The role of regular physical activity on proteasome complex in traumatic brain injury

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Content

Abbreviations

ROS-Reactive oxygen species
BER-Base excision repair
OGG1 -8-oxoguanine DNA glycosylase
GPX-Gluthation peroxidase
SOD-Superoxide dismutase
CAT-Cathalase
RCD-Reactive carbonyl derivates
NMDA-N-methyl-D-aspartic acid
BDNF-Brain derived neurotrophic factor
CREB- cAMP response element binding
L-NAME- L-Nitro-Arginine Methyl Ester
IGF-1-Insulin like growth factor
VEGF-Vascular endothelial growth factor
UCP3- Uncoupling protein 3
FPI- Fluid percussion injury
NGF-Nerve growth factor
HRP- Horseradish peroxidase
EPR- Electron paramagnetic resonance
HB-Homogenization buffer
TPCK- *N-p*-tosyl-L-phenylalanine chloromethyl ketone
DTT- Dithioerythritol
NOS-Nitrogen monoxide sinthase
LTP-Long term potentiation

1. Introduction

1.1. ROS and oxidative damage and repair of the damage

Reactive oxygen species (ROS) are normal product of aerobic organisms. The redox state of the cell is reductive in nature at normal condition, and when the cell is stimulated with different kinds of stimuli there is a shift to oxidized state. Hence, ROS at low doses play an important role at physiological processes, such as signaling, transcriptional regulation, etc. ROS are generated by different sources in the cells and the mitochondrial electron transport chain is suggested to be one of the most significant ones. Cells are equipped with enzymes which readily generates ROS similar to NADPH oxidize, xanthine oxidize or cytochrome P450. It cannot be excluded that multiple redox-active flavoproteins all contribute a small portion to the overall production of oxidants under normal conditions. On the other hand, ROS at large concentrations could jeopardize the viability of the cells. Indeed, during oxidative stress, the increased production of ROS, including highly reactive hydroxyl radicals, overwhelm the capability of antioxidant systems and their scavenging activities on oxidatively modified molecules. One of the processes behind oxidative stress could be due to decreased efficiency of repair activities that lead to the accumulation of oxidative damage together with the processes mentioned earlier. ROS can modify oxidized lipids, proteins and DNA. Accumulation of oxidized proteins appears to occur at a much higher extent (i.e. on the order of 5–10% of total cellular proteins on average, Stadtman et al.1992) than that of oxidatively modified lipids (Miyazawa et al. 1990) or DNA (Richter 1988), which is far below 0.1% as a steady state level. DNA suffers from a wide range of oxidative modifications; more than 20 different alterations have been reported so far as a result of ROS attack on DNA (Radak and Goto 2000). This damage could have very serious consequences, such as mutation, cell arrest, apoptosis, necrosis, etc. Therefore, organisms developed a multi level DNA repair system to soften the effects of these oxidative DNA damages. Base excision repair (BER) is specialized to repair oxidative modifications on DNA. Guanine is one of the main targets of ROS, since it has the lowest redox potential, which means that it attracts ROS. A recent review suggests that oxidation of guanine can be also

regarded as a potential buffer to attenuate the consequences of DNA oxidation (Radak and Boldogh 2010). Indeed, 8-oxoG, which is the most well known product of DNA oxidation, is repaired efficiently by OGG1. The background/base level of 8-oxoG is estimated to be 1-2 per 10^6guanine residue in nuclear DNA and about 1-3 per 10^5 in mitochondrial DNA (Nakamoto et al. 2008). Recent studies have shown that 8-oxoG could be just an intermediate product of guanine oxidation. Hence, 8-oxoG, which has a reduction potential lower than that of guanine, is prone to undergo further oxidation upon exposure to various oxidants including peroxynitrite or singlet oxygen, producing lesions such as oxaluric acid, guanidinohydantoin (Gh/Ia), and spiroiminodihydantoin. Spiroiminodihydantoin could have massive mutagenic effects since it induces much higher levels of G·C → T·A transversions than 8-oxoG in addition to causing G·C → C·G transversions.

8-oxoG is excised from DNA by formamidopyrimidine-DNA glycosylase in *Escherichia coli* and by the homolog 8-oxoguanine DNA glycosylase (OGG1) or MutM in mammals. Human and mouse OGG1 sequences include Lys249 and aspartic acid residues at the active site. Human cDNA sequence analysis showed two kinds of mRNAs with open reading frames encode the 345 and 424 amino acid peptides of alpha and beta hOGG1, respectively. The two human forms of OGG1 have 316 identical amino acids and the N-terminus of both contains a mitochondrial targeting signal essential for mitochondrial localization. Data revealed that both forms are a result of alternative splicing, in addition to a sequence analysis which suggested that human OGG1contains typical housekeeping gene promoter. Human OGG1 is ubiquitously expressed in all kinds of tissues, though with significantly different levels. Probably due to the differences in amino acid sequences, alpha hOGG1 is almost exclusively present in nucleus, while the beta form is detected in the inner membrane of mitochondria. Human OGG1 recognizes and cleaves 8-oxoG with different efficiencies. The cleavage is the fastest with C in the opposite site of the substrate rather than T, and the lowest efficiency measured occurs when A is in the opposite site of 8-oxoG.

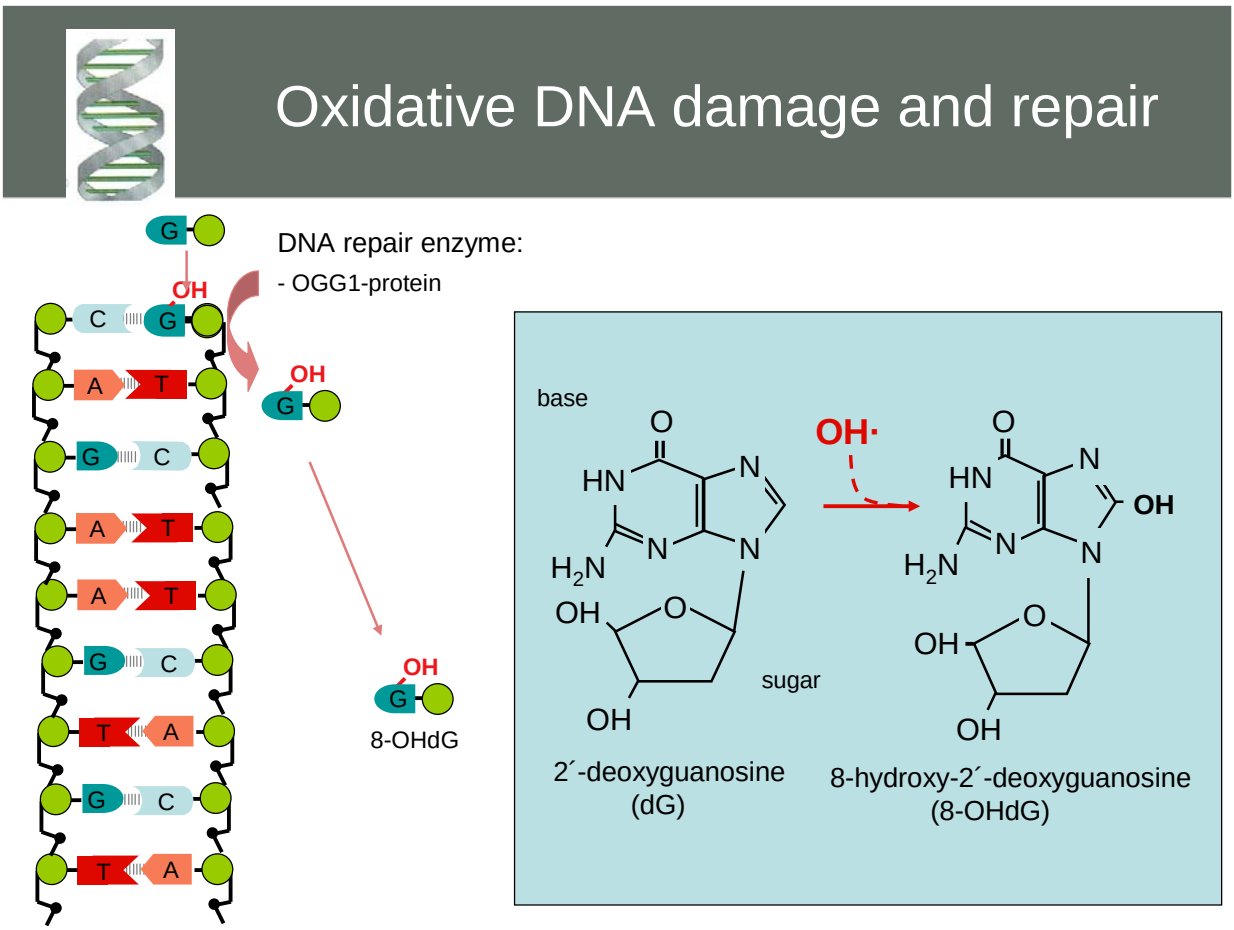

Fig 1. The figure shows the oxidation of guanine, and the repair process of 8-oxoG by OGG1. Guanine has a low redox potential, hence it attracts hydroxyl radical, which results in the generation of 8-oxoG. Then 8-oxoG is recognized and cleaved by OGG1 in mammalian systems. Unrepaired 8-oxoG can lead to transversion and cause mutation.

Structural analysis of OGG1 has revealed that upon DNA binding, which might be charge dependent, the enzyme undergoes a massive local structural conformational change to strengthen its affinity for C in the opposite strand. This creates a powerful bond allowing the removal of 8-oxoG from the DNA helix. The activity of OGG1 is readily modified by phosphorylation and acetylation. Mild oxidative stress, such as regular physical

exercise, has been shown to increase the activity of OGG1 in the crude cell extract of human and rat skeletal muscle (Radak et al. 2002).

Oxidative modifications of amino acid residues in antioxidant and repair enzymes would curb their biological activities resulting in further accumulation of oxidative damage (Levine and Stadtman 2001). Therefore, the degree of oxidative modifications of these enzymes is considered to have significant deteriorating consequences on cell function.

Indeed, ROS are modifying the structure of lipids, resulting in lipid peroxidation which has the potential to initiate significant change of permeability of biological membranes and cell death. ROS interacting with DNA and the oxidized bases could easily result in mutation, cell cycle arrest. In comparison of damages to different components of the cell, amino acid residues are the subjects of a larger degree of oxidative modification (Radak et al. 2002). All amino acid residues can be oxidized by ROS, although, the sensitivity varies significantly (Radak et al. 2002).Moreover, some oxidants-induced damage is limited and specific to certain residues; whereas, other species such as the hydroxyl radical, give rise to widespread, relatively non-specific damage (Davies 2005). Some other reactive species such as peroxides can induce further oxidation and chain reactions (within proteins and via damage transfer to other molecules) and stable products. The oxidative damage to cysteine and methionine residues, two amino acids which are highly susceptible to oxidation, can be repaired by various enzymatic systems that catalyze the reduction of cysteine disulfide bridge, cysteine-sulfenic and -sulfinic acids as well as methionine sulfoxide (Mary et al. 2004). However, most of the oxidative modification of amino acids leads to changes in hydrophobicity of the proteins (Stadtman 2001). This modification in hydrophobicity can serve as a special tag of the proteins, which can act as ubiquitiniation of proteins. Hence, severely oxidized proteins are marked for proteolytic degradation for the enzyme complex of proteasome.

1. 2. Proteasome

Proteasome is a huge proteinase-complex containing multiplate catalytic sites (Coux et al. 1996). Proteasomes are responsible for the degradation of most cytosolic proteins and specific regulatory proteins such as transcription factors associated proteins and cell regulators. There are two forms of the enzymes. A 20S proteasome possessing a molecular weight of approximately 700 kDa and consisting of seven different subunits and seven different beta subunits. A 26S proteasome is approximately 2,000 kDa and contains additional regulatory subunit, (19S regulatory subunit also called as the cap). There are at least five peptidase activities of this enzyme complex, and the major activities include trypsin-like, peptidylglutamyl peptide-hydrolyzing and chymotrypsin-like activities. Many, but not all proteins to be degraded by proteasomes, are marked by a covalent linkage with ubiquitin. While the proteasomes are involved in the degradation on cytoplasmic proteins in general, they have a prominent role in eliminating altered proteins such as oxidatively damaged, misfolded, or unassembled proteins that are potentially harmful to cells (Grune et al. 1997).

Degradation of Altered Proteins by Proteasomes

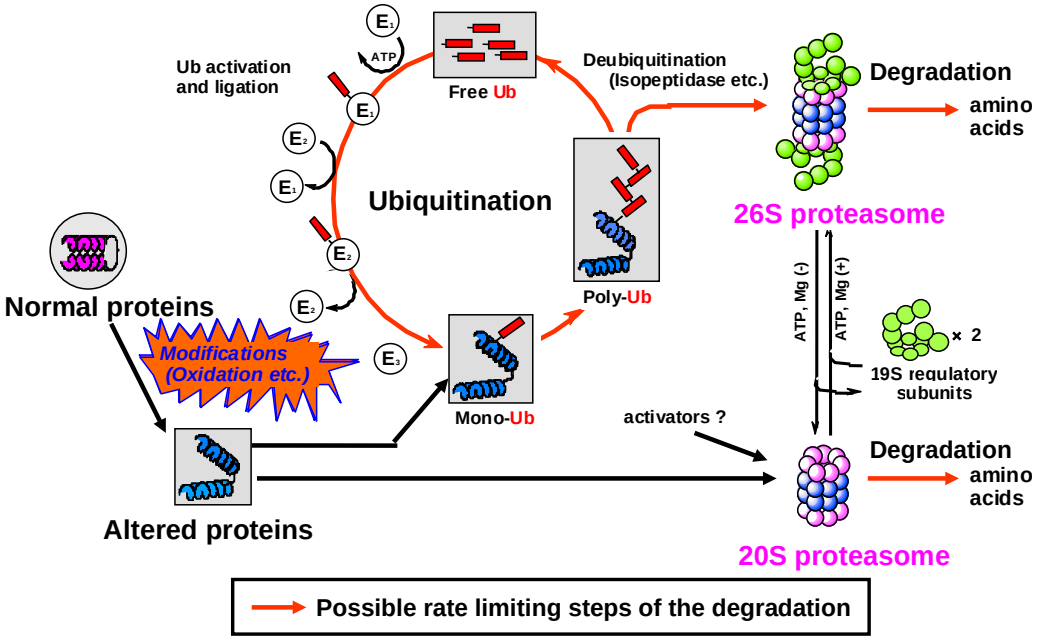

Fig 2. This figure shows the degradation of altered proteins. Incomplete assembly of proteins, or oxidative damage on proteins could cause either ubiquitination or changes in hydrophobicity, which serves as a tag for the degradation by the proteasome complex.

1.3. Exercise

There is accumulating evidence that exercise training affects the activity of proteasome enzyme complex. Nine weeks of swimming training increased the trypsin-like activity of proteasome in the brain of rats (Radak et al. 1999). In a related study, hydrogen peroxide was administered every other day to sedentary and exercise trained rats. Both hydrogen peroxide and physical exercise training increased the activity of proteasome complex in the myocardium of rats. Swimming training further up-regulated the effects of hydrogen peroxide administration on the activity of the proteasome complex (Radak et al. 2000). These finding suggest that moderate increase in ROS production can up-regulate proteasomal activity responsible for the removal of damaged proteins. Wakshlag and coworkers (2002) studied the effect of exercise on proteolytic capacity of skeletal muscle of dogs. The results indicated pronounced up-regulation of ubiqitinated conjugates and the p31 regulatory capping subunit during the period of high physical activity. A human study applying eccentric muscle action causing muscle damage revealed that eccentric exercise caused a decrease in calpain 3 mRNA immediately after the exercise, whereas the calpain 2 mRNA level increased at the day 1. In contrast, cathepsin B+L and proteasome enzyme activities were increased at the day 14 (Feasson et al. 2002). These findings nicely demonstrate that proteolytic processes occur in a selective manner and play an important role in muscle remodeling after injury. The role of proteasome complex in brain remodeling after injury is still unknown.

1.4. Exercise effects on Brain

Regular exercise is known to improve the physiological performance of skeletal and cardiac muscle and decrease the incidence of a wide range of diseases, including heart and vascular diseases, certain kind of cancer, diabetes II, etc. (Radak 2005). In the last decade, it became evident that regular exercise beneficially affects brain function, as well as having the potential to play an important preventive and therapeutic role in stroke, Alzheimer and Parkinson diseases (Matson 2006, Greisbach et al. 2004, Stummer et al. 1995). However, this point can be viewed differently, namely that physical inactivity has very serious consequences on the body, including the structure and function of the brain. In fact, human sapience is designed for physical activity and our DNA is constructed to hunting, gathering, fighting, escaping, etc. using our muscles. The genetic adaptation of DNA needs several thousand years, while our environment became very civilized at the last several decades. Therefore, our DNA is designed to stone-age and we are living within an age of technology. This discrepancy has serious consequences, including inactivity, which is "forced" on us by our modern environment. The theory of "use it or lose it" was developed more than 2500 years ago, and this is very much true for our brain. Since we have lost our natural physical activity (hunting, gathering, walking, fighting, physical work, etc) we use exercise to try to keep our body fit. Specifically, physical activity, namely, exercise, is the natural condition for our genetic heritage and physical inactivity is un-natural condition. In fact, it jeopardizes our health in addition to the physiological performance of our brain.

The effects of exercise and physical activity appear to be very complex and may include neurogenesis via neurotrophic factors, increased capillarization, decreased oxidative damage, and increased proteolytic degradation by proteasome and neprilysin (Cotman and Bertold 2002, Cotman and Engesser-Cesar 2002, Johnson and Mitchell 2003, Molteni et al. 2000, Neeper et al. 1995, Oliff et al. 1998, Lazarov et al. 2005). However, the effect of exercise on brain damage recovery and prevention is studied extensively; although the underlying mechanisms are not well known.

1.5 Exercise, antioxidants and oxidative damage

There are conflicting data on the effect of exercise on the activities of antioxidant enzymes. It has been suggested that, for instance, in the case of DNA, the damage can be reduced from 109 to 106 in a daily base/cell as a result of the antioxidant scavenging system (Beckmand and Ames 1998).

The findings of an early study suggested that exercise, such as voluntary running, results in oxidative damage to low vitamin E fed animals (Suzuki 1983). Swimming- exposed rats suffered significant increase in lipid peroxidation, and increased gluthatione peroxidase (GPX) activity (Hara et al 1997), while 6-hydroxymelatonin supplementation prevented oxidative lipid damage. On the other hand, it has been noted that the activities of antioxidant enzymes were dependent on brain region, and the effects of exercise were also dependent on the brain portion (Somani et al., 1995). In certain brain parts such as the brain stem and corpus striatum, exercise training resulted in increased activities of superoxide dismutase (SOD), and glutathione peroxidase (GPX) (Somai et al. 1995,). Simultaneously, it was reported that a single bout of exercise, which caused oxidative damage to skeletal muscle (Radak et al. 1995), liver and kidney (Radak et al. 1996), did not cause damage to the brain (Radak et al. 1995). Further, the activities of antioxidant enzymes (Cu,Zn-SOD, Mn-SOD, catalase (CAT), GPX) were not significantly altered by an exercise session. A similar phenomenon has been reported after exercise training. Treadmill running did not alter the activities of SOD, CAT, or GPX in the brain of rats. However, exercised rats with diabetes have shown decreased Cu, Zn-SOD and GPX activities (Özkaya et al. 2002).

The available information on brain antioxidant status for exercise suggests that exercise training selectively regulates the activity of antioxidant enzymes in different brain regions. The activity response of antioxidant enzymes in the brain, with exercise, is probably dependent on the type of physical activity, the intensity and duration of exercise training, and the age, sex, and strain of rats.

The first (age-related) study outlined the relationship between the accumulation of oxidative damage to proteins, reactive carbonyl derivative (RCD), and certain brain functions (Carney et al. 1991). A spin trapping agent of PBN was administered for two

weeks to aged and young gerbils. Following this period, the activities of glutamine synthase and proteasome increased while the level of RCD decreased significantly. These changes were accompanied by improved brain function, as measured by the Morris maze test. Although the findings of this study were questioned at the time by Cao and Cuttler (1995a, b), the results of the original study were later confirmed by other laboratories (Foster et al. 1996).

Liu et al. (1996) immobilized rats overnight, which resulted in increased oxidative damage of lipids, proteins and DNA in the brain of animals. We applied the same immobilizing method and measured brain function two hours after immobilization using the passive avoidance test and found performance to be impaired (Radak et al., 2001A). We then added groups, which were exposed to a single bout of exhaustive swimming or swimming after immobilization. The oxidative damage of macromolecules increased as a result of immobilization, in accordance with Liu and co-workers, and revealed that exercise after immobilization appeared to decrease damage.

Oxidative damage has been associated with poor physiological function of the brain. We have also shown that regular exercise training attenuated the age-related accumulation of RCD in the brain, increased the activity of proteasome complex, and improved brain function (Radak et al. 2001B). Chronic exercise training, using the rat model, did not cause significant alteration of lipid peroxidation levels in the brain. On the other hand, the supplementation of vitamin C elevated the oxidative damage of lipids (Cuskun et al. 2005). Ogonovszky et al. (2005) subjected rats to moderate-, very hard- and over-training, and found beneficial effects on brain function and lowered accumulation of RCD, even with very hard training and over-training. The content of 8-oxoG was not significantly altered by the overtraining protocol, and activity of OGG1 (this enzyme specifically targets the 8-oxoG modification on the DNA) was also not changed in the crude cell extract (Ogonovszky et al. 2005). We recently evaluated the activity of the DNA damage/ repair enzyme of OGG1 in the nucleus and mitochondria of trained and detrained rats, and did not detect any significant alterations (Radak et al. 2006).

The findings of several studies indicate that regular exercise acts as a pre-conditioner against oxidative stress. Hence, trained rats suffered less damage during stroke or other oxidative stress- associated challenges (Ding et al. 2006). We attempted to mimic some

characteristics of Alzheimer disease, i.e. causing lesions by the injection of NMDA into the brain (Toldy et al. 2005). Exercise and nettle supplementation significantly reduced the ROS content, decreased oxidative protein damage and modulated the activity of redox sensitive transcription factors (Toldy et al. 2006, Toldy et al. 2009). Data indicated that the accumulation of oxidative damage impairs brain function, and exercise, under certain conditions, can attenuate the accumulation of damage causing a decline in function.

1.6. Neurotrophins, trophic factors and physiological function

Brain-derived neurotrphic factor (BDNF) is one of the most versatile and important neurotrophic factors in the brain. It plays a curricular role in the learning process, memory, locomotion, behavior, and a wide range of stress responses (Barde 1989). It has been suggested that BDNF regulates brain development, neuroplasticity, neurogenesis, neurite outgrowth, synaptic plasticity, and cell survival (van Praag et al. 1999). The expression and protein content of BDNF have been shown to be up-regulated by exercise, and oxidative stress (Matson et al. 2002). Exercise does not simply up-regulate the content and expression of BDNF in different brain regions, but also impacts downstream effectors of BDNF, namely the transcription factor cAMP response element binding protein (CREB). DNA binding of CREB does not directly translate to gene transcription but activates inducible transcription factors, such as NF-kB, cFos, and Jun and this trans-activation causes persistent expression of genes. CREB DNA binding sites contribute the activation of mRNA of BDNF transcription and this process can be regulated by ROS. It was recently reported that glutamate neurotoxicity and treatment with hydrogen peroxide decreased the DNA binding of CREB and increased the DNA binding of NF-kB (Zou and Crews 2006). Moreover, it appears that BDNF acts through TrkB receptors that activate CREB, thus creating a positive loop for the cascades (Zou and Crews 2006). Exercise, which enhances the content of BDNF, and TrkB, activates CREB and increases the expression of BDNF to make the neurons more resistant to oxidative stress, probably by the alteration of redox state in the neurons. On the other hand, when BDNF was blocked, the exercise-induced increase in CREB mRNA levels, as well as the phosphorilation of CREB, were prevented (Vaynman et al. 2004a,b). It has been shown that ROS stimulate the expression of BDNF, at least in cell culture, and antioxidants prevent this increase (Wang et al. 2006). Relatively short exposure (6 hr) of neurons to ROS resulted in activation of CREB, while a longer exposure (24 hrs) suppressed the protein content and mRNA levels of CREB (Pugazhenthi et al. 2003). In some brain regions, exercise training increases the level of ROS, although the level of oxidative damage does not increase (Ogonovszky et al. 2005, Toldy et al. 2006, Szabo et al. 2009, Siamilis et al. 2009).

In addition to ROS, nitric oxide might also act as a modulator of exercise-induced changes in BDNF levels. L-NAME, non-selective nitric oxide synthase inhibitor administration has been shown to decrease the activation of CREB (Park et al. 2004,), and the exercise-induced BDNF mRNA expression seems to be related to nitric oxide production. On the other hand, we could not detect increased nNOS protein content in the brain of exercise- trained and caloric restricted animals (Szabo et al. unpublished). Thus, exact regulation pathways by which exercise increases the content and expression of BDNF and CREB are vague, but it appears that the redox homeostasis could play a curricular role in the regulation process.

Among the other trophic factors elevated by exercise include the insulin-like growth factor (IGF-1), and vascular endothelial growth factor (VEGF). It is well established that exercise increases neurogenesis and this is one of the processes by which exercise benefits brain function (van Praag 1998). It has been suggested that BDNF is one of the major regulators of neurogenesis. However, the findings of a recent paper indicate that VEGF is also heavily involved in neurogenesis (Fabel et al. 2003, Ding et al. 2004). The exercise effects seem to be dependent on the dose of exercise on VEGF content and mRNA expression (Ding et al. 2004). Recent reports suggest that ROS play an important role in angiogenesis; however, its underlying molecular mechanisms remain unknown. But it is known that VEGF induces angiogenesis by stimulating endothelial cell proliferation and migration (Ushio-Fukai and Alexander 2004). Therefore, it seems that exercise training could result in better oxygen and fuel supply to the brain.

IGF-1 is essential for nerve growth, neurotransmitter synthesis and release (Anlar et al. 1999), and it is believed to be functionally associated with the action of BDNF (Ding et al. 2006). IGF-1 may protect from hyperglycemia-induced oxidative stress and neuronal injuries by regulating MMP, possibly by the involvement of UCP3 (Gustafsson et al. 2004). The main functional effects of IGF-1 are not dependent on redox homeostasis, however, observations indicate that IGF-1 could act as a regulator of oxidative challenge. Exercise is a very potent modulator of certain neutroptrophins and these agents could be significantly involved in the beneficial effects of exercise on the function of the nervous

system. Moreover, exercise-induced alteration of redox balance might be delicately engaged in some of the regulatory pathways.

1.7. Fluid percussion injury (FPI)

Head injuries are one of the most frequent cause of death for young adults. Around the world high percentage of these chronically disabled individuals become dependent on care due to their head injury. It is suggested that after severe head injury 70% of the patients die and 10-20% remain dependent (Bullock et al.1990). About 40% of those who die as a consequence of traumatic brain injury were able to speak at a certain extent after the injury/impact, which suggest that the second stage of the injury occurs when the patients are under medical treatment. Therefore, experimental animal models were created to learn more about the detailed molecular consequences of traumatic brain injury. Other fluid percussion injury (FPI) are widely used and this model fluid percussion model produce brain injury by rapid injecting fluid volumes into cranial cavity. This model of brain trauma generates both focal and diffuse injury which includes a focal cerebral contusion and subarachnoid hemorrhage at the site of impact as well as more diffuse neuronal loss within the ipsilateral hippocampus, thalamus, striatum, amygdala, and medial septum. A distinct pattern of cerebellar damage has been observed following FPI. Lateral FPI, the one which was used in our study, to the frontoparietal cortex, resulted in Purkinje cell loss within the cerebellar vermis beginning 1 day post-injury and continuing through 7 days post-injury (Fukuda et al. 1996). This finding was associated with microglial activation seen in a distinct pattern characterized by “stripes” of microglia extending from the Purkinje cell layer outward to the cerebellar cortex. The results suggest that Purkinje cell death may not be mediated through activation of climbing fibers from the inferior olive. Further studies revealed activation of the stress protein heme oxygenase-1 within Bergmann glia in a similar “striped” distribution within the cerebellar vermis (Fukuda et al. 1996), as well as evidence of Purkinje cell degeneration through 14 days post-injury (Sato et al. 2001). Heme oxygenase-1 expression in the cerebellum mirrored the pattern of Purkinje cell loss, suggesting an association. These findings demonstrate prolonged glial induction of HO-1 in the traumatized brain. Such a response may reflect a protective role of these cells against secondary insults including oxidative stress. The existence of oxidative stress following FPI was demonstrated by the induction of the pyruvate dehydrogenese complex and the

related oxidative stress (Sharma et al. 2009). Oxidative stress is readily measured by the accumulation of oxidative damage, which can cause loss of brain function (Radak et al. 2001), and the severe stress leads to cell death. We have demonstrated that oxidative modifications of proteins have larger impacts on the brain function than oxidative alteration of lipids or DNA (Radak et al. 2001). Although, it is known that FPI causes oxidative damage, there are limited investigations aimed at studying FPI-induced oxidative stress. As it mentioned before, a functional view on the oxidation of proteins and the degradation of oxidized proteins by proteasome complex could be an important player in FPI-induced oxidative stress and recovery. Generally, it is interesting to note that proteasome is very important to remodeling after injury. Up to date, according to our knowledge, there is no investigation on the possible involvement of proteasome complex in FPI-induced oxidative stress and stress recovery.

2. Aims

In our investigations we wanted to see how the regular physical activity influences the brain functions, with a special focus on the role of proteasome system.
We used two kinds of models to study the role of exercise on proteasome system, which occur in healthy/normal conditions and in damaged circumstances.
In our first model, we used healthy animals and the applied exercise program was swimming training, where we expected better performance in behavior test and changed protein turn over in memory related proteins.

1. In our study we asked the question whether that the effects of exercise on cognitive function, free radicals generation and neurotrophins could be reversible.
2. We wanted to explore whether exercise could attenuate the level of oxidative stress, and detraining could result in enhanced degree of protein and DNA damage in the brain.
3. Our aims was to illustrate that the activity of 8-oxoG repair enzyme, OGG1 is induced by exercise.

Our second model was done to study the effects of voluntary exercise on FPI model in order to get more information about the possible molecular mechanisms that take place in this experimental model. This model was developed to mimic the traumatic brain injury that often occurs as a result of accidents, which kills many individuals in every year. Therefore, the present investigation was carry out in order to study the molecular mechanisms involved in traumatic brain injury, and find some acting point to facilitate the treatment of traumatic brain injuries, with special focus on the following issues:

4. We wanted to evaluate whether voluntary exercise could attenuate the damage caused by traumatic brain injury.
5. Since traumatic brain injury results is oxidative damage, we wanted to examine whether the proteasome complex would play an important role in the recovery process.
6. We wanted explore whether early gene expression of Zif268 could change in concert to proteasome in FPI model.

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3. Methods

Model "A", to study the effects of regular swimming training in brain function and molecular mechanism including the role of proteasome system.

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 the three groups: control (C), exercise trained (ET) and detrained (DT) group. 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 it was increased to 120 minutes daily for 5 days. Swimming was selected because no electric shock was required to promote this exercise protocol, meaning the stimuli of exercise would not interfere with the stimuli used during passive avoidance test. After the 8 week exercise training the DT group was kept as the control group for another 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.

Retention of passive avoidance test

The passive avoidance behavior was investigated in a one-trial step-through paradigm. The measurement was done as described earlier (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 minute 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).

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 4ºC. 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 mouse tissue. For both the standards and the samples, 100 µl was added to each well in duplicate, and incubated for 6h (NGF) or 2h (BDNF) at room temperature. The wells were then incubated with a secondary monoclonal anti-NGF (overnight at 4ºC) or antihuman BDNF polyclonal antibody (1h at room temperature). Next, the wells were incubated with anti-rat IgG (NGF) or anti-IgY (BDNF) conjugated to HRP for 2.5h (NGF) or 1h (BDNF) at room temperature. A TMB solution was utilized to develop color in the wells for 10 minutes at room temperature. The reaction was stopped with the addition of 1N HCl to the wells. The absorbance was read at A45 (Molecular Devices ThermoMax microplate reader, with SOFTmax PRO v3.1)

Electron paramagnetic resonance

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

Excision assay

The 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, a (1000g for 10 min at 4ºC), the pellet was re-suspended in HB and centrifuged again. Then after the pellet was re-suspended in HB with 0.5% NP40 and centrifuged. Then 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 was aliquoted and stored at -80ºC. The protein levels were measured by the BCA method. For the separation of mitochondria the supernatant of the first centrifugation was centrifuged at 14 000g for 30 min at 4ºC. Then the sup was re-suspended in HB and re-centrifuged three times. After dissolving the pellet in 0.5 ml HB, the final pellet was suspended in HB containing 0.5% Triton X –100, and was kept on ice for 20 min. The protein levels were measured by the BCA method. The assay was carried out according to the protocol described (Radak et al. 2002) 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 (2μg) 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 30ºC for 5-15 min and stopped by placing the solution in ice. Next, 30 μl chloroform was 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 minutes of heating at 95ºC, samples were chilled and loaded into polyacrylamide gel (20%) with 7 M urea and 1 x 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. The activity to repair 8-oxodG was determined and expressed as a percentage of substrate cleaved.

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 emissions, respectively (Hayashi and Goto 1998) from the cytosolic and nuclear fraction of brain homogenates. Blue native gel mitochondria were prepared as described by earlier. Briefly, the brain was homogenized in buffer A (500 mM Tris-HCl (pH. 7.4), 0.25 m sucrose, 500mm 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 minutes. 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 minute. 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 was 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 densitometer.

Model "B" to assess the role of proteasome system and the related molecular pathways in FPI with and without voluntary exercise.

Animals

A total of 33 male Sprague–Dawley adult rats (250–300 g) were utilized in these experiments. Rats underwent lateral fluid percussion injury (FPI; *n*=14) or sham injury (*n*=16) and were housed with or without access to a running wheel from post injury day 0 to 14. All animals were continually monitored and cared for by an IACUC-approved veterinary care staff upon arrival at UCLA. During the experiments, rats were single housed in opaque plastic bins (20×10×10 in.) lined with bedding materials. Each animal was checked for weight loss, loss of stereospecific behavior, and any changes in coat color. All procedures were approved by the UCLA Chancellor's Animal Research Committee.

Voluntary running wheel exercise

Rats were individually caged with or without access to a running wheel (RW) from post injury day 0 to 14 [Sham-RW (*n*=8) or FPI-RW (*n*=8)]. This post injury period was selected given that it has previously been associated with behavioral deficits. Exercising animals were placed in standard cages equipped with running wheels (diameter=31.8 cm, WIDTH=10 cm; Nalge Nunc International) that rotated against a resistance of 100 g. Wheel revolutions were recorded using an appropriate software (VitalViewer Data Acquisition System; Mini Mitter, Sunriver, OR). Sedentary animals (Sed) were left undisturbed in their home cages [Sham-Sed (*n*=8) or FPI-Sed (*n*=6)]. All rats had ad lib access to food and water and were maintained on a 12/12-h light–dark cycle. The mean number of revolutions was calculated each night, given that this was the most active period.

Fluid percussion injury

Lateral fluid percussion injury has been done accordingly to the method described previously (Griesbach et al.2004). In brief, rats were initially anesthetized with 4% isofluorane (in 100% O_2) and were then maintained to 1.5–2% isofluorane (in 100% O_2). The head was secured in a stereotactic frame, shaved and prepped with betadine and

ethanol. Body temperature was monitored and maintained at 37°C with a heating pad (Braintree Scientific; Braintree, MA). A midline sagittal incision was made. With the aid of a microscope (Wild; Heerburg, Switzerland), a 3-mm diameter craniotomy was made with a high-speed drill (Dremel; Racine, WI), 3-mm posterior to bregma and 6-mm lateral to the midline, on the left side. A plastic injury cap was placed over the craniotomy with silicone adhesive, cyanoacrylate, and dental cement. When the dental cement hardened, the cap was filled with 0.9% saline solution. Anesthesia was discontinued and the animal was removed from the stereotaxis device. The injury cap was attached to the fluid percussion device. At the first sign of hind-limb withdrawal to a paw pinch, a mild fluid percussion pulse (1.5 atm) was administered. Apnea times were determined as the time from injury to the moment of spontaneous breathing. Time of unconsciousness was operationally defined as the time from the injury to the return of a hind-limb withdrawal reflex. Sham animals underwent an identical preparation with the exception of the FPI. Immediately upon responding to a paw pinch, anesthesia was restored, the injury cap removed, and the scalp was sutured. Neomycin was applied on the suture and the rat was placed in a heated recovery chamber for approximately 1 hour before returning to its cage (Fig 3).

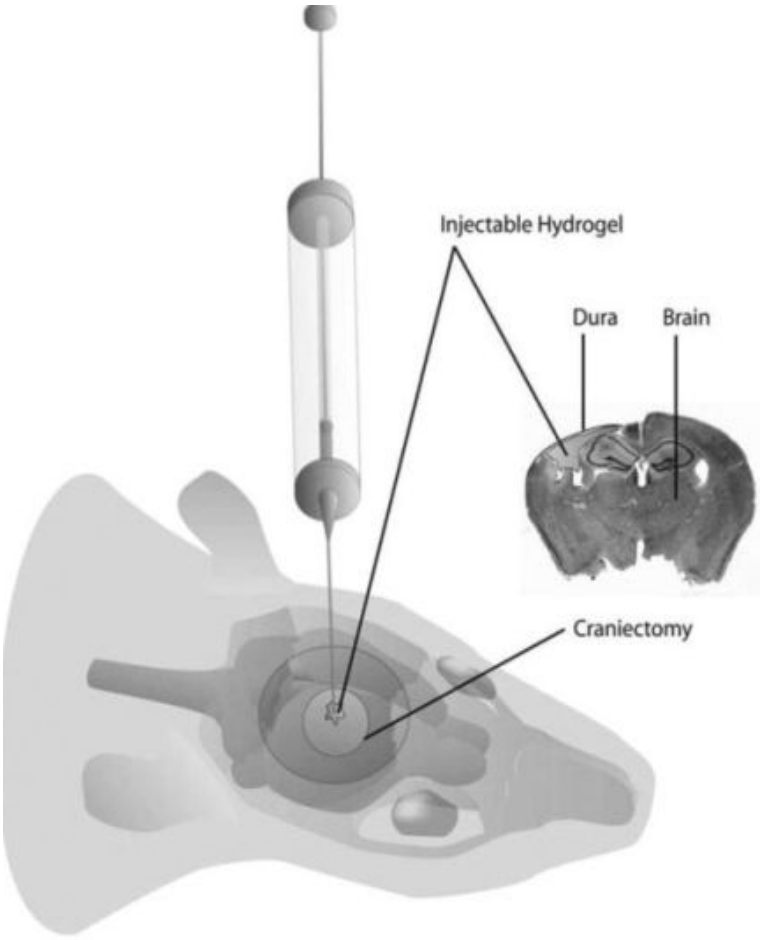


Fig. 3 The figure shows the method how FPI was induced.

Proteasome Activity measurement

The chymotripsin-like activity of proteasome was measured as described earlier (Hayashi and Goto 1998). In brief, caudal cortex was homogenized (100mg) in a 10x volume lysis buffer containing 10mM Tris, 0.25M Sucrose, 1.5 mM MgCl2, 1 mM DTT, 10% Glycerol, 10 KCl, 5mM ATP, complete solution should be around 7.3-7.5. The homogenates were centrifuged, the supernatants were collected and total protein concentration was determined by Micro BCA procedure (Pierce, Rockford, IL) using bovine serum albumin as a standard. The samples were diluted to same concentration (1mg/ml). For the assay, we mixed 5x reaction buffer (500mM Tris-HCl pH 8.0, 5 mM DTT, 25 mM MgCl2), homogenization buffer, BSA, SDS, sample, and substrate (Suc-leu-Leu-Val-Tyr-AMC) to measure cymotripsin like activity of proteasome complex. Incubated the samples at 37 C for 30 min, blocked the reaction with ice-cold methanol, centrifuged the samples at 10,000 g for 5 min we took the supernatant and mixed with Tris-HCl buffer (pH 9.0) and we read the fluorescent intensity at Ex: 320nm Em: 460nm. The activity was calculated as described previously (Hayashi and Goto 1998).

Protein Measurements by western blotting

Synapsin I, Zif 268, 20S alfa proteins were analyzed by Western blot as previously described (Griesbach et al.2004). Membranes were incubated with the following primary antibodies: anti-synapsin I (1:2000; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-total Zif 268 (1:2000; Cell Signaling Technology, Inc., Beverly, MA, USA), anti-20 alfa subunit of proteasome (1:2000; Cell Signaling Technology, Inc.), Biotechnology), and anti-actin (1:2000; Santa Cruz Biotechnology) followed by anti-goat IgG horseradish peroxidase conjugate for synapsin I, and actin.

Statistical analysis

At the model A.

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

At the model B.

An analysis of variance (ANOVA) with repeated measures was used. A Fischer-test was used for cross group comparisons. Results were expressed as the mean percent of control values for graphic clarity and represent the mean ± standard error of the mean (SEM).

4. Results

Model A.

The brain performance, assessed by passive avoidance test, was improved significantly by exercise training, but after de-training, the control and detraining animals showed similar results (Fig. 4).

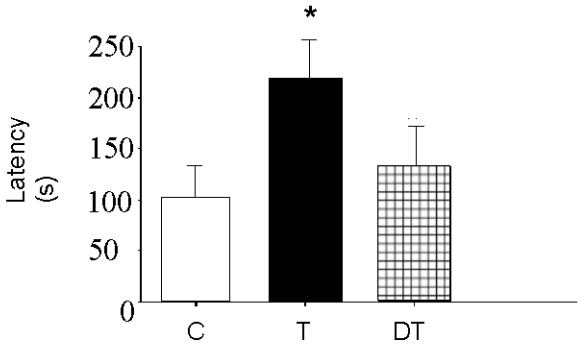


Fig. 4. The memory of test of the rats showed that exercise training (T) resulted in improved function compared to control © and detrained (DT) animals. Data was expressed as mean +/- SD. (P<0.05, n=6).

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 5).

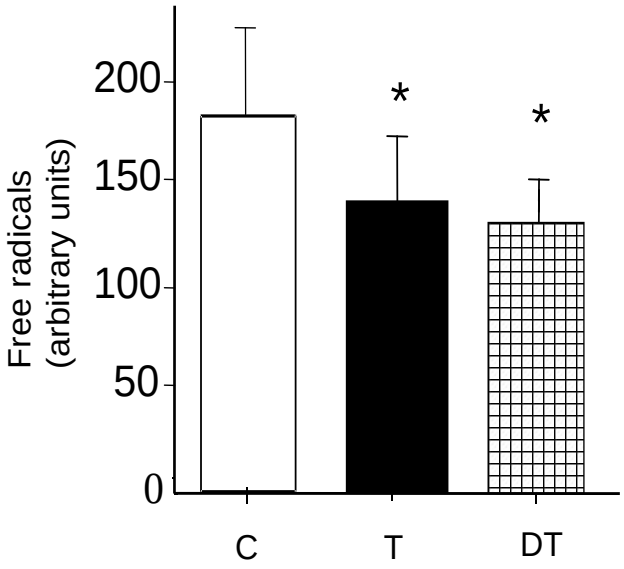


Fig. 5. The evaluation of EPR data is shown on this figure, which revealed that both exercise training and detraining decreased the level of ROS. Data were expressed as mean +/- SD. (P<0.05, n=6).

Since, the activity of DNA repair enzyme, OGG1, did not change either in nucleus or in mitochondria by training or detraining (Fig. 6), lower level of free radicals production and unchanged OGG1 activity suggest better protection of nuclear and mitochondrial DNA. OGG1 is the major enzyme, which cleaves 8-oxoG from the helix in order to minimize the risk of mutation is often caused by 8-oxoG.

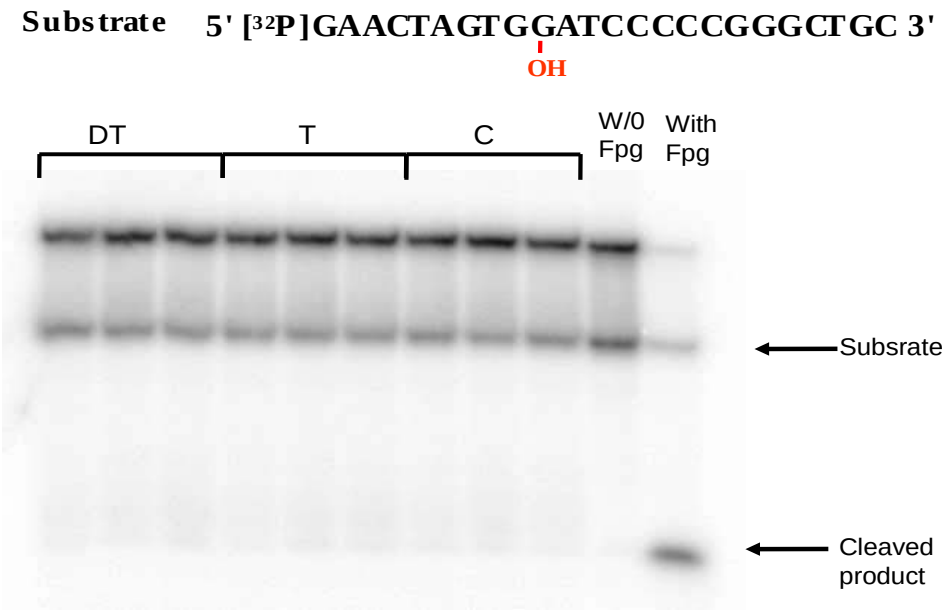


Fig. 6. The activity of nuclear OGG1 is displayed in this graph. Significant alteration was not observed. The lane marked as "with Fpg was loaded with the bacterial form of OGG1, called Fpg. The lane without Fpg is blank with enzyme or tissue samples. C, T, or DT lanes were loaded with nuclear extracts gained from control ©, trained (T) or detrained animals (DT). Representative data are shown. Data were expressed as a mean +/- SD. (P<0.05, n=6).

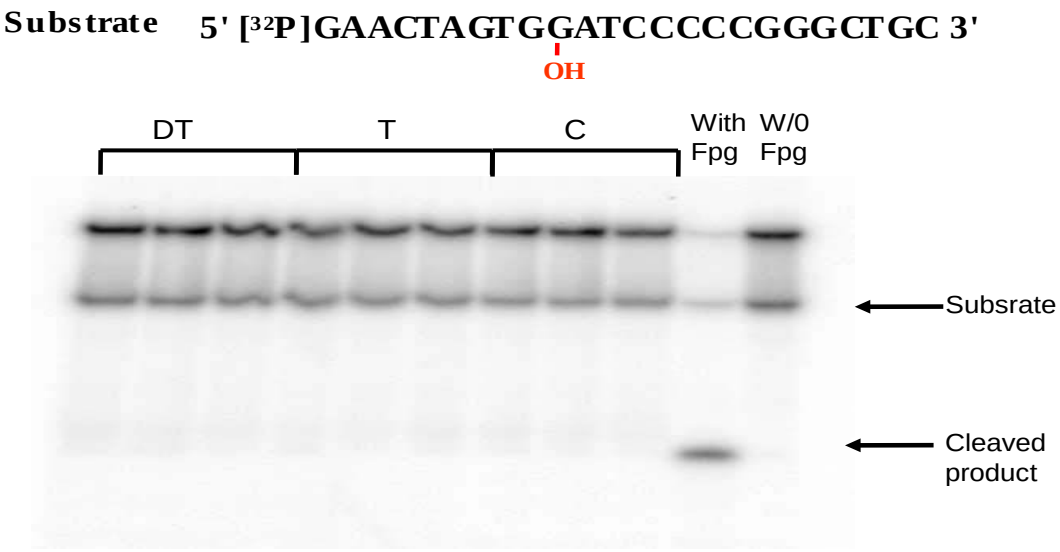


Fig. 7. The activity mitochondrial OGG1 is displayed in this graph. Significant alteration was not observed. The lane marked as "with Fpg) was loaded with the bacterial form of OGG1, called Fpg. The lane "w/0 Fpg is blank with enzyme or tissue samples. C, T, or DT lanes were loaded with mitochondrial extracts gained from control ©, trained (T) or detrained animals (DT). Representative data are shown. Data was expressed as a mean +/- SD. (P<0.05, n=6).

The proteasome activity and content were measurable in nucleus, but none of them was altered by training or detraining (Fig. 8). Proteasome is the major enzyme complex responsible for the degradation of oxidatively altered proteins, and our current observation reveals that exercise or detraining do not impact the activity and content of this enzyme complex.

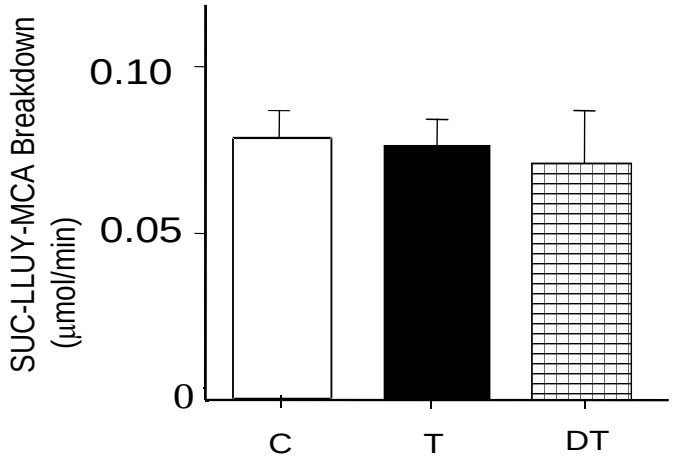


Fig. 8. The activity of proteasome is shown in this graph. The activity of proteasome was measured as described in the method section by fluorometric method using synthetic probe. Significant alteration was not observed. Data was expressed as a mean +/- SD. (P<0.05, n=6).

Finally, training significantly increased the protein content of BDNF compared to control animals, while detraining resulted in significantly decreased level of BDNF compared to sedentary rats (Fig. 9). BDNF is a powerful neurotrophin, which is implicated in neurogenesis, cell survival, memory, and brain plasticity. Here we confirmed that exercise is a stimulating factor for BDNF and our date revealed that this process is reversible, since detraining decreased the protein concentration of BDNF.

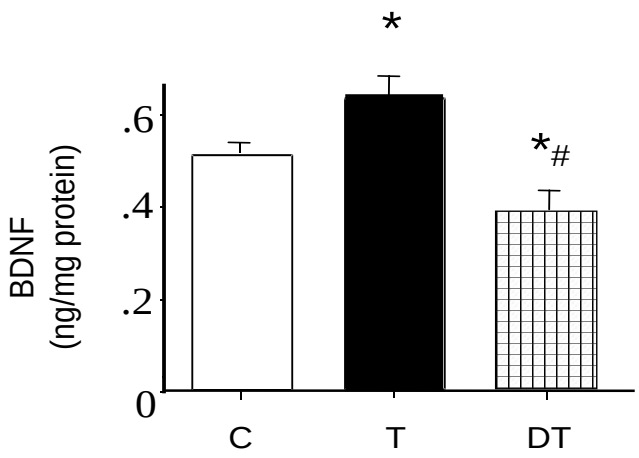


Fig. 9. The level of BDNF was measured by ELISA and the data showed that exercise training increases (T) and detraining (DT) decreases the amount of BDNF protein compared to control value. Data were expressed as mean +/- SD. (P<0.05, n=6).

The protein level of NGF was not induced by exercise training, but decreased due to the detraining which suggested adverse effects of sedentary life (Fig. 10). Detraining resulted in decreased level of NGF and BDNF, compared to control levels, suggesting that the termination of exercise training could have some negative effects on brain, at least in a short term.

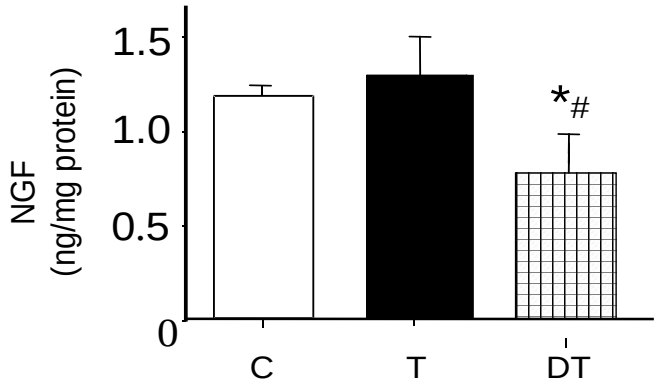


Fig. 10. The level of NGF was measured by ELISA and the data show that exercise training did not affect the amount (T), while detraining (DT) decreased the concentration of NGF compared to control value. Data were expressed as mean +/- SD. (P<0.05, n=6).

The protein concentration of mitochondrial electron chain complexes was evaluated by blue native gel method. This is was of the first study according to our knowledge, which used this method in exercise study, and there is a lack of information from brain samples in general as well. This method does not use any powerful detergents, such as SDS, hence proteins are functional even after the separation with electrophoreses. Our observation shows that training or detraining did not significantly alter the protein levels of the complexes (Fig. 11).

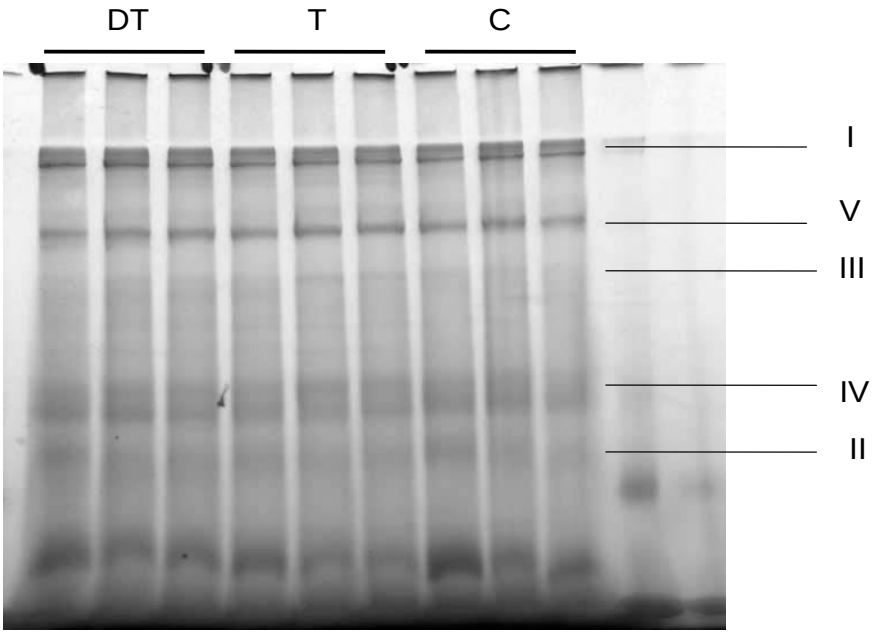


Fig.11. The protein levels of mitochondrial electron transport complexes were assessed by blue-native gel. Significant training or detraining mediated alteration was not found. Representative data is shown. Data was expressed as a mean +/- SD. (P<0.05, n=6).

Model B.

Our data suggests that FPI induces oxidative stress, since the carbonyl concentration increased significantly in caudal cortex of these animals in comparison to the control (Fig. 12). On the other hand, voluntary exercise decreased the accumulation of carbonyl groups in both sham and FPI groups, suggesting either an increased resistance or increased repair of oxidative protein damage. Accumulation of carbonyl groups in the amino acid residues of proteins is used as a marker for oxidative stress, as a result of its generation by the interaction of hydrogen peroxide, iron and amino acid residues. Therefore, carbonyl level of proteins is used as a marker for oxidative stress on proteins.

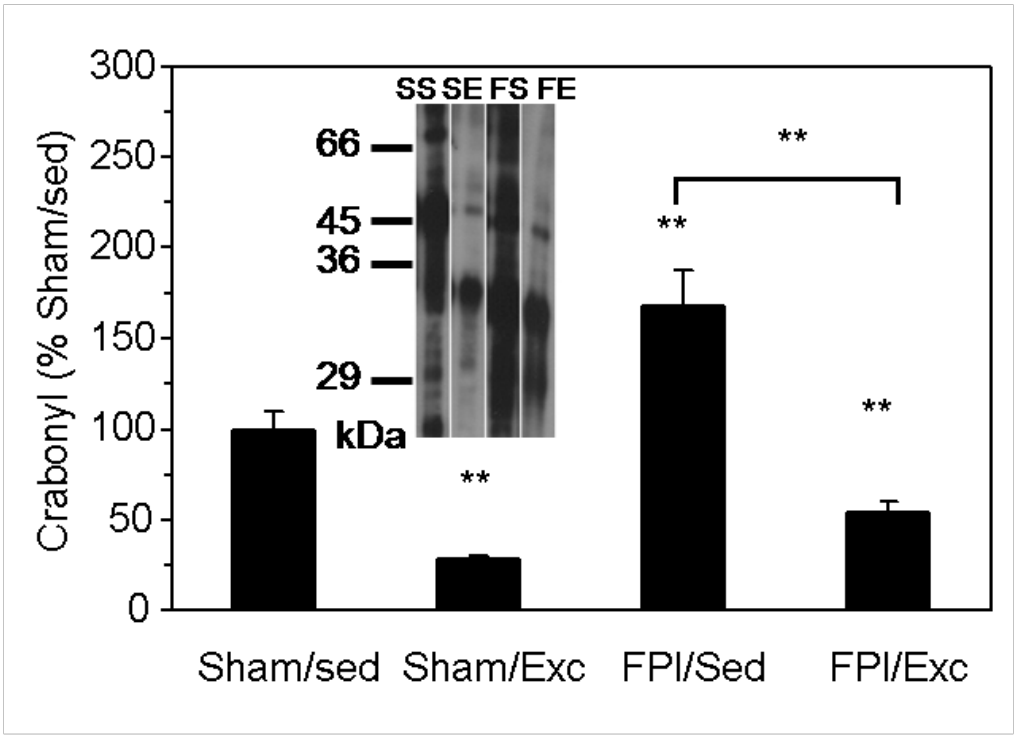


Fig. 12. The effect of exercise on level of carbonyl show densitometric data of the level of carbonyl groups in amino acid residues. The levels of carbonyls were increased significantly in FPI/Sed animals, which suggest that FPI resulted in a massive oxidative stress to proteins. Values are mean±SEM percentage of control group. **p<0.01, *p<0.05 representative bands of Western Blots are shown (N=19).

Synapsin is one of the well accepted markers of brain plasticity. Here we evaluated whether FPI and/or exercise changed the protein concentration of synapsin I. Our data revealed that as a result of FPI, the content of synapsin I decreased significantly; whereas, exercise induced the protein level of synapsin I. The rats that exercised showed no significantly altered synapsin I levels compared to the control rats. This suggests a beneficial effect of voluntary exercise on FPI via synapsin I (Fig. 13).

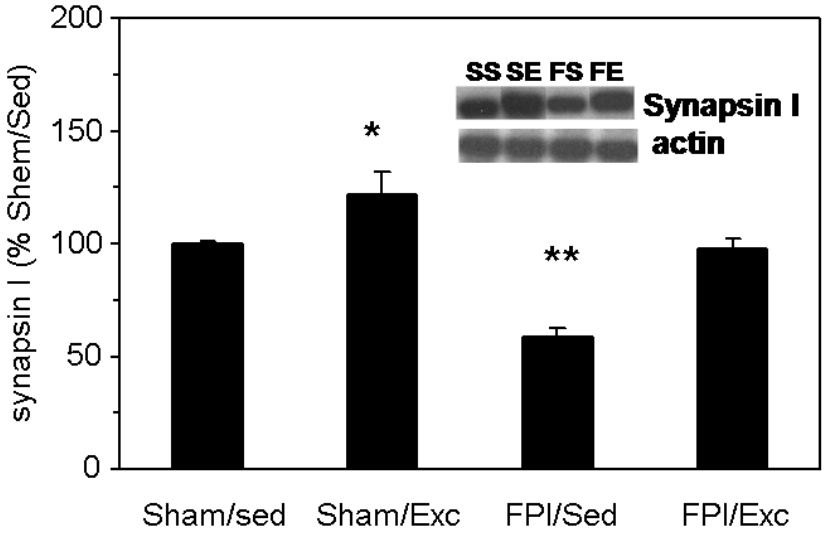


Fig. 13. Shows that FPI decreased levels of synapsin I (FPI-Sed), and that exercise counteracted the effects of FPI (FPI/exc). Exercise also elevated levels of synapsin I in sham animals (Sham/exc). Values are mean±SEM percentage of control group. **p<0.01, *p<0.05, representative bands of Western Blots are shown (N=19).

Based on this suggestion, we have checked the correlation between oxidative protein damage assessed by the level of carbonyl groups in amino acid residues and synapsin protein concentration (Fig. 14). Our data displays a strong negative relationship, which is in accordance to the belief that oxidative stress results in oxidative damage, which further causes decreased brain plasticity.

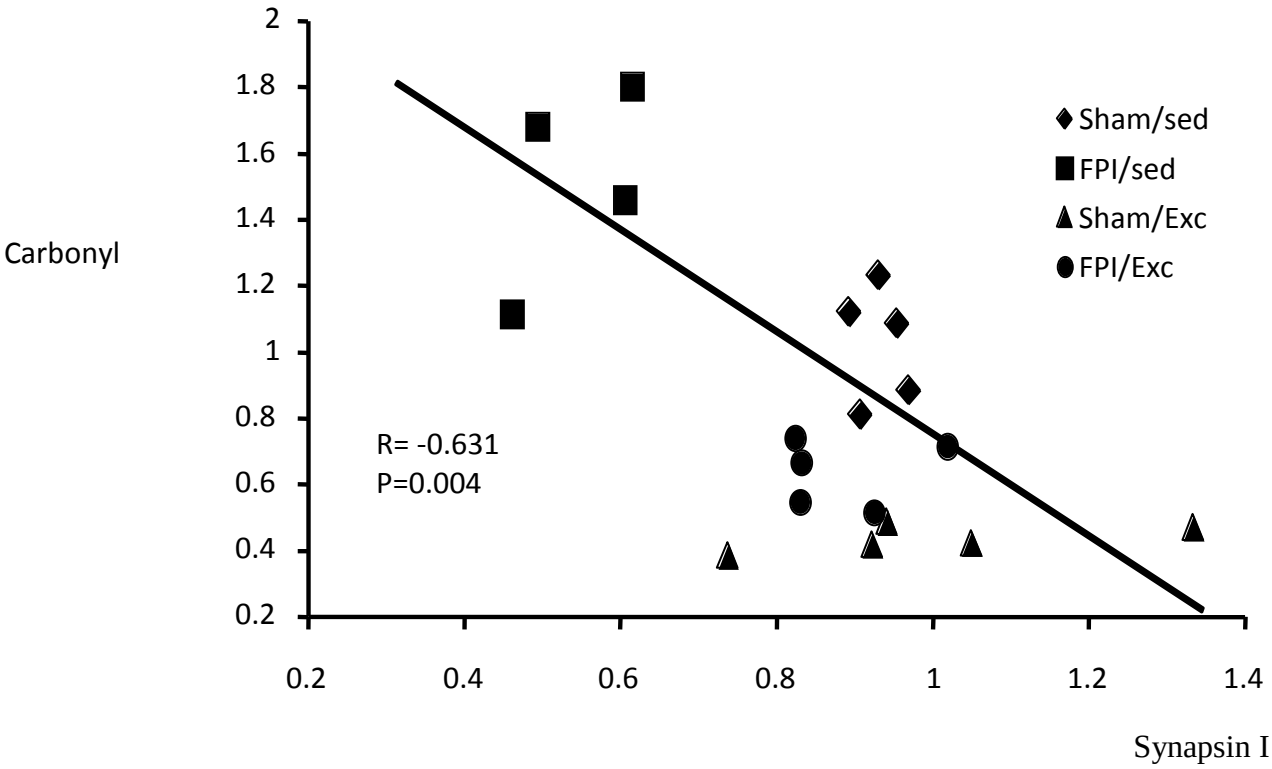


Fig. 14. Reveals a significant negative correlation between the level of oxidative protein damage (carbonyl groups) and synapsin I protein concentration. Values were calculated from the densitometric data obtained by Western blot analysis. (N=19, p<0.05, r=-0.63).

Upon further research on the possible reason of increased level of carbonyl group accumulation, we tested the activity of proteasome, which is a powerful housekeeping

enzyme leading to the degradation of oxidized proteins. We found the activity of proteasome was reduced by exercise and FPI attenuated this decrease (Fig. 15).

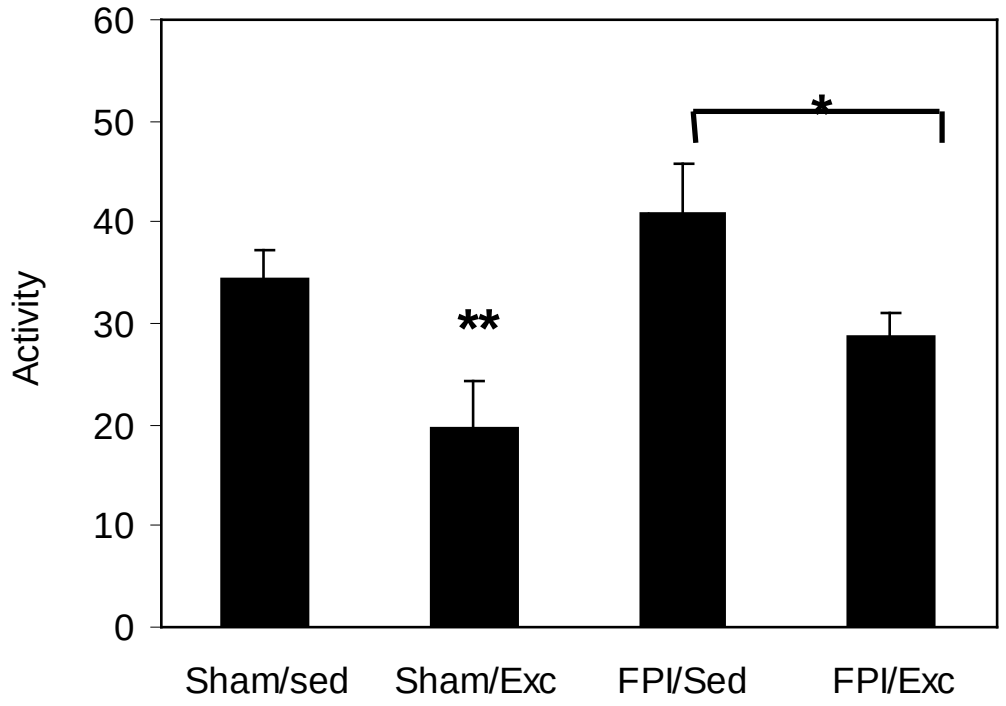


Fig. 15. Reveals the effects of FPI and exercise on proteasome activity. It shows that exercise (FPI/EXC) reduces the FPI-induced elevation FPI/Sed) of the chymotripsin-like activity of proteasome. Exercise also reduced chymotripsin like activity in sham animals (Sham/Exc). Values are mean±SEM percentage of control group. p*<0.05, p<0.01 representative bands of Western Blots are shown (N=6).**

The correlation between carbonyl group concentration and proteasome activity establish a positive relationship, suggesting that lower level of oxidative stress and accumulation of carbonyl groups is associated with decreased activity of proteasome enzyme. Lack of damage decreases the activity of housekeeping enzymes (Fig 16).

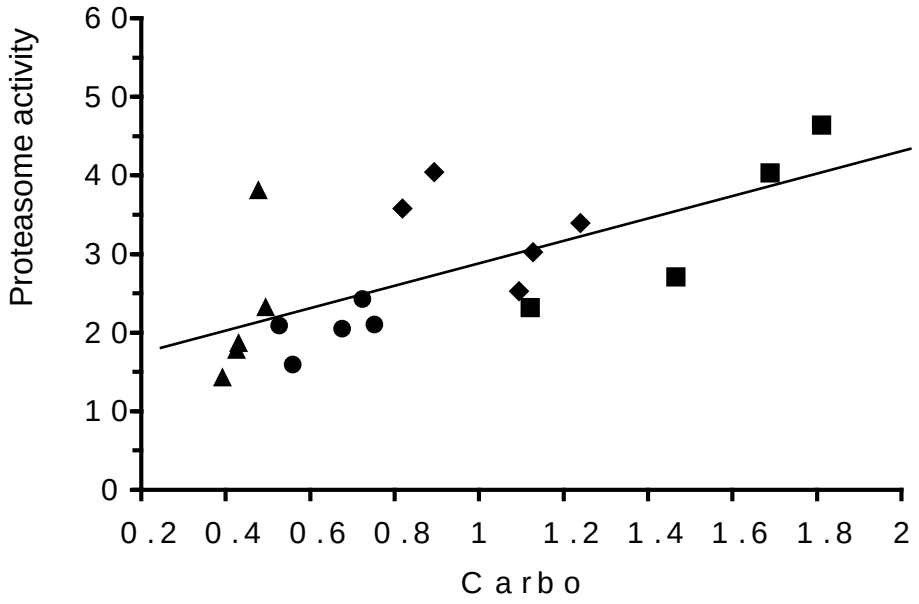


Fig.16. Scatter plot reveals a positive correlation between the activity of proteasome and the level of carbonyl groups. (N=19, p<0.05, r=0.68) Values are mean±SEM percentage of control group .

Not only the activity, but the protein content of 20S subunit of proteasome complex was also reduced by the lack of the accumulation of carbonyl groups (Fig 17).

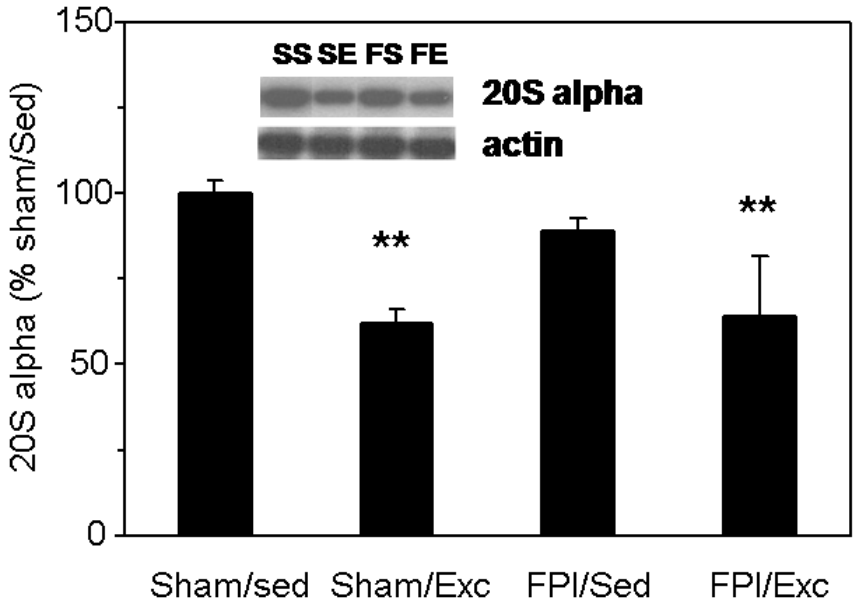


Fig.17. Shows the effects of FPI and exercise on the contents of the proteasome subunit. The voluntary exercise decreased the 20S subunit of proteasome complex in Sham (Sham/Exc) and injured animals (FPI/Exc). Values are expressed as mean±SEM percentage of control group. p*<0.05, p<0.01 representative bands of Western Blots are shown (N=19).**

Zif 268 is an early gene, believed to regulate the content of proteasome. It was established for the first time according to our knowledge in exercise model, that the protein level of Zif 268 showed very similar changes as the content and activity of proteasome (Fig. 18).

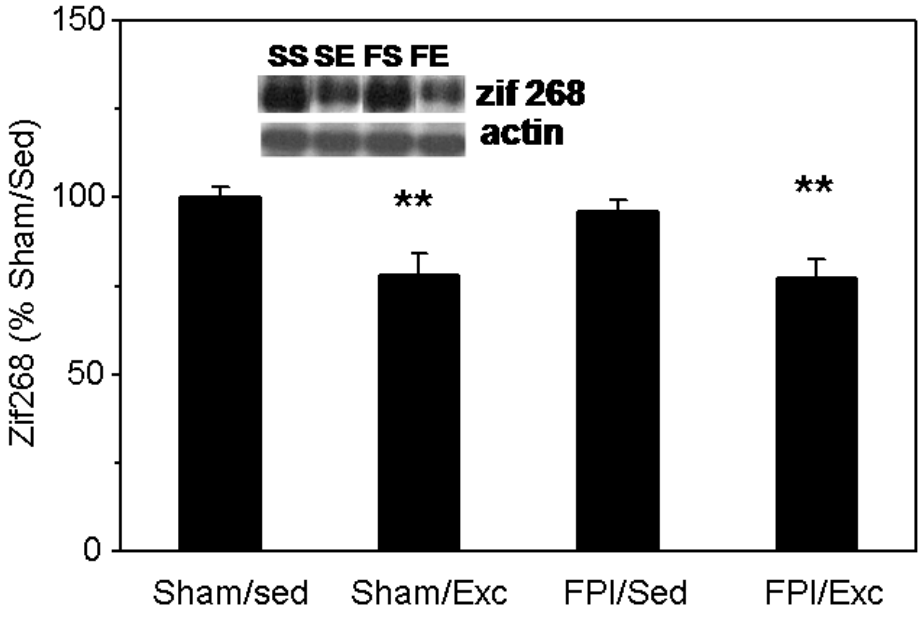


Fig.18. It shows the exercise reduced the content of Zif 268 in a similar fashion to observe the 20S alfa subunit. Values are expressed as a mean±SEM percentage of the control group. p*<0.05, p<0.01, representative bands of Western Blots are shown (N=6).**

The suggested pathway dependent relationship was strengthened by mathematical relationship gained from correlation matrix (Fig. 19).

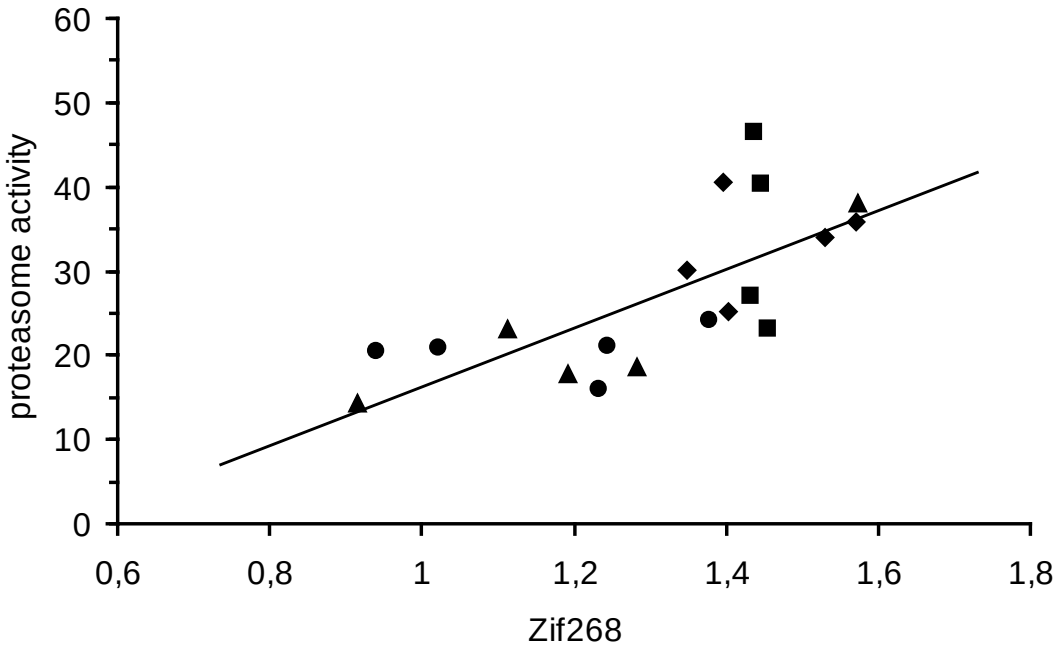
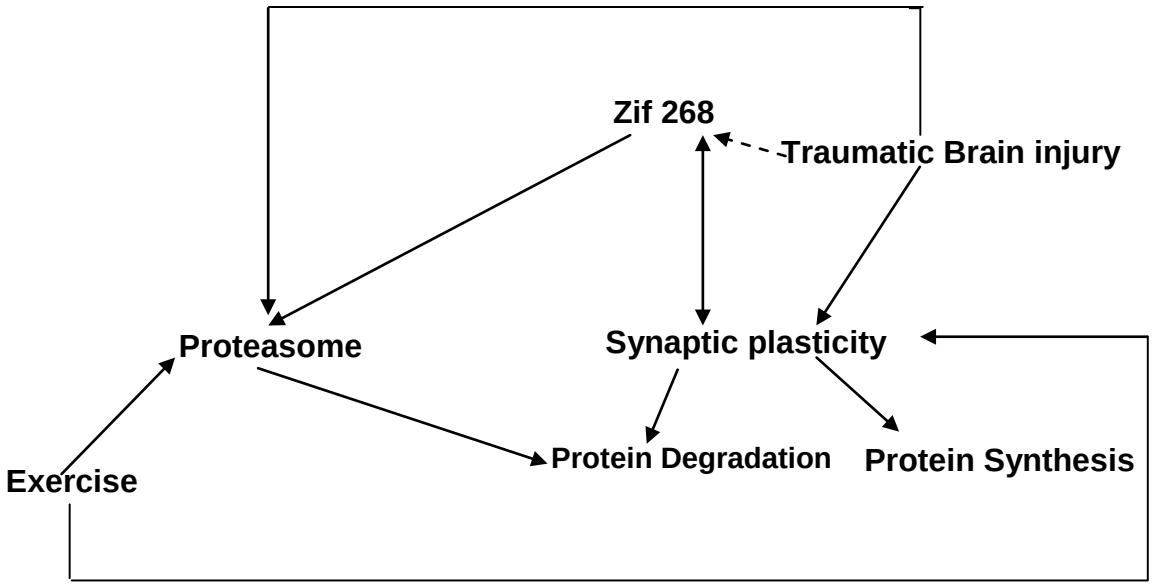


Fig.19. Scatter plots reveal a significant positive correlation between Zif 268 and proteasome activity (r=0. 71, p<0. 01), which suggests an association between Zif 268 and proteasome function.

Based upon the findings of the second experiment, we have drawn a diagram about the suggested relationships which take place in FPI and exercise (Fig. 20).



Zif 268-Transcription factor can influence the expression of proteins of specific subunits of the proteasome.

Proteasome (located in dendrites) degrades select proteins and can strenghten synaptic plasticity.

Synaptic plasticity is the result of concurrent protein synthesis and degradation.

Exercise has the power to facilitate synaptic plasticity and help to eliminate the damaged proteins by the proteasome.

TBI may damage synaptic function and expose damaged proteins to the proteasome system.

Fig. 20. Diagrammatic representation for the potential effects of brain trauma and exercise on proteasome function and synaptic plasticity.

5. Discussion

It is well demonstrated that regular exercise has beneficial effects on brain function, including increased memory (Radak et al. 2001), capillarization (Fabel et al. 2003), brain plasticity (Cotman and Brechtold 2002), proteasome activation and up-regulation of antioxidant system (Radak et al. 2000). In our model A, we have discovered some of these changes are in fact reversible, such as the improvement in memory. Moreover, there is a negative re-bound in BDNF and NGF content after detraining. Reduction in BDNF and NGF concentration occurred even compared to the control level, while no significant differences were noted in the memory of control and detrained animals. This may be an indication that change in memory is not entirely dependent on the neurotrophin concentration. Therefore, other control factors cannot be ruled out. The concentration of free radicals assessed by ESR suggests that exercise can reduce the free radical concentration. Moreover, lower levels of radicals were observed in detrained brain. On the other hand In or model B we found that voluntary exercise reduces the levels of protein oxidation elevated after fluid percussion injury . Indeed, the complexity of cognitive function may exclude the fact that it is driven by a single molecular process. We have the strong reason to believe unlike any other complex physiological processes such as aging, that cognitive function also affects the number of different molecular pathways, where neurotrophis are important proteins.

The induction of BDNF (NGF) could be strongly dependent on the activation of the cyclic AMP response element binding protein (CREB), which has been shown to be very sensitive to the redox state (El Jamali et al. 2004). In the current experimental conditions (model A), it seems unlikely that free radicals-associated induction of CREB played a significant role in the increased BDNF and NGF level as a result of exercise. On the other hand, the exercise mediated increase in BDNF mRNA level was prevented by L-N-nitro-L-arginine-methyl ester (L-NAME), 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 nucleus. The results of the present study cannot elucidate the mechanism by which the BDNF and NGF levels were regulated, but similar changes in memory

and neurotrophin levels may represent a causative relationship. Paralelly in model B is interesting that exercise was able to reverse all the studied parameters associated with the effects of FPI on the brain. It appears that FPI and exercise alter the activity and protein levels of proteasome, Zif 268, synapsin 1. It is suggested that these changes may affect synaptic plasticity.

ROS and the associated oxidative damage have been suggested to be one of the possible regulating factors of brain function (Carney et al. 1991, Radak et al. 2001). Oxidative damage can be a stimulation factor of the damage repairing enzymes (Radak et al. 2005). The integrity of cellular DNA has a vital importance for life. Base excision repair enzymes (BER) is specialized to remove the oxidatively damaged bases. The first step of BER is executed by DNA glycosylases that recognize and remove the damaged base. The 8oxoguanonine is primary repaired by 8-oxoG-DNA glycosylase (OGG1), and if it remains un-repaired it leads to increased mutation. As a result, this may jeopardize the viability of the cells. To our knowledge this is the first study measuring the nuclear and mitochondrial activity of OGG1 in after exercise (model A). 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 resulted in increased activity of OGG1 in skeletal muscle (Radak et al. 2001), and it has been shown that regular exercise decreased the DNA damage and up-regulated the activity of OGG1 in skeletal muscle of trained animals (Radak et al. 2002). The earlier data was obtained from crude cell extracts; therefore the site of increase remained unknown. The present data suggest that the DNA in nucleus and mitochondria of neuron cells in the brain remained un-effected by exercise training.

To our knowledge, this is the first exercise related study in which the nuclear activity of proteasome complex has been measured (model A), although no alteration was found by exercise training or detraining. Altered protein degradation plays a role in the development and of Alzheimer's and Parkinson diseases (Mattson 2000). Exercise has been show to increase the activity of proteasome and nephrilisin (enzyme involved in beta-amyloid degradation) and can significantly increase the degradation and decrease the content of beta-amyloid (Lazarov et al. 2004). 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. For the first time, it is shown that the nuclear activity of proteaosme complex did not change by exercise training and de-training. In contrast of model A we found important changes in proteasome activity and content as well in model B. The results further show that exercise normalized levels of proteasome components and activity following brain trauma. The fact that changes in protein oxidation and proteasome activity were paralleled with changes in the synaptic protein synapsin I, seem to suggest that exercise can affect synaptic plasticity in the traumatically injured brain *via* protein modifications.

Therefore, one of the novel findings of the present study is that brain trauma elevated levels of oxidative stress, 20S proteasome subunit, and proteasome activity, which may reflect the effects of FPI on protein turnover. FPI is known to induce necrosis, apoptosis and the related inflammation (Chen et al.2008). The clearance of damaged proteins is indispensable in order to provide the necessary conditions to cellular regeneration. Indeed, accumulation of damaged proteins could lead to aggregation and depressed proteolytic degradation. Accumulation of damaged proteins readily results in impaired cellular and organ functions, hence proper houskeeping is an important means to maintain normal physiological function. Proteasome complex is the major housekeeping system, therefore it is not surprising that its activity and content induced by FPI. In a similar phenomenon, induction of proteasome has been reported after different traumatic stressors, such as hyperoxia, radiation or oxidative damage (Chambellan et al.2006, Choudhury et al.2006, Radak et al.2000).

There are a few studies reporting that exercise may affect the activity and content of proteasome in other brain regions such as the caudal cortex, but none of them used voluntary exercise (Radak et al.2001, Ogonovszky et al.2005). It is known that the activity of proteasome complex can be induced by oxidative protein damage in cell culture (Sitte et al.1998). This complex is responsible for the degradation of oxidatively modified proteins, and according to this, our results suggest that in vivo, similar phenomenon could take place.

Besides the important housekeeping function of proteasome, they play an important regulatory role by targeting degradation of transcription factors important for synaptic plasticity (Hegde and Upadhya 2007). Product of degradation are transported to class I

major histocompatibility complex, which supplies peptides to synapse and which has been shown to strongly alter hippocampal long term potentiation (Huh et al2001). Along with this line, it has been suggested that Zif 268 is one of the important regulatory proteins to tune the function of proteasome by altering the expression of several subunits of the complex. Although, it has been shown that the postsynaptic density is regulated by the proteasome (Ehlers 2003), exact regulatory mechanism of proteasome complex, protein specificity excluding the damaged proteins, is vague. A data of a recent microarray study revealed that 4 subunits and the activity responsible element of the complex were dependent of Zif 268 gene (James et al.2005). Here, we propose upon the very similar pattern of the proteasome activity, content and Zif268 content data that causative relationship is possible among these proteins at least in this model condition.

The involvement of Zif 268 in LTP has been well demonstrated in mice with genetic deletion of Zif 268, which showed a deficit in memory test (Jones et al.2001). Synapsin 1, one of the major player in LTP is regulated by Zif268 (Petersohn et al.1995). It was found that ZiF268 and synapsin 1 content changed in inverse manner, which is simply according to the generally accepted gene suppressor effects of Zif268 (James et al.2005)

It has been shown that accumulation of carbonyl groups in amino acid residues of brain proteins results in loss of cognitive function measured by Morris water maze, passive avoidance and active avoidance tests (Radek et al.2001, Carney et al.1991). Hence, the decrease of carbonyls groups as an effect of exercise could be an important therapeutic mean following FPI.

Mitochondrial electron chain complexes suggested being an active contributor of free radical release (model A). The method of blue native gel allows the study of protein content of mitochondrial complexes. This is one of the first investigations to apply blue native gel method in exercise study, and no differences as a result of exercise or detraining were detected in the protein content of electron transport complexes in neurons mitochondria.

Taking together our observations, the data of our investigations suggests that exercise training only temporarily increases the memory of rats, since the memory falls by detraining. It appears that the exercise-induced improvement in memory is partly independent from the concentration of BDNF and NGF. Due to the decreased free radical

concentration by exercise training, the repair or degradation of oxidative DNA and protein damage was unaffected, suggesting that training or detraining did not result in oxidative challenge to brain.

Ultimately, it appears that voluntary exercise following FPI promotes recovery by decreasing the level of oxidative protein damage and by modulation of proteasome activity with the possible involvement of Zif 268. The stimulating effect of exercise on proteasome function could translate into modifications of synaptic plasticity, i.e., levels of synapsin I, which may have the potential help to reduce the deleterious effects of brain trauma.

6. Summary

In our current investigations, we used laboratory rats as a model for traumatic brain injury and regular physical activity, we have confirmed that exercise has beneficial effects on brain function. On the other hand, we have generated some novel information by our studies. We have proved that the effects of exercise on brain are reversible, since the functional parameters were declined by detraining. This is expected, but still very important information, which suggest the importance of lifelong exercise, similarly to the lifelong learning. Our study further revealed the importance of oxidative stress and neurotrophins as potential regulatory factors in cognitive function. Upon our finding, number of relevant questions may be asked about the positive or negative effects of antioxidant supplementation on non-deficient subjects. We further gain new information about the DNA damage repair mechanisms. The quality control of DNA is one of the highest priorities of the living cells, since the accumulation of the oxidative damage readily jeopardizes the fate of the cells. Here we have shown, that neither exercise training nor detraining results in that size of challenge to nuclear or mitochondrial DNA, which could result in induction of OGG1, which is one of the most important enzymes in the base excision repair process of 8-oxoG. Similarly, with this observation, it is safe to conclude that neither exercise nor detraining was powerful enough to alter the protein content of the mitochondrial electron chain complexes. In our second study, we have reached also some important conclusions. The most important one could be that mimicked traumatic brain injury by fluid percussion injury results in oxidative stress and accumulation of carbonyl groups, which could be significantly prevented by voluntary exercise. In addition, further observation on the involvement of proteasome in remodeling of brain after FPI was gained. Simply put, the effects of exercise on proteasome complex appear to be very important to reduce the oxidative damage caused by FPI and regulate brain plasticity. We also have shown that this process includes synapsin I and Zif 268.

Összefoglalás

Kutatásunkban, melyben laboratóriumi patlányok voltak a vizsgálatban szereplő modellek alanyai, megerősíthetjük a mozgás jótékony hatását az agyi funkciókra. Másfelől újszerű információkat szereztünk a vizsgálatunkkal. Bizonyítottuk, hogy a mozgás hatásai az agyra reverzibilisek, mivel a funkcionális paraméterek romlottak az edzés megszűnését követően. Ez várható volt, de még mindig fontos információ, mivel azt sugallja, hogy mennyire fontos az élethosszig tartó mozgás, csakúgy, mint az állandó tanulás. Vizsgálatunk rámutat az oxidatív stressz és a neurotrophinok fontosságára, mint potenciális faktorok a kognitív funkciókban. Eredményeink alapján számos kérdés merült fel az antioxidánsok pozitív avagy negatív hatásaival kapcsolatban. Továbbá új információkat nyertünk a DNS sérülés javító mechanizmusokkal kapcsolatban. Minőség ellenőrzés a DNS-en a legmagasabb prioritást élvezi az élő sejtekben, mivel a felhalmozódó oxidatív károsodás könnyedén veszélybe sodorja a sejtek további sorsát. Ahogy kimutattuk, sem a fizikai aktivitás, sem az edzés elhagyása nem eredményez olyan mennyiségű kihívást sem a nukleáris sem a mitokondriális DNS-ben, amely OGG1 indukciót váltana ki , amely az egyik legfontosabb enzim az 8-oxoG bázis „kivágás" javítás folyamatában. Hasonlóan ezzel a megfigyeléssel azt a következtetést vonhatjuk le, hogy sem az edzés, sem az edzés megszűntetése nem volt elegendő, hogy befolyásolja a fehérje mennyiséget a mitokondriális elektronlánc komplexben. A második vizsgálatunkban szintén néhány fontos eredményt értünk el. A legfontosabb, hogy a modellezett traumás agysérülés a oxidatív stresszt eredményez, carbonylációt, amellyel szemben a mozgás preventívnek minősült. Továbbá, újszerű megfigyelésnek számít a proteaszóm szerepe az agy remodellezésében FPI után. Egy másik megvilágításban, a mozgás hatása a proteaszóm komplexen nagyon fontos hatással bír, az oxidatív sérülés csökkentésében, amit az FPI okoz, valamint az agyi plaszticitás szabályozásában. Kimutattuk, hogy ez a folyamat magába foglalja a synapsin I-et és a Zif 268-at.

Conclusions

At the beginning of the study based on the available information on the related literature we have stated different aims. After the study, here we show whether our aims meet with our results of our two investigations.

1.

Our results showed, except the concentration of free radicals, that the effects of exercise training, in general, reversible. Here we also have to note that the time frame does completely allow studying the complex reversibility.

2.

One of the novel observations of our study was that the level of free radicals which cause oxidative damage in DNA and proteins, decreased as a result of exercise training, measured by ESP, but the level of oxidative damage did not increase.

3.

Neither exercise training nor detraining altered the activity of main excision repair enzyme- OGG1.

4.

The exercise attenuates the damage caused by traumatic brain injury
by our experimental data and indeed voluntary exercise had beneficial effect on FPI induced damage.

5.

Proteasome complex plays an important role in the recovery process.
Here we have shown first time, according to our knowledge, that voluntary exercise effects proteasome activiy, which is involved in the remodeling process of the brain after FPI.

6.

The early gene expression of Zif268 changed in concert to proteasome in FPI model.

Indeed, we have shown, according to our suggestion that the early gene of Zif268 plays in important role in the induction of proteasome in FPI model.

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