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The effects of training and detraining on memory, neurotrophins and oxidative stress markers in rat brain

Zsolt Radak [a,*], Anna Toldy [a], Zsofia Szabo [a], Savvas Siamilis [a], Csaba Nyakas [a], Gabriella Silye [a], Judit Jakus [b], Sataro Goto [c]

[a] *Institute of Sport Science, Faculty of Physical Education and Sport Sciences, Semmelweis University, Budapest, Hungary*
[b] *Institute of Chemistry, National Academy of Science, Budapest, Hungary*
[c] *Department of Biochemistry, Faculty of Pharmaceutical Sciences, Toho University, Funabashi, Japan*

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Abstract

In the current investigation we tested how swimming training (T) (8 week, 5 times/week, 2 h/day), and detraining (DT) affects brain functions and oxidative stress markers in rat brain. The free radical concentration, measured by electron paramagnetic resonance, decreased in brain of T and DT rats compared to controls (C). The level of brain-derived neurotrophic factor (BDNF) increased as a result of training, but decreased below the control level after 6 weeks of detraining. In addition, the concentration of nerve growth factor (NGF) also declined with DT. The passive avoidance test was used to assess the memory of rats, and training-induced improvement was observed but the enhancement disappeared with detraining. When the content of mitochondrial electron transport complexes, as a potent free radical generator, was evaluated by the blue native gel method, no significant alterations were observed. The repair of nuclear and mitochondrial 8-oxodeoxyguanosine, as measured by the activity of OGG1, showed no significant difference. Therefore, the results suggest that regular exercise training improves memory, decreases the level of reactive oxygen species, and increase the production of BDNF and NGF. On the other hand, it appears that the beneficial effects of training are reversible in the brain, since detraining down-regulates the neurotrophin level, and memory. It is suggested that exercise training is more likely to beneficially effect the production of reactive oxygen species and the related oxidative damage.

Keywords: Exercise; Oxidative stress; Brain-derived neurotrophic factor; Detraining; Memory

1. Introduction

Regular physical exercise has been shown to maintain health, improve quality of life and decrease the incidence of a number of life-style related diseases (Radak et al., 2004, 2005a,b). Recently, it has become clear that exercise related adaptation processes have beneficial effects on brain function, including learning, long-term potentiation, and memory (van Praag et al., 1999; Radak et al., 2001a; Ogonovszky et al., 2005). Exercise training appears to maintain cerebrovascular integrity (McFarland, 1963), increase capillary growth (Black et al., 1987), increase dendritic connections (Pysh and Weiss, 1979), and enhance the efficiency of processing functions of the central nervous system (Dustman et al., 1990). Recently, it has been shown that voluntary exercise induces the production of the protein content of neurotrophic factors, especially brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF). This has been associated with an improved learning process (van Praag et al., 1999). Trophic factors are implicated in cell survival and differentiation, alteration of synaptic strength, memory, and increased resistance to oxidative stress (Guo and Mattson, 2000; Leeds et al., 2005; Klumpp et al., 2005).

Accumulating evidence suggests that reactive oxygen species (ROS)-induced alteration of redox milieu and/or the associated oxidative damage to lipids, proteins and DNA are associated or causative factor in aging and in a variety of brain diseases (reviewed by Mattson, 2002). It appears that the accumulation of oxidative damage in the brain is associated with impaired brain function (Radak et al., 2001b). Immobilization-induced oxidative stress in the brain and impaired

* Corresponding author at: Institute of Sport Science, Faculty of Physical Education and Sport Sciences, Semmelweis University, Alkotas u. 44, Budapest H-1123, Hungary. Tel.: +36 1 3 565764; fax: +36 1 3 566337.
E-mail address: radak@mail.hupe.hu (Z. Radak).

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memory were attenuated by exercise, and regular exercise has been shown to decrease the level of reactive carbonyl derivative, a marker of oxidative protein damage, and to prevent the age-related decline in memory and learning (Radak et al., 2001a,b).

Therefore, available information from separate studies suggests that exercise has a capability to alter the level of BDNF, NGF, memory and the rate of oxidative damage in brain of animals. On the other hand, the relationship between the oxidative damage/repair and trophic factors, have not been studied. In addition, most of the investigation on exercise and trophic factors applied voluntary exercise as a model, and the effects of enforced exercise, which is more similar to human exercise training, are not clear. The reversibility of the exercise-induced adaptive process is well known for cardiovascular function and mitochondrial enzyme activity (Henriksson and Reitman, 1977). The effects of detraining on brain function and oxidative stress- related processes are not known.

Therefore, we have designed an investigation to study the effect of regular exercise and detraining on memory, assessed by a passive avoidance test, BDNF and NGF content. The generation of free radicals, the oxidative damage of mitochondrial and nuclear proteins, and the activity of the DNA repair enzyme, OGG1, as well as the activity and content of nuclear proteasome complex, were measured to obtain information about the oxidative status of the cells. In addition, the content of mitochondrial electron transport complexes, a potential source of ROS generation, was investigated using Blue Native Gel polyacrylamide gel electrophoresis (BN-PAGE).

2. Methods

2.1. Animals

Twenty one male Wistar rats (13 month old) were used in the study and were cared for according to the guiding Principles for the Care and Use of Animals based upon the Helsinki Declaration, 1964. The study was approved by the local Animal Welfare Committee. Seven rats were randomly assigned to each of three groups: control (C), exercise trained (ET) and detrained (DT). ET and DT rats were subjected to swimming exercise for 8 weeks. Water temperature was maintained at 32 °C and swimming duration was 60 min per day, 5 days a week for 4 weeks. Then, for the remaining 4 weeks, exercise was increased to 120 min a day for 5 days a week. Swimming was selected because no electric shock was required to promote this exercise protocol, and therefore, the stimuli of exercise would not interfere with the stimuli used during the passive avoidance test. After the 8 week exercise training the DT group was kept as the control group for an additional 8 weeks. One day after the last training session of the ET group and at the end of the 8 week detraining period, a passive avoidance test was used to assess the memory. Then the animals were decapitated and the brain was quickly removed, washed and stored at −80 °C.

2.2. Retention of the passive avoidance test

The passive avoidance behavior was investigated in a one-trial step-through paradigm (Ader et al., 1972). The measurement has been described previously (Radak et al., 2001a). In brief, the assessment of the passive avoidance response was done by the registration of latency in entering the dark compartment one day following the electric foot-shock. Avoidance to enter the dark compartment during a 3 min period was set to 100% and a time-related percentage was given when the animals entered (50% was given to the animal if it entered the chamber after 1.5 min).

2.3. Biochemical assays

The concentrations of BDNF and NGF were determined, from the hippocampal section of the brain, using the E-Max ImmunoAssay System (Promega, Madison, WI). Standard 96-well flat-bottom Corning ELISA plates were incubated with carbonate coating buffer containing either polyclonal anti-NGF or monoclonal anti-BDNF over-night at 4 °C. The next day, the plates were blocked with 1 * B&S buffer for 1 h at room temperature. Serial dilutions of known amounts of NGF and BDNF, ranging from 500 to 0 pg, were performed in duplicate for the standard curve for each set of tissue. For both the standards and the samples, 100 μl was added to each well in duplicate, and incubated for 6 h (NGF) or 2 h (BDNF) at room temperature. The wells were then incubated with a secondary monoclonal anti-NGF (overnight at 4 °C) or antihuman BDNF polyclonal antibody (1 h at room temperature). Then, the wells were incubated with anti-rat IgG (NGF) or anti-IgY (BDNF) conjugated to HRP for 2.5 h (NGF) or 1 h (BDNF) at room temperature. A TMB solution was used to develop color in the wells for ten min at room temperature. The reaction was stopped with the addition of 1 N HCl to the wells. The absorbance was read at A450 (Molecular Devices ThermoMax microplate reader, with SOFTmax PRO v3.1.

2.4. Electron paramagnetic resonance

The electron paramagnetic resonance (EPR) measurements were carried out as described by Stadler et al. (2003). In brief, the measurementswere carried out using an X-Band computer-controlled EPR spectrometer, constructed by Magnettech GmbH (Berlin, Germany). Approximately 100 mg of cerebellum were frozen into a rod-shaped form and spectra of the samples were recorded at 77 K using a quartz finger Dewar filled with liquid nitrogen. Instrument settings were: 100 kHz modulation frequency, 0.7050 mT modulation amplitude, 18 mW microwave power, 1 min scan time, and 20.63 mT field sweep. For evaluation, a double integration method of the EPR signals, with Mn/MnO as an internal standard, was used, and the data were expressed as arbitrary units.

2.5. Excision assay

Whole brain samples were homogenized with buffer (HB) containing 20 mM of Tris (pH 8.0), 1 mM of EDTA, 1 mM of dithiothereitol, 0.5 mM of spermidine, 0.5 mM spermine, 50% glycerol and protease inhibitors. The nuclear and mitochondrial fraction was separated by centrifugation. After centrifugation (1000 × *g* for 10 min at 4 °C), the pellet was re-suspended in HB and centrifuged again. Then, the pellet was re-suspended in HB with 0.5% NP40 and centrifuged. Next, the pellet was washed twice in HB. After centrifugation the pellet was rocked for 30 min after the addition of a 1/10 vol/vol of 2.5 M KCl and centrifuged at 14 000 rpm for 30 min. The supernatant aliquots were and stored at −80 °C. The protein levels were measured by the BCA method. For the separation of mitochondria, the supernatant of the first centrifugation was centrifuged at 14 000 × *g* for 30 min at 4 °C. Then the supernatant was re-suspended in HB and re-centrifuged three times. The pellet was dissolved in 0.5 ml HB. The final pellet was suspended in HB containing 0.5% Triton X –100, and kept on ice for 20 min. The protein levels were again measured by the BCA method.

The assay was carried out according to the protocol described by Cardozo-Pelaez et al. (2000). In brief, twenty picomoles of synthetic probe containing 8-OHdG (Trevigen, Gaithersburg, MD, USA) were labeled with P^{32} at the 5′ end using polynucleotide T4 kinase (Boeringer Mannheim, Germany). For the nicking reaction, protein extract (2 μg) was mixed with 20 μl of a reaction mixture containing 0.5 M of *N*-[2-hydroxyethel]piperazine-*N*′ –[ethanesulfonic acid], 0.1 M EDTA, 5 mM of dithiolthreitol, 400 mM KCl, purified BSA and labeled probe (approximately 2000 cpm). The reaction was carried out at 30 °C for 5–15 min and stopped by placing the solution in ice. Then 30 μl chloroform were added and samples were centrifuged and 15 μl taken and mixed with loading buffer containing 90% formamide, 10 mM NaOH, and blue–orange dye. After three min heating at 95 °C, samples were chilled and loaded into polyacrylamide gel (20%) with 7 M urea and 1 × TBE and run at 400 mV for 2 h. Gels were quantified using BAS 2000 Bioimaging Analyzer (Fuji Film Co., Japan). Radioactive

signal densities were determined using the software designed for this system.

2.6. Proteasome activity and content

The chymotrypsin-like activity of proteasome complex from nucleus was determined fluorometrically by measuring the release of 7-amino-4-methyl-coumarin from the peptides succinyl-Leu-Leu-Val-Tyr-MCA (SUC-LLVY-MCA) at 380 nm excitation and 440 nm emission, respectively, (Hayashi and Goto, 1998) from the cytosolic and nuclear fraction of brain homogenates. The generation of antiserum against RC2 subunit of the proteasome complex was done according to the protocol described earlier by Hayashi and Goto in our laboratory (1998). Nucelar proteins were separated by SDS–PAGE using 4% stacking and 15% separation gels. After electrophoresis the proteins were transferred to PVDF membrane (Millipore, Bedford, MA) with 0.025 M Tris–HCl buffer (pH 8.3) containing 0.192 M glycine. The membrane was soaked in 5% dried milk in phosphate-buffered saline (PBS)-0.1% Tween 20 to block non-specific binding of the antibodies, incubated for 2 h at room temperature with the rabbit proteasome antiserum at a dilution of 1:5000 and then reacted with ^{125}I-Protein A (2 μCi). The membrane was then washed with PBS-0.1% Tween 20 and exposed to an imaging plate overnight. The immunological signals were visualized in a BAS 2000 Bioimaging Analyzer (Fuji Film Co., Japan).

2.7. Blue native gel

Mitochondria were prepared as described by Schagger, 1996. Briefly, brain was homogenized in buffer A (500 mM Tris–HCl (pH. 7.4), 0.25 M sucrose, 500 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride (PMSF) at a ratio of 25 μL/mg tissue. Homogenized samples were centrifuged at 20 000 × *g* for 20 min. Mitochondria-enriched pellets were dissolved in buffer 2 [1 M aminocaproic acid, 50 mM bis–Tris–HCl, 1 μg/mL pepstatin, 1 μg/mL leupeptin, 10 μL/mL PMSF, and 20 μm *N*-*p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), pH 7.0] at a ratio of 4 μL/mg tissue and freshly prepared 10% dodecyl maltoside was then added at 2 μL/mg tissue to dissolve membrane proteins. These homogenates were centrifuged at 100 000 × *g* for 15 min. The supernatants were collected and used for ETC enzymatic assays. Protein concentrations in the supernatants were determined by the BCA assay. The methods have been described in detail (Jung et al., 2000). The proteins were separated on a gradient gel (5–13% polyacrylamide) with a 4% polyacrylamide stacking gel. Then, micrograms of each sample were loaded onto the gels and then electrophoresed with 1.5 mA of constant current per gel at 4 °C. Finally, the gels were stained with Coomassie blue for protein content measurement by densitometry.

2.8. Statistical analysis

The statistical significance was assessed by ANOVA, followed by Tukey's posthoc test and Pearson's correlation. The significance level was set at $p < 0.05$.

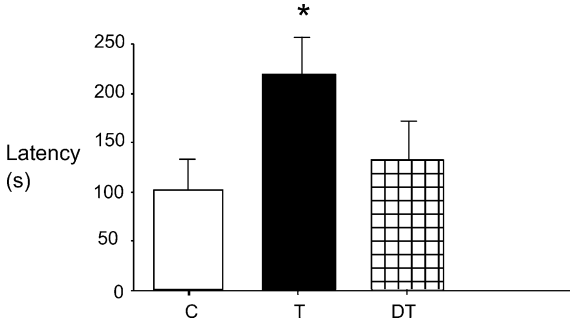

Fig. 1. On the second day of the passive avoidance test electric shock was delivered to the rats when they entered the dark chamber. The panel shows the latency time of control (C) trained (T) and detrained (DT) animals. Exercise increased the latency significantly, which could represent better memory. Values are mean ± S.D. for six animals per group. * $P < 0.05$ vs. control.

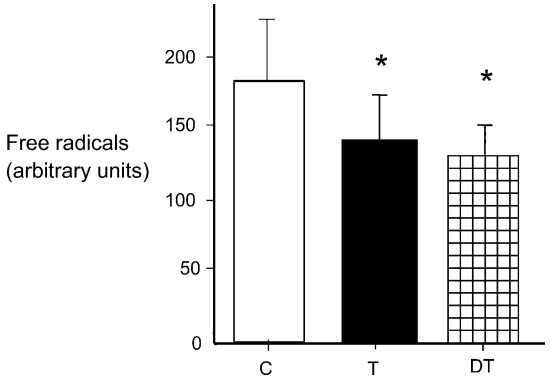

Fig. 2. The EPR data revealed that the free radical concentration was decreased by training and detraining in the cerebellum of the experimental animals. Values are mean ± S.D. for six animals per group. * $P < 0.05$ vs. control.

3. Results

The brain performance, assessed by a passive avoidance test, improved significantly with exercise training. But, after detraining, the control and detraining animals did not differ (Fig. 1). On the other hand, the level of free radical species in the cerebellum decreased as a result of exercise training and this beneficial effect was not eliminated, by detraining (Fig. 2). Since the activity of the DNA repair enzyme, OGG1, did not change, either in nucleus (Fig. 3) or mitochondria, with training or detraining (Fig. 4), the lower level of free radical production and unchanged OGG1 activity suggest better protection of nuclear and mitochondrial DNA. The proteasome activity and content were measurable in the nucleus, but were not altered by training or detraining (data are not shown), which suggests that significant modifications of proteins did not occur with training and/or detraining. Exercise training significantly increased the protein content of BDNF, compared to control animal results, while detraining resulted in a decreased level of BDNF, when compared to

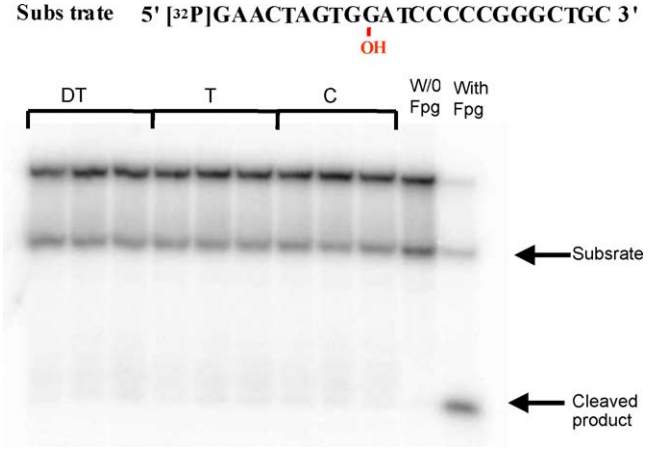

Fig. 3. The repair of nuclear 8-oxoguanonine was measured by the activity of OGG1, which was not altered by training or detraining. Representative data of three animals from each group is shown in the panel.

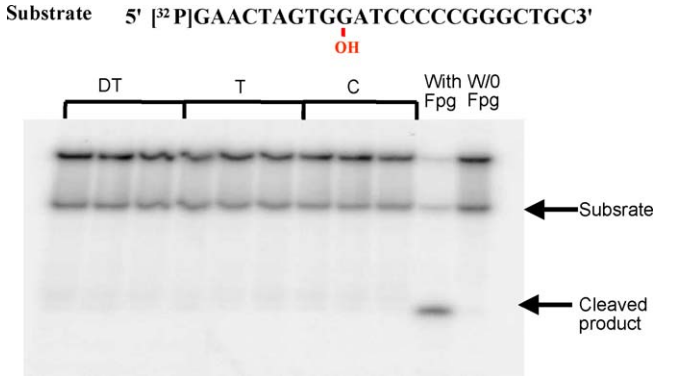

Fig. 4. The activity of OGG1 in the mitochondria was not significantly changed by training or detraining. Representative data of three animals from each group is shown in the panel.

sedentary rat data (Fig. 5). The protein level of NGF was not induced by exercise training, but decreased due to the detraining, suggesting adverse effect of sedentary life (Fig. 6). The blue native gel data revealed that exercise did not significantly alter the content of electron chain complexes (Fig. 7).

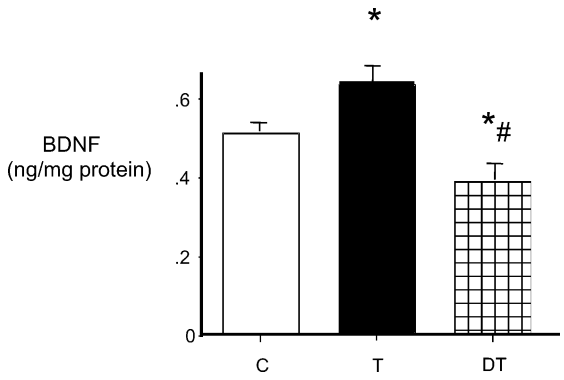

Fig. 5. Exercise training increased and detraining decreased the protein content of BDNF measured by ELISA method. Values are mean ± S.D. for six animals per group. * $P < 0.05$ vs. control. $^{\#}P < 0.05$ vs. trained.

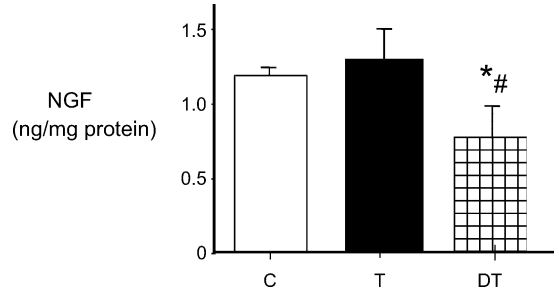

Fig. 6. The protein content of NGF was decreased as a result of detraining compared to control and trained rats. Values are mean ± S.D. for six animals per group. * $P < 0.05$ vs. control. $^{\#}P < 0.05$ vs. trained.

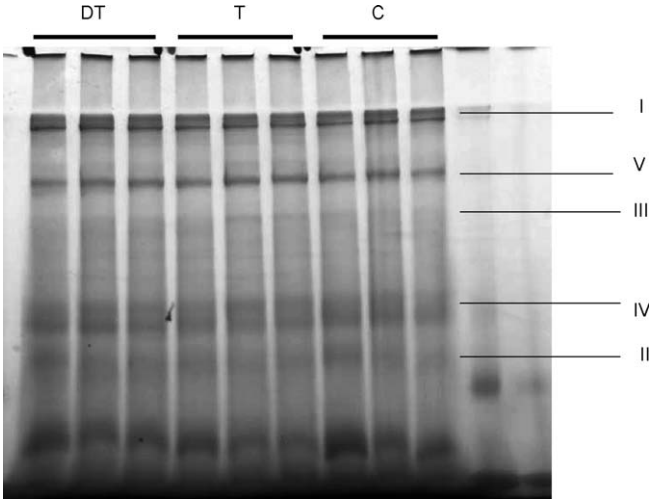

Fig. 7. The blue native gel data revealed that the content of mitochondrial electron transport chain complexes did not change by training or detraining. Representative data of three animals from each group are shown displayed. The numbers of complexes are on the right.

4. Discussion

It is well demonstrated that regular exercise has beneficial effects on brain function, including better memory (Radak et al., 2001a,b), increased capillarization (Fabel et al., 2003), brain plasticity (Cotman and Berchtold, 2002), proteasome activation and up-regulation of the antioxidant system (Radak et al., 2000). In the present study, we have shown, that some of these changes are reversible, such as an improvement in memory. Moreover, there is a negative re-bound in BDNF and NGF content after detraining. Reduction in BDNF and NGF concentration occurred when compared to control levels, while no significant differences were noted in the memory of control and detrained animals. This may indicate that the changes in memory are not entirely dependent on neurotrophin concentration, and therefore, other controlling factors cannot be ruled out. The concentration of free radicals, as assessed by ESR, suggests that exercise can reduce the free radical concentration and moreover, lower level of radicals as was observed in the detrained brain.

The induction of BDNF (NGF) could be strongly dependent on the activation of cyclic AMP response element binding protein (CREB), which has been shown to be very sensitive to redox state (El Jamali et al., 2004). In the current experimental conditions, it seems unlikely that free radical-associated induction of CREB played a significant role in the increased BDNF level caused by exercise. On the other hand, since an exercise- mediated increase in BDNF mRNA level was prevented by *L-N*-nitro-L-arginine-methyl ester, a nitric oxide synthase (NOS) inhibitor, this suggests that NO plays an important role in the regulation of BDNF. A recent study by Zheng et al. (2005) indicates that exercise up-regulates the mRNA expression of NOS in paraventricular nuclei. The results of the present study cannot elucidate, the mechanisms by which the BDNF and NGF levels were regulated.

ROS and the associated oxidative damage have been suggested as one of the possible regulating factors of brain function (Carney et al., 1991; Radak et al., 2001a,b). Oxidative damage can be a stimulating factor for the damage repair enzymes (Radak et al., 2005a,b). The integrity of cellular DNA has a vital importance for life. Base excision repair enzymes (BER) are specialized to remove the oxidatively-damaged bases. The first step of BER is executed by DNA glycosylases, which recognize and removes the damaged base. The 8-oxoguanonine is primary repaired by 8-oxoG-DNA glycosylase (OGG1), if it remains un-repaired it leads to increased mutation, hence jeopardizing the viability of the cells. To our knowledge, this is the first study, which measures the nuclear and mitochondrial activity of OGG1 after exercise. The activity did not change in either cell components, suggesting that exercise did not increase the concentration of DNA damage in nucleus and mitochondria. Marathon running has been shown to result in an increased activity of OGG1 in skeletal muscle (Radak et al., 2001a,b), and it has been demonstrated that regular exercise decreases DNA damage and up-regulates the activity of OGG1 in skeletal muscle of trained animals (Radak et al., 2002). These earlier data were obtained from crude cell extracts and therefore the site of increase remains unknown. The present data suggest that the DNA in nucleus and mitochondria of neuro cells in the brain is not affected by exercise training.

To our knowledge this is the first exercise-relating study in which, the nuclear activity of proteasome complex has been measured, although no alteration was found by exercise training or detraining. Altered protein degradation plays a role in the development of Alzeheimer's and Parkinson diseases (Mattson, 2000). Exercise has been show to increase the activity of proteasome and nephrilisin (enzyme involved in beta-amyloid degradation), which can significantly increase the degradation, and decrease the content of beta-amyloid (Lazarov et al., 2005). The degradation of damaged proteins in the cytosol is very important, but the activity of proteasome in the nucleus is also vital for cell survival. Here we show, for the first time, that the nuclear activity of proteaosme complex did not change with exercise training and de-training.

Mitochondrial electron chain complexes have been suggested to be an active contributors of free radical release. The blue native gel method allows the study of the protein content of mitochondrial complexes. This is one of the first investigations, which has applied the blue native gel method in an exercise study. However, no differences which were caused by exercise or detraining, in the protein content of electron transport complexes were detected. One should point out, that for the separation of nuclear and mitochondrial fractions the whole brain was used, which naturally contains neurons and other cells as well, not allowing to restrict the findings to the population of neurons.

The data from the present study suggest that exercise training increases the memory of rats, but this increase is temporary, since the memory fails with detraining. It appears that the exercise-induced improvement in memory is partially independent from the concentration of BDNF and NGF. Due to the decreased free radical concentration with exercise training, the repair or degradation of oxidative DNA and protein damage was unaffected, suggesting that training or detraining did not result in an oxidative challenge to the brain.

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