cells, this NOS isoform had been also detected in neurons and glial cells [42,44].

Astrocytes with elevated levels of iNOS or eNOS are found in direct association with A β -deposits, and experimental AD is associated with astrocytosis accompanied by significant increases of both iNOS and eNOS [43]. Thus, increased expression of these NOS isoforms contributes to the maintenance and progression of the neurodegenerative process. Contrary to iNOS, the isoforms eNOS and nNOS are constitutively expressed, and their activation does not require new enzyme protein synthesis. However, these constitutive forms of NOS are also inducible, since synthesis of new enzyme protein is stimulated by pathological conditions [45,46].

All three isoforms of NOS, as well as 3-NT, are found in sections of temporal cortex taken from postmortem brains of patients with AD [47]. Aberrant expression of nNOS in cortical pyramidal cells and of iNOS and eNOS in glial cells is highly co-localized with nitrotyrosine. Therefore, it is likely that increased expression of all NOS isoforms in astrocytes and neurons contributes to generation of nitrotyrosine and severe damage to brain neurons [48]. Indeed, loss of synaptic contacts is observed early in the brain of patients with AD [49,50], and this process significantly correlates with cognitive decline [51]. Damage and shrinkage of neurons follows, with subsequent selective loss of neurons in the hippocampus, cortex, neocortex and limbic structures [51,52].

The primary site of damage in AD is cholinergic neurons in the nucleus basalis of Meynert. These neurons project to the cerebral cortex and to similar neurons located in the diagonal band of Broca and the medial septum. From there, neurons project to the hippocampus [52,53] and parietotemporal cortex [25,54], the regions that are most severely affected in the brains of patients with AD [55]. Thus, in the present study, we used a commonly accepted rat model of AD: injection of A β into the *nucleus basalis magnocellularis*, a structure homologous to the nucleus basalis of Meynert [56] with subsequent morphological analysis of neurodegeneration in the parietotemporal cortex. Harkany et al. showed that A β injection into the *nucleus basalis magnocellularis* resulted in damage to cholinergic neurons and concomitant, significant loss of their cholinergic projection fibers in the cortex [57]. These authors then demonstrated that A β -induced cholinergic denervation of the rat cortex closely correlated with NOS activation [58]. They suggested that NO overproduction and subsequent formation of peroxynitrite might damage additional cortical neurotransmitter systems. Thus, increased cortical nNOS activity may be responsible, at least in part, for both the primary (cholinergic) and secondary (generalized) neurodegeneration of AD.

Dark neurons in brain sections stained by Niesl are known to represent a typical morphological change indicative of degenerating neurons [59]. Since dark neurons are massively shrunken, abnormally hyperchromic, and acquire an angular shape, they can be clearly distinguished from normal neurons [59]. In our study, we found multiple neurons with these characteristic signs

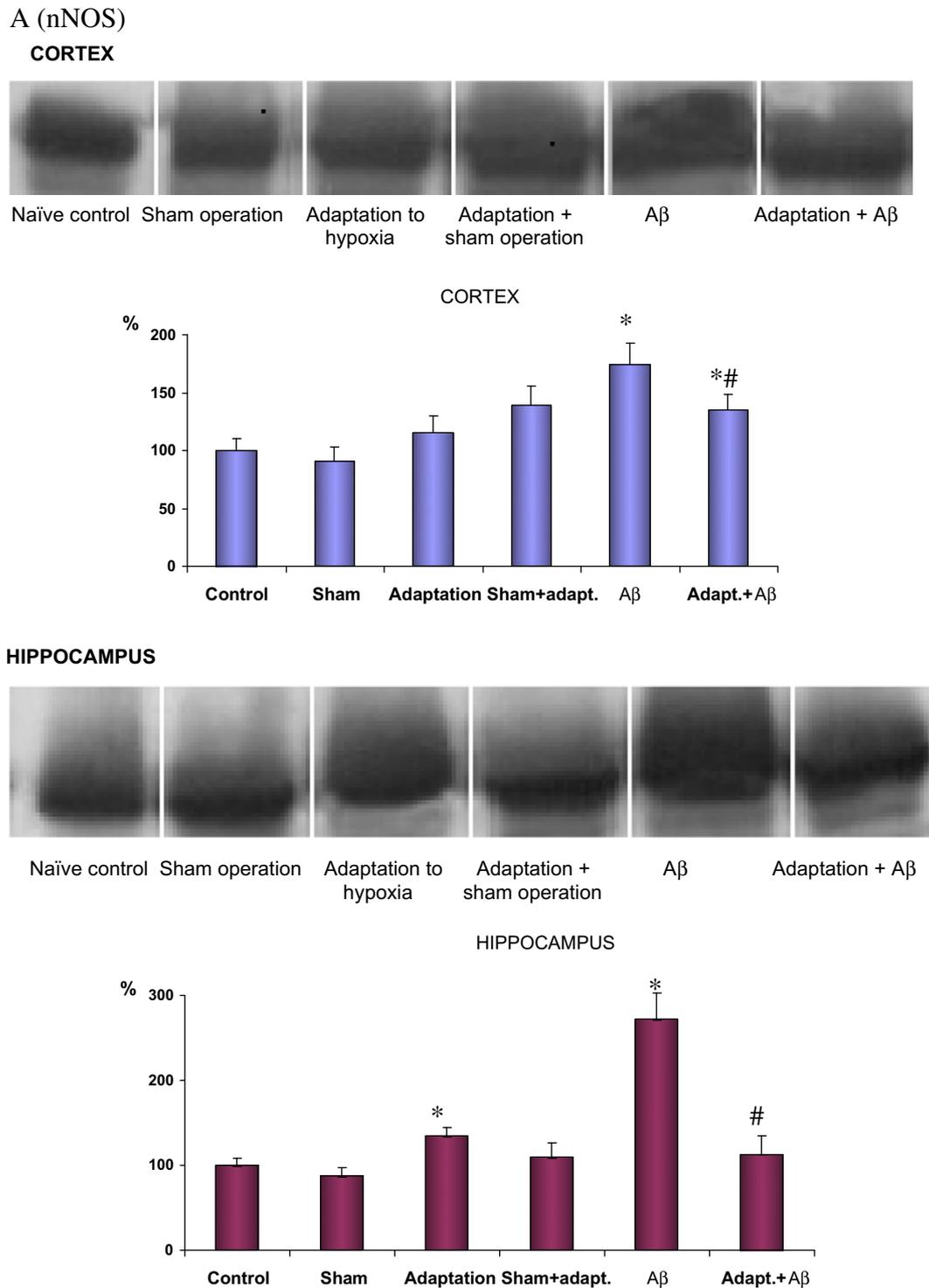


Fig. 4. Representative Western blots and diagrams showing expression of nNOS (5A), iNOS (5B) and eNOS (5C) in rat brain cortex and hippocampus. The data are mean \pm SEM ($n \geq 5$ each). Results are expressed as relative densitometric units in per cent of the value for naïve control. *greater than control control and/or sham operation, $p < 0.05$. #less than A β , $p < 0.05$.

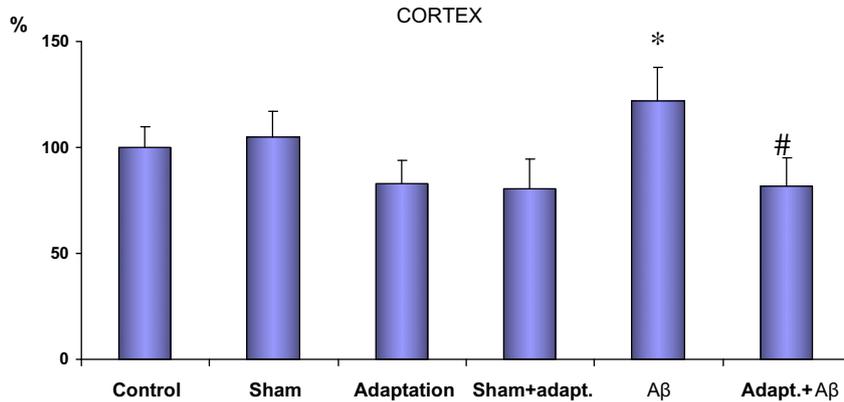
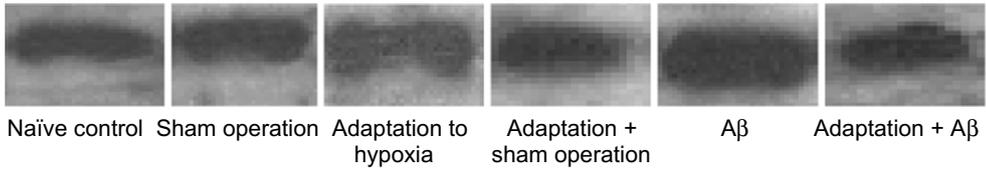
of degeneration in brains of rats injected with A β . These degenerating neurons, typical for experimental AD, were not observed in sham-operated control rats.

In this investigation, we confirmed the protective effect of AIH on the cognitive function of rats, as we had previously reported [21–23]. Our data suggest that this protective effect on cognitive function is due to less vulnerability of brain neurons to A β -induced neurodegeneration. Thus, more functional neurons were present in rats with experimental AD and adapted to intermittent hypoxia.

Our results allow speculation about possible mechanisms responsible for the protective effect of AIH and the role of NO-dependent mechanisms in this protection. Although adaptation to hypoxia *per se* stimulates NO production [60,61], the current study shows that this adaptation prevented NO overproduction in experimental AD and alleviated adverse effects of AD. Severe hypoxia produced by a simulated altitude of 11,000 m is another condition that causes brain damage, which is associated with NO overproduction [62]. Earlier we found that an 8-day program of

B (iNOS)

CORTEX



HIPPOCAMPUS

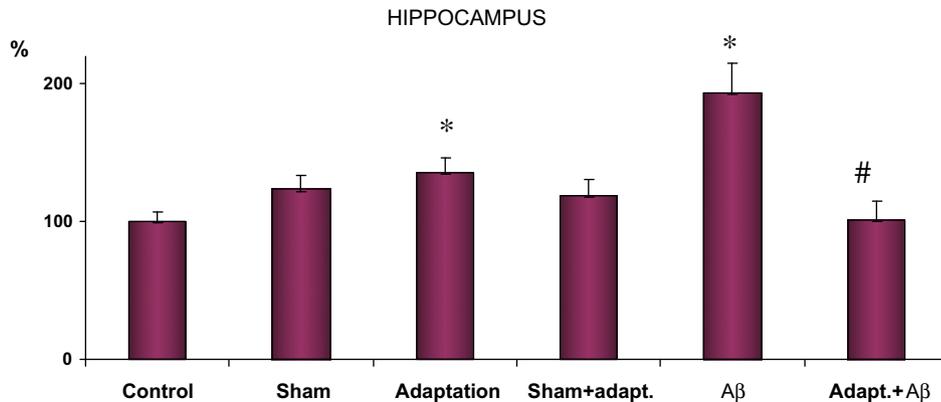
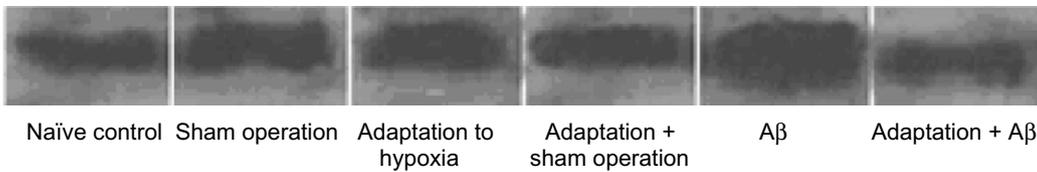


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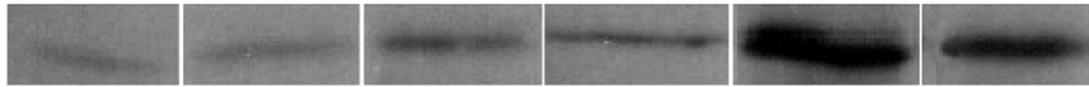
intermittent hypobaric hypoxia prevented subsequent NO overproduction in the brain and prolonged survival when these rats were then exposed to severe hypoxia [62]. This protection appeared to be related to a moderate, prior increase in NO synthesis during the adaptation process, since inhibition of NOS during adaptation abolished the protection. We also demonstrated that inhibition of NOS blunted AIH-induced improvement in memory retention of rats with experimental AD, whereas an NO donor mimicked the protective effect of AIH [22]. Findings of the present study that AIH reduced expression of NOS isoforms and accumulation of 3-NT support the hypothesis that restriction of NO overproduction may underlie the neuroprotective effect of AIH in

experimental AD. However, the causal relationship between restriction of NO overproduction and AIH-induced neuroprotection remains to be proved.

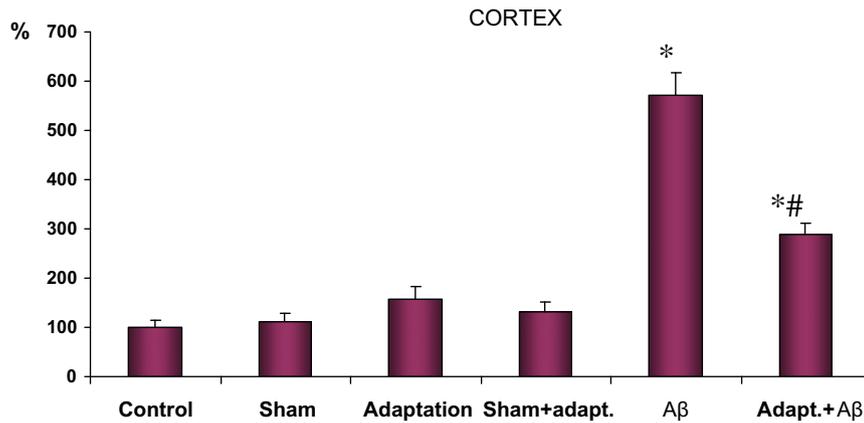
At least two mechanisms might account for the AIH-induced restriction of NO overproduction demonstrated in this study. First, NO overproduction can be directly restricted by NO synthesized during adaptation by a negative feedback mechanism due to binding of NO to NOSFe²⁺, which restricts further NO production [63]. Second, adaptation to hypoxia facilitates binding of NO to complexes that form NO stores. Formation of NO stores is apparently an adaptive mechanism to protect cells from the toxic effects of excessive NO [64,65]. Indeed, in our previous experiments we have

C (eNOS)

CORTEX



Naïve control Sham operation Adaptation to hypoxia Adaptation + sham operation A β Adaptation + A β



HIPPOCAMPUS



Naïve control Sham operation Adaptation to hypoxia Adaptation + sham operation A β Adaptation + A β

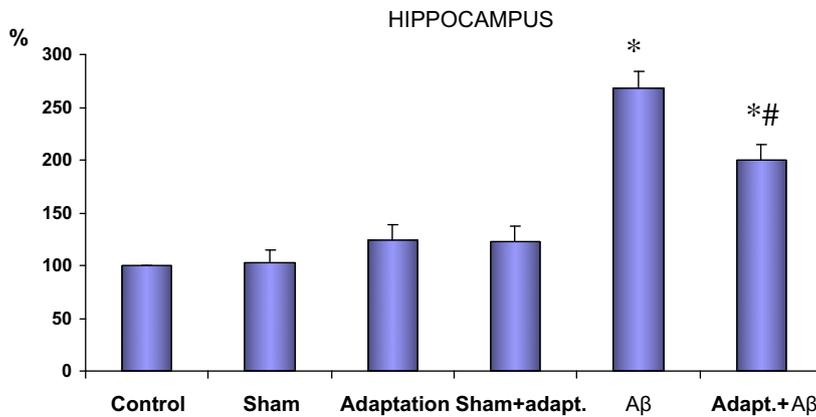


Fig. 4 (continued)

demonstrated that the amount of NO stores in blood vessels of hypoxia-adapted rats with experimental AD was significantly greater than in non-adapted rats [66].

Another important neuroprotective mechanism of adaptation to hypoxia may involve restriction of oxidative stress in brain tissue due to the activation of antioxidant defenses. Indeed, it has been shown that oxidative stress damages the brain during withdrawal from chronic ethanol consumption [11] or following injection of iron citrate [67]. Brain content of the oxidative stress marker, superoxide anion, was reduced by daily AIH in rats withdrawn from ethanol consumption decreased [11]. Reduced concentrations of lipid peroxidation products, increased activities of superoxide

dismutase, catalase and glutathione peroxidase were found in red blood cells of patients with dyscirculatory encephalopathy after 10 days of AIH [68]. These patients also had improved well-being and memory. In our earlier experiments, adaptation to hypoxia effectively prevented oxidative stress induced by A β in rats [21,23]. AIH may also exert a direct protective and reparatory effect on brain neurons by facilitating the proliferation of neural stem cells [69].

The hypoxia-inducible transcription factor, HIF-1, may also protect against A β toxicity [70]. In fact, the rapid dynamics of synthesis and degradation of HIF may be especially important for adaptation to intermittent hypoxia [71–73]. Therefore, it would

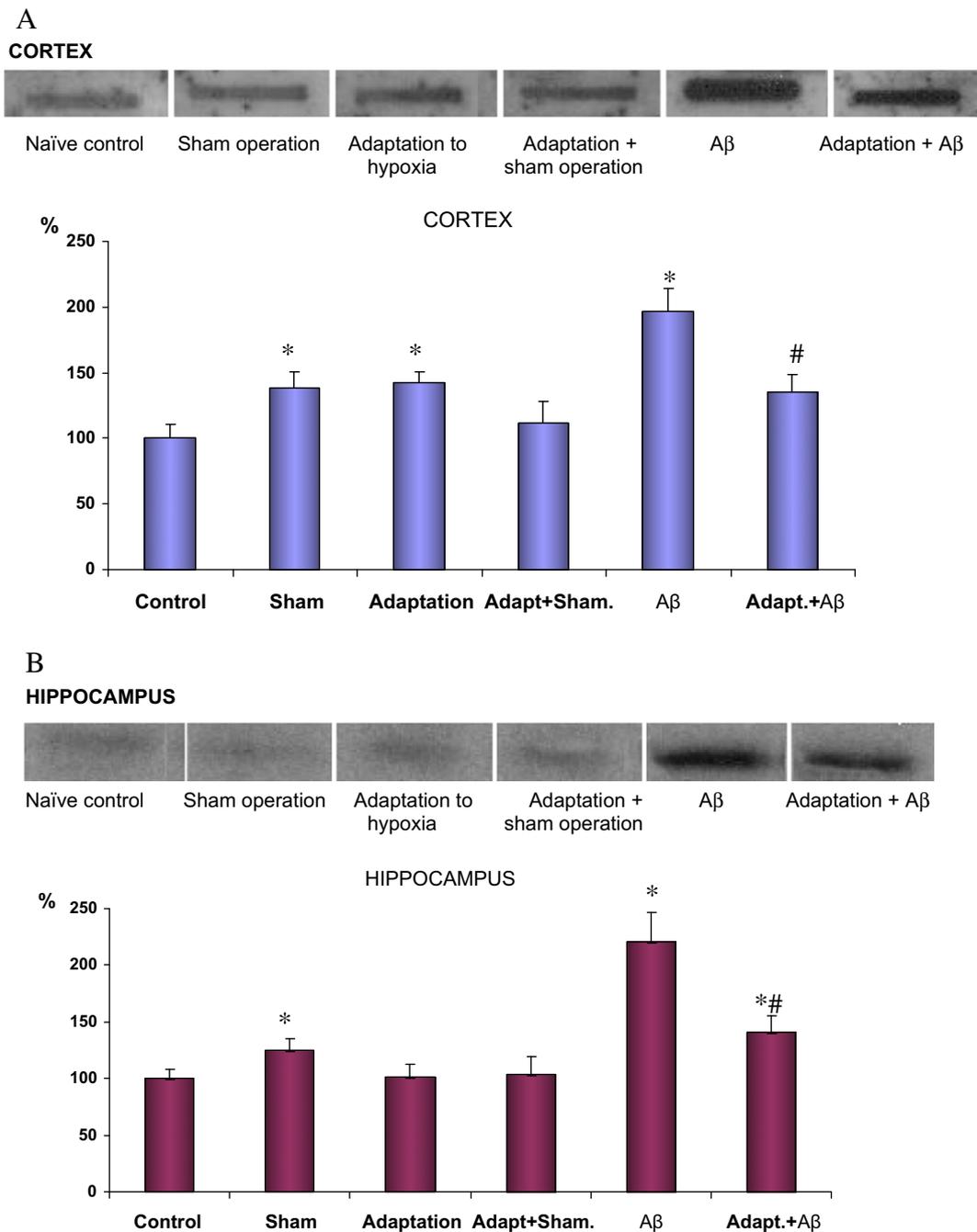


Fig. 5. Representative slot-blot and diagrams showing expression of 3-nitrotyrosine in rat brain cortex (A) and hippocampus (B). The data are mean \pm SEM ($n \geq 5$ each). Results are expressed as relative densitometric units in per cent of the value for naïve control. *greater than control and/or sham operation, #greater than A β , $p < 0.05$.

be interesting to investigate in future studies a potential role of HIF-1 in the neuroprotective effect of adaptation to hypoxia and any possible interactions between HIF-1 and NO overproduction. Clearly, by elucidating the protective mechanisms of adaptation to hypoxia, new, clinically relevant, approaches to prevention and treatment of AD may be developed.

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References

- [1] R.T. Mallet, M.-G. Ryou, E.B. Manukhina, H.F. Downey, Intermittent hypoxic conditioning of canine myocardium: robust protection against ischemia-reperfusion injury, in: L. Lukyanova, N. Takeda, P.K. Singal (Eds.), *Adaptation Biology and Medicine: Health Potentials*, Narosa Publishers, New Delhi, 2008, pp. 59–78.
- [2] R.T. Mallet, M.-G. Ryou, E.B. Manukhina, H.F. Downey, Beta-adrenergic signaling and ros: pivotal roles in intermittent, normobaric hypoxia-induced cardioprotection, in: L. Xi, T.V. Serebrovskaya (Eds.), *Intermittent Hypoxia: From Molecular Mechanisms to Clinical Applications*, Nova Science Publishers, Hauppauge, 2008, pp. 189–212.
- [3] T.V. Serebrovskaya, E.B. Manukhina, M.L. Smith, H.F. Downey, R.T. Mallet, Intermittent hypoxia: cause of or therapy for systemic hypertension?, *Exp Biol. Med.* 233 (2008) 627–650.
- [4] E.B. Manukhina, A.V. Lapshin, F.Z. Meerson, Adaptation to intermittent hypoxia prevents disorders of endothelium-dependent relaxation and fall of blood pressure in experimental myocardial infarction, *Hypox. Med. J.* 1 (1994) 15–18.

- [5] M.G. Pshennikova, E.B. Manukhina, T.G. Sazontova, P.A. Prodius, N.E. Golantsova, I.Yu. Malyshev, M.G. Pshennikova, M.G. Pshennikova, Molecular mechanisms of adaptation to environmental factors: new achievements of the Meerson school, in: B.B. Pandolf, N. Takeda, P.K. Singal (Eds.), *Adaptation Biology and Medicine*. vol. 2, Narosa Publishers, Delhi, 1999, pp. 199–213.
- [6] A.L. Krushinskii, V.P. Reutov, V.S. Kuzenkov, E.G. Sorokina, V.B. Koshelev, O.E. Fadiukova, L.M. Baïder, Z.V. Kuropteva, T.T. Zhumambaeva, L.Kh. Komissarova, T.V. Riasina, N.S. Kositsyn, V.G. Pinelis, Nitric oxide is involved in the protective effects of short-term adaptation to hypoxia in the course of stress-induced disorders in Krushinsky-Molodkina rats, *Izv. Akad. Nauk. Ser. Biol.* 3 (2007) 329–335 (in Russian).
- [7] D. Maresová, K. Jandová, J. Bortelová, S. Trojan, B. Trnková, Functional and morphological changes of the brain in rats exposed to intermittent hypobaric hypoxia after the repetitive magnesium administration, *Prague Med. Rep.* 106 (2005) 61–69.
- [8] F.Z. Meerson, V.A. Isabaeva, A.Z. Ivanshina, Acceleration of the rate of elaboration and degree of retention of conditioned reflexes in animals adapted to altitude hypoxia, *Dokl. Akad. Nauk. SSSR* 202 (1972) 982–984 (in Russian).
- [9] F.Z. Meerson, *Essentials of Adaptive Medicine: Protective Effects of Adaptation*, Hypoxia Medical Ltd., Moscow, 1994.
- [10] J.C. López-Ramos, P.J. Yi, L. Eleore, N. Madroñal, A. Rueda, J.M. Delgado-García, Classical eyeblink conditioning during acute hypobaric hypoxia is improved in acclimatized mice and involves Fos expression in selected brain areas, *J. Appl. Physiol.* 103 (2007) 1479–1487.
- [11] M.E. Jung, J.W. Simpkins, A.M. Wilson, H.F. Downey, R.T. Mallet, Intermittent hypoxia conditioning prevents behavioral deficit and brain oxidative stress in ethanol-withdrawn rats, *J. Appl. Physiol.* 105 (2008) 510–517.
- [12] M. Bernaudin, A.S. Nedelec, D. Divoux, E.T. MacKenzie, E. Petit, P. Schumann-Bard, Normobaric hypoxia induces tolerance to focal permanent cerebral ischemia in association with an increased expression of hypoxia-inducible factor-1 and its target genes, Erythropoietin and VEGF, in the adult mouse brain, *J. Cereb. Blood Flow Metab.* 22 (2002) 393–403.
- [13] F.R. Sharp, R. Ran, A. Lu, Y. Tang, K.I. Strauss, T. Glass, T. Ardizzone, M. Bernaudin, Hypoxic preconditioning protects against ischemic brain injury, *NeuroRx* 1 (2004) 26–35.
- [14] N.S. Samoilenkova, S.A. Gavrilova, V.B. Koshelev, Protective effect of hypoxic and ischemic preconditioning in rat brain local ischemia, *Dokl. Biol. Sci.* 414 (2007) 183–184.
- [15] K. Prass, A. Scharff, K. Ruscher, D. Lowl, C. Muselmann, I. Victorov, K. Kapinya, U. Dirnagl, A. Meisel, Hypoxia-induced stroke tolerance in the mouse is mediated by erythropoietin, *Stroke* 34 (2003) 1981–1986.
- [16] N.V. Gulyaeva, M.V. Stepanichev, M.V. Onifriev, I.V. Sergeev, O.S. Mitrokhina, Yu.V. Moiseeva, E.N. Tkatchouk, Interval hypoxia training prevents oxidative stress in striatum and locomotor disturbances in a rat model of parkinsonism, in: A. Fisher, I. Hanlin, M. Yoshida (Eds.), *Progress in Alzheimer's and Parkinson's Diseases*, Plenum Press, New York, 1998, pp. 717–723.
- [17] S. Gauthier, P. Scheltens, Can we do better in developing new drugs for Alzheimer's disease?, *Alzheimers Dement* 5 (2009) 489–491.
- [18] M.P. Mattson, Neuroprotective signaling and the aging brain: take away my food and let me run, *Brain Res.* 886 (2000) 47–53.
- [19] N. Mahendra, S. Arkin, Effects of four years of exercise, language, and social interventions on Alzheimer discourse, *J. Commun. Disord.* 36 (2003) 395–422.
- [20] D.F. Swaab, E.J. Dubelaar, E.J. Scherder, E.J. Van Someren, R.W. Verwer, Therapeutic strategies for Alzheimer disease: focus on neuronal reactivation of metabolically impaired neurons, *Alzheimer Dis. Assoc. Disord.* 17 (2003) S114–S122.
- [21] M.G. Pshennikova, E.V. Popkova, D.A. Pokidyshev, I.P. Khomenko, O.M. Zelenina, S.V. Kruglov, E.B. Manukhina, M.V. Shimkovich, A.V. Goriacheva, Iu.I. Malyshev, The effects of adaptation to hypoxia on the resistance to neurodegenerative disorders in the brain of different genetic strains, *Vestn. Ross. Akad. Med. Nauk.* 2 (2007) 50–55 (in Russian).
- [22] E.B. Manukhina, M.G. Pshennikova, A.V. Goryacheva, I.P. Khomenko, S.Yu. Mashina, D.A. Pokidyshev, I.Yu. Malyshev, Role of nitric oxide in prevention of cognitive disorders in neurodegenerative brain injuries in rats, *Bull. Exp. Biol. Med.* 146 (2008) 391–395.
- [23] E.B. Manukhina, A.V. Goriacheva, I.V. Barskov, I.V. Viktorov, A.A. Guseva, M.G. Pshennikova, I.P. Khomenko, S.Yu. Mashina, D.A. Pokidyshev, I.Yu. Malyshev, Prevention of the brain neurodegeneration in rats with experimental Alzheimer's disease by adaptation to hypoxia, *Russ. Fiziol. Zh. Im. I M Sechenova.* 95 (2009) 706–715 (in Russian).
- [24] T. Harkany, S. O'Mahony, J. Kelly, K. Soos, I. Toro, B. Penke, P.G.M. Luiten, C. Nyakas, K. Gulya, B.E. Leonard, β -Amyloid (Phe(SO₃H))^{24–25} in rat nucleus basalis induces behavioral dysfunctions impairs learning and memory and disrupts cortical cholinergic innervation, *Behav. Brain Res.* 90 (1998) 133–145.
- [25] G. Paxinos, C. Watson, *The Rat Brain in Stereotaxic Coordinates*, Academic Press, Sydney, 1986.
- [26] E.A. van der Zee, B.R. Douma, B. Bohus, P.G. Luiten, Passive avoidance training induces enhanced levels of immunoreactivity for muscarinic acetylcholine receptor and coexpressed PKC gamma and MAP-2 in rat cortical neurons, *Cereb. Cortex* 4 (1994) 376–390.
- [27] T. Harkany, G.I. De Jong, K. Soós, B. Penke, P.G.M. Luiten, K. Gulya, β -Amyloid(1–42) affects cholinergic but not parvalbumin-containing neurons in the septal cortex of the rat, *Brain Res.* 698 (1995) 270–274.
- [28] A.B. Clippingdale, J.D. Wade, C.J. Barrow, The amyloid-beta peptide and its role in Alzheimer's disease, *J. Pept. Sci.* 7 (2001) 227–249.
- [29] M. Aslan, T. Ozben, Reactive oxygen and nitrogen species in Alzheimer's disease, *Curr. Alzheimer Res.* 1 (2004) 111–119.
- [30] C. Bertoni-Freddari, P. Fattoretti, T. Casoli, G. Di Stefano, M. Balietti, B. Giorgetti, G. Perretta, Neuronal apoptosis in Alzheimer's disease: the role of age-related mitochondrial metabolic competence, *Ann. NY Acad. Sci.* 1171 (2009) 18–24.
- [31] M. Kirsch, H.G. Korth, R. Sustmann, H. De Groot, The pathobiochemistry of nitrogen dioxide, *Biol. Chem.* 383 (2002) 389–399.
- [32] O. Augusto, M.G. Bonini, A.M. Amanso, E. Linares, C.C. Santos, S.L. De Menezes, Nitrogen dioxide and carbonate radical anion: two emerging radicals in biology, *Free Radic. Biol. Med.* 32 (2002) 841–859.
- [33] S. Pfeiffer, A. Lass, K. Schmidt, B. Mayer, Protein tyrosine nitration in mouse peritoneal macrophages activated in vitro and in vivo: evidence against an essential role of peroxynitrite, *FASEB J.* 15 (2001) 2355–2364.
- [34] M.L. Brennan, W. Wu, X. Fu, Z. Shen, W. Song, H. Frost, C. Vadseth, L. Narine, E. Lenkiewicz, M.T. Borchers, A.J. Lulis, J.J. Lee, N.A. Lee, H.M. Abu-Soud, H. Ischiropoulos, S.L. Hazen, A tale of two controversies: defining both the role of peroxidases in nitrotyrosine formation in vivo using eosinophil peroxidase and myeloperoxidase-deficient mice and the nature of peroxidase-generated reactive nitrogen species, *J. Biol. Chem.* 277 (2002) 17415–17427.
- [35] J.M. Souza, E. Daikhin, M. Yudkoff, C.S. Raman, H. Ischiropoulos, Factors determining the selectivity of protein tyrosine nitration, *Arch. Biochem. Biophys.* 371 (1999) 169–178.
- [36] A.J. Gow, D. Duran, S. Malcolm, H. Ischiropoulos, Effects of peroxynitrate-induced protein modifications on tyrosine phosphorylation and degradation, *FEBS Lett.* 385 (1996) 63–65.
- [37] A. Castegna, V. Thongboonkerd, J.B. Klein, B. Lynn, W.R. Markesbery, D.A. Butterfield, Proteomic identification of nitrated proteins in Alzheimer's disease brain, *J. Neurochem.* 85 (2003) 1394–1401.
- [38] G. Cenini, R. Sultana, M. Memo, D.A. Butterfield, Elevated levels of proapoptotic p53 and its oxidative modification by the lipid peroxidation product, HNE, in brain from subjects with amnesic mild cognitive impairment and Alzheimer's disease, *J. Cell Mol. Med.* 12 (2008) 987–994.
- [39] D.A. Butterfield, T.T. Reed, M. Perluigi, C. De Marco, R. Cocca, J.N. Keller, W.R. Markesbery, R. Sultana, Elevated levels of 3-nitrotyrosine in brain from subjects with amnesic mild cognitive impairment: implications for the role of nitration in the progression of Alzheimer's disease, *Brain Res.* 1148 (2007) 243–248.
- [40] M.A. Smith, P.L. Richey Harris, L.M. Sayre, J.S. Beckman, G. Perry, Widespread peroxynitrite-mediated damage in Alzheimer's disease, *J. Neurosci.* 17 (1997) 2653–2657.
- [41] M.N. Wallace, J.G. Geddes, D.A. Farquhar, M.R. Masson, Nitric oxide synthase in reactive astrocytes adjacent to β -amyloid plaques, *Exp. Neurol.* 144 (1997) 266–272.
- [42] S.M. de la Monte, B.X. Lu, Y.K. Sohn, D. Etienne, J. Kraft, N. Ganju, J.R. Wands, Aberrant expression of nitric oxide synthase III in Alzheimer's disease: relevance to cerebral vasculopathy and neurodegeneration, *Neurobiol. Aging* 21 (2000) 309–319.
- [43] H.J. Lüth, M. Holzer, U. Gärtner, M. Staufenbiel, T. Arendt, Expression of endothelial and inducible NOS-isoforms is increased in Alzheimer's disease, in APP23 transgenic mice and after experimental brain lesion in rat: evidence for an induction by amyloid pathology, *Brain Res.* 913 (2001) 57–67.
- [44] P.L. Gabbott, S.J. Bacon, Localisation of NADPH diaphorase activity and NOS immunoreactivity in astroglia in normal adult rat brain, *Brain Res.* 714 (1996) 135–144.
- [45] U. Förstermann, H. Kleinert, Nitric oxide synthase expression and expression control of the three isoforms, *Naunyn Schmiedeberg's Arch. Pharmacol.* 352 (1995) 351–364.
- [46] P. Aguilera, M.E. Cháñez-Cárdenas, E. Floriano-Sánchez, D. Barrera, A. Santamaría, D.J. Sánchez-González, F. Pérez-Severiano, J. Pedraza-Chaverri, P.D. Jiménez, Time-related changes in constitutive and inducible nitric oxide synthases in the rat striatum in a model of Huntington's disease, *Neurotoxicology* 28 (2007) 1200–1207.
- [47] P. Fernández-Vizorra, A.P. Fernández, S. Castro-Blanco, J.M. Encinas, J. Serrano, M.L. Bentura, P. Muñoz, R. Martínez-Murillo, J. Rodrigo, Expression of nitric oxide system in clinically evaluated cases of Alzheimer's disease, *Neurobiol. Dis.* 15 (2004) 287–305.
- [48] H.J. Lüth, G. Münch, T. Arendt, Aberrant expression of NOS isoforms in Alzheimer's disease is structurally related to nitrotyrosine formation, *Brain Res.* 953 (2002) 135–143.
- [49] C.A. Davies, D.M. Mann, P.Q. Sumpter, P.O. Yates, A quantitative morphometric analysis of the neuronal and synaptic content of the frontal and temporal cortex in patients with Alzheimer's disease, *J. Neurol. Sci.* 78 (1987) 151–164.
- [50] D.J. Selkoe, Alzheimer's disease is a synaptic failure, *Science* 298 (2002) 789–791.
- [51] S.T. De Kosky, S.W. Scheff, Synapse loss in frontal cortex biopsies in Alzheimer's disease: correlation with cognitive severity, *Ann. Neurol.* 27 (1990) 457–464.
- [52] H. Braak, U. Rüb, C. Schultz, K. Del Tredici, Vulnerability of cortical neurons to Alzheimer's and Parkinson's diseases, *J. Alzheimers Dis.* 9 (3 Suppl.) (2006) 35–44.
- [53] P.J. Whitehouse, D.L. Price, A.W. Clark, J.T. Coyle, M.R. DeLong, Alzheimer disease: evidence for selective loss of cholinergic neurons in the nucleus basalis, *Ann. Neurol.* 10 (1981) 122–126.

- [54] B.T. Stuver, B.R. Douma, R. Bakker, C. Nyakas, P.G. Luiten, In vivo protection against NMDA-induced neurodegeneration by MK-801 and nimodipine: combined therapy and temporal course of protection, *Neurodegeneration* 5 (1996) 153–159.
- [55] A. Nishimura, S. Fujiwara, Y. Mizoi, Y. Tatsuno, Morphometric, pathologic studies of the brain in autopsied cases of Alzheimer-type Dementia, *Nihon Hoigaku Zasshi* 46 (1992) 141–151.
- [56] D. Boyd-Kimball, R. Sultana, H.F. Poon, B.C. Lynn, F. Casamenti, G. Pepeu, J.B. Klein, D.A. Butterfield, Proteomic identification of proteins specifically oxidized by intracerebral injection of amyloid beta-peptide (1–42) into rat brain: implications for Alzheimer's disease, *Neuroscience* 132 (2005) 313–324.
- [57] T. Harkany, S. O'Mahony, J. Keijser, I.P. Kelly, C. Kónya, Z.A. Borostyánko, T.J. Görcs, M. Zarándi, B. Penke, B.E. Leonard, P.G. Luiten, Beta-amyloid(1–42)-induced cholinergic lesions in rat nucleus basalis bidirectionally modulate serotonergic innervation of the basal forebrain and cerebral cortex, *Neurobiol. Dis.* 8 (2001) 667–678.
- [58] S. O'Mahony, T. Harkany, A.A. Rensink, I. Abrahám, G.I. De Jong, J.L. Varga, M. Zarándi, B. Penke, C. Nyakas, P.G. Luiten, B.E. Leonard, Beta-amyloid-induced cholinergic denervation correlates with enhanced nitric oxide synthase activity in rat cerebral cortex: reversal by NMDA receptor blockade, *Brain Res. Bull.* 45 (1998) 405–411.
- [59] J. Cammermeyer, The importance of avoiding Dark Neurons in experimental neuropathology, *Acta Neuropathol.* 1 (1961) 245–270.
- [60] E.B. Manukhina, I. Yu. Malyshev, Role of free NO and NO stores in protective effects of adaptation to hypoxia, in: P.K. Singal, N. Takeda (Eds.), *Adaptation Biology and Medicine. vol. 4: New Concepts*, Narosa Publishing House, New Delhi, 2005, pp. 82–94.
- [61] E.B. Manukhina, H.F. Downey, R.T. Mallet, Role of nitric oxide in cardiovascular adaptation to intermittent hypoxia, *Exp. Biol. Med.* 231 (2006) 343–365.
- [62] I.Yu. Malyshev, T.A. Zenina, L.Yu. Golubeva, V.A. Saltykova, E.B. Manukhina, V.D. Mikoyan, L.N. Kubrina, A.F. Vanin, NO-dependent mechanisms of adaptation to hypoxia, *Nitric Oxide* 3 (1999) 105–113.
- [63] H.M. Abu-Soud, J. Wang, D.L. Rousseau, J.M. Fukuto, L.J. Ignarro, D.J. Stuehr, Neuronal nitric oxide synthase self-inactivates by forming a ferrous-nitrosyl complex during aerobic catalysis, *J. Biol. Chem.* 270 (1995) 22997–23006.
- [64] E.B. Manukhina, A.F. Vanin, K.M. Markov, I.Yu. Malyshev, Formation and role of nitric oxide stores in adaptation to hypoxia, *Adv. Med. Biol.* 578 (2006) 35–40.
- [65] E.B. Manukhina, A.F. Vanin, I.Yu. Malyshev, R.T. Mallet, H.F. Downey, Intermittent hypoxia-induced cardio- and vasoprotection: Role of NO stores, in: L. Xi, T.V. Serebrovskaya (Eds.), *Intermittent Hypoxia: From Molecular Mechanisms To Clinical Applications*, Nova Science Publishers, Hauppauge, 2009, pp. 113–146.
- [66] S.Y. Mashina, V.V. Aleksandrín, A.V. Goryacheva, M.A. Vlasova, A.F. Vanin, I.Yu. Malyshev, E.B. Manukhina, Adaptation to hypoxia prevents disturbances in cerebral blood flow during neurodegenerative process, *Bull. Exp. Biol. Med.* 142 (2006) 169–172.
- [67] A.M. Lin, C.F. Chen, L.T. Ho, Neuroprotective effect of intermittent hypoxia on iron-induced oxidative injury in rat brain, *Exp. Neurol.* 176 (2002) 328–335.
- [68] S.A. El'chaninova, N.A. Koreniak, I.V. Smagina, L.E. Pinegin, B.Ia. Varshavskii, Intermittent hypoxia in the treatment of dyscirculatory encephalopathy, *Zh Nevrol Psikhiatr Im S S Korsakova* 102 (2002) 29–32 (in Russian).
- [69] L.L. Zhu, T. Zhao, H.S. Li, H. Zhao, L.Y. Wu, A.S. Ding, W.H. Fan, M. Fan, Neurogenesis in the adult rat brain after intermittent hypoxia, *Brain Res.* 1055 (2005) 1–6.
- [70] T. Soucek, R. Cumming, R. Dargusch, P. Maher, D. Schubert, The regulation of glucose metabolism by HIF-1 mediates a neuroprotective response to amyloid beta peptide, *Neuron* 39 (2003) 43–56.
- [71] J.A. Neubauer, Physiological and pathophysiological responses to intermittent hypoxia, *J. Appl. Physiol.* 90 (2001) 1593–1599.
- [72] G.L. Semenza, O₂-regulated gene expression: transcriptional control of cardiorespiratory physiology by HIF-1, *J. Appl. Physiol.* 96 (2004) 1173–1177.
- [73] T.E. Walshe, P.A. D'Amore, The role of hypoxia in vascular injury and repair, *Annu. Rev. Pathol.* 3 (2008) 15–43.