The effects of regular physical activity on brain ageing in animal models

Ph.D. Thesis

Krisztina Marosi

Semmelweis University
Doctoral School of Sport Science

Supervisor: Dr. Csaba Nyakas, DSc

Official reviewers: Dr. Béla Székács, DSc
Dr. István Szabolcs, DSc

Head of Comprehensive Exam Committee: Dr. Kornél Sipos, CSc
Members of Comprehensive Exam Committee: Dr. Péter Osváth, PhD
Dr. József Pucsok, DSc
Dr. Csaba Sós, PhD

Budapest
2012

Introduction

Ageing of the brain is accompanied by several structural and neurophysiological changes associated with various degrees of cognitive impairment. It is known that the progress of neuronal decline can be influenced by both endogenous and exogenous factors. In females, among the endogenous factors, gonadal steroid hormones, especially estrogens, seem to be potent biomodulators. As an exogenous factor physical exercise can be accentuated. Estrogens have been shown to exert beneficial influences on the ageing brain. Estrogens affect neurotransmission functions, especially by enhancing the activity of cholinergic neurons. Neurogenesis, neuronal survival, and synaptogenesis are also influenced by estrogen treatment in the hippocampus. In addition, estrogens can reverse the age-related oxidative stress in different tissues, indicating its potential role in the prevention of certain neurological disorders.

Despite the beneficial effects of estrogen on the brain functions, there are drawbacks regarding its clinical use being due to potential cardiovascular and oncological side-effects.

In addition to potential health benefits of pharmacological interventions, numerus clinical and animal studies have supported the role of enhanced physical activity in the promotion of cognitive health during ageing. Because of the suspected similarities and overlap between the molecular actions of E2 and physical exercise the question has been raised whether exercise can replace E2 treatment in older individuals and in what dimensions.

Exercise has been shown to upregulate the levels of brain derived nerve growth factor (BDNF), the intensity of neurogenesis and the power of synaptic plasticity. BDNF activates PKA/Akt/CREB and MAPK/CREB pathways indicating convergence in the molecular actions with estrogen.

Physical exercise may also play a preventive role against age-associated oxidative stress as well. It has been proposed that regular physical training induces an adaptation process in reactive oxygen species (ROS)-detoxifying systems, resulting in increased resistance of cells to oxidative challenges. The adaptive response to long-term exercise is rather complex and not fully elucidated; it may involve the modulation of redox-sensitive transcriptional factors.

Furthermore, the animal model for lifelong exercise can provide novel data on the neurobiological impacts of exercise during ageing. Since similar animal model has not been used in earlier studies, the effects of lifelong exercise on brain ageing are largely unknown.

Aims

Experiment A: The neurobiological effects of long-term estradiol treatment and long-term aerobic physical exercise in aging and aged female rats.

In this study I aimed at comparing the neurobiological effects of long-term estradiol treatment and long-term physical exercise in ageing and aged female rats.
In the current study I have hypothesized that

- the actions of exercise and estrogen are convergent to modulate intracellular signal transduction-related biochemical markers associated with improved cognitive performance.
- physical activity – similarly to estrogen treatment – maintains the redox and energy balance in the neurons by reducing the oxidative stress and regulating the energy metabolisms.

The action of combined exposure of ageing and aged rats to physical activity and estradiol treatments on cognition and neurotrophic intracellular pathways has not been explored yet. Therefore, I examined whether exercise combined with estrogen administration might exert different actions as compared to the individual treatment effects alone.

Experiment B. The effects of lifelong exercise on brain ageing in male rats

The aim was to investigate the neuropsychological and neurobiological effects of life-long exercise in the hippocampus of aged male rats.
According to the hypothesis, lifelong exercise can play a preventive role in brain aging because it may improve cognitive functions and it may exert neurotrophic and neurogenetic actions. Moreover it may affect the redox and energy balance of the neurons. In addition it may influence the cholinergic neurotransmission as well.

Materials and methods

Animals and treatments

Experiment A: The neurobiological effects of long-term estradiol treatment and long-term aerobic physical exercise in aging and aged female rats.

Thirty-two middle-aged (12 months old) and 32 aged (24 months old) female Wistar rats were selected for the study. The ageing and old animals were divided into four experimental groups that were subjected to the following treatments: 1. sham-injected controls (C): only the sesame oil vehicle was injected twice weekly, 2. estradiol treatment (E2) alone: subcutaneous injection of 17β-estradiol for 15 weeks (30 μg/kg/week, divided into two injections per week; E2 was dissolved in sesame oil), 3. exercise treatment (EX) included 30 minutes of moderate intensity running (speed: 18 m/minutes) on a rodent treadmill for 15 weeks, five times a week, 4. estradiol treatment combined with exercise (E2+EX). After the 15 weeks period, the cognitive functions of the animals were tested.

Experiment B. The effects of lifelong exercise on brain ageing in male rats

16 male Wistar rats were used in this experiment. The rats were randomly divided into two groups (8 animals per group): 1. control group (C): sedentary cage controls, 2. physically trained group (EX): the rats were trained on rodent treadmill from their age of three month to their age of 24 months. The rats run 60 minutes with 18 m/min velocity. There were 3 training sessions per week.

Cognitive testing

The memory functions were investigated in Novel Object Recognition Test (NOR). Attention and working memory were assessed by measuring spontaneous alternation in an Y-maze. Spatial learning was tested in the Morris water maze test.

Measurement of plasma 17β-estradiol and corticosterone levels

2 ml of trunk blood samples obtained with decapitation were heparinized with 50 µl heparin. The plasma was separated by centrifugation at 15,300 *g* for 20 minutes at room temperature. The plasma 17β-estradiol and corticosterone levels were measured using the 96 well enzyme immunoassay kits. The assays were carried out according to the manufacturer's instructions.

Western blots

The hippocampi of animals were homogenized in lysis buffer containing protease inhibitors. The concentration of protein was determined using the Bradford assay. Twenty µg of protein were electrophoresed on 8-15% (v/v) polyacrylamide SDS-PAGE gels. Proteins were electro-transferred onto PVDF membranes. The nonspecific binding of immunoproteins was blocked with 5% non-fat dry powdered milk dissolved in TBST for two hours at room temperature. After blocking, the membranes were incubated with primary antibodies overnight at 4^{o}C. Antibodies were dissolved in TBST containing 5% non-fat powdered milk. The membranes were rinsed in TBS-T followed by 1 h incubation with HRP-conjugated secondary antibody at RT. After incubation the membranes were repeatedly washed in TBS-T and incubated with an enhanced chemiluminescens reagent. The protein bands were visualized on X-ray films. The bands were quantified by Image J software, and standardized to β-actin.

Detection of Reactive Oxygen Species (ROS)

The overall ROS was determined by using modifications of the dichlorodihydrofluorescein diacetate staining method. For fluorescence reactions, 96-well black microplates were loaded with phosphate buffer (pH 7.4) to a final concentration of 152 µM/well. Then 8 µl diluted freshly-prepared hippocampus homogenate and 40 µl of 125 µM dye were added. The changes in the fluorescent signal of the oxidized H_2DCF-DA were recorded at three time points (0, 1 and 30 min), using a micro plate fluorescence reader.

Detection of protein carbonyls

The levels of oxidized proteins were determined using an Oxyblot kit. Proteins were derivatized with 4-dinitrophenylhydrazine (DNPH) for 15 min followed by incubation at room temperature with a neutralization buffer. Derivatized proteins were electrophoresed on a 10% SDS-PAGE

and blotted on PVDF membranes. Blots were blocked with 5% non-fat dry milk in Dulbecco's PBS containing 0.05% Tween 20 (PBS-T) for 3 h at 4^{o}C followed by incubation with anti-DNP primary antibody overnight at 4 °C. After three washes with PBS-T, membranes were incubated for 1 h at room temperature with HRP- secondary antibodies. Immunocomplexes were visualized using ECL plus reagent. The bands were quantified by Image J software, and standardized to β-actin.

Immunohistochemistry

One hippocampi hemisphere of each animal was fixed in 4% paraformaldehyde solution. After fixation the tissues were dehydrated with 30% sucrose solution in +4 ^{o}C. Then the samples were taken into liquid nitrogen and cut into sections with 20 μm thickness. The sections were washed in PBS (pH 7.4). The endogen peroxidase activity was blocked during 20 minutes of incubation with 1% H_2O_2. The sections were rinsed for 3X20 min in PBS, The nonspecific binding of immunoproteins were blocked in 5% bovine serum albumin dissolved in PBS. After blocking, the sections were incubated with primary antibodies for 5 nights at 4^{o}C. The sections were rinsed in PBS for 5X20 min followed by 3-4 h incubation with secondary antibody at RT. The sections were incubated in ABC-Peroxidase Solution for 30 minutes at room temperature followed by DAB reaction.

Statistical analysis

In the experiment A, comparing treatments at different ages, two-factorial ANOVA was used followed by the Fisher post hoc t-test. In the experiment B, the results of experimental groups were compared by paired t-test. Statistical significance was set at $p<0,05$.

Results

Experiment A: The neurobiological effects of long-term estradiol treatment and long-term aerobic physical exercise in aging and aged female rats.

Cognitive tests

The Novel Object Recognition test showed that exercise (EX), estrogen (E2) and combined treatments (E2EX) increased the recognition of novel object. Both E2 and the combined treatment enhanced NOR performance in the old rats. In the spontaneous alteration test, E2 and combined treatments increased the number of alternations in both age groups. Exercise improved performance in the 15 months old animals. In the Morris water maze test E2 treatment in the old animals shortened the escape latency to the platform against controls on the fourth, sixth and seventh days of the test.

Plasma 17β-estradiol and corticosterone levels

The treatments of 15 and 27 months old rats with 17β-estradiol markedly elevated plasma estradiol levels. No effect of exercise could be obtained in either age on the level of estradiol. No significant treatment effect was found in either age on the corticosterone levels.

Neurotrophic effects in the hippocampus.

Estrogen receptor alpha (ERα) proteins were increased in the hippocampus in both ages exposed to E2 and combined treatments. Interestingly, exercise also enhanced the ERα protein level but only in the ageing (15 months) animals. There was no effect of exercise in the old rats on the expression of ERα protein. Similarly to ERα, BDNF immunoreactivity was increased by EX, E2 and E2+EX treatments resulted in the ageing groups. BDNF levels also increased in the estradiol treated (E2, E2+EX) old animals compared to their control group.

Effects on intracellular signaling pathways

The activation of signaling molecules (MAPK, Akt, AMPK) was investigating by evaluating their phosphor-specific form. The phosphorylation of MAPK, Akt and CREB was significantly increased in the aged animals after E2, EX and E2+EX treatments. All treatments increased the phosphorylation of Akt in the younger groups. AMPK activation was enhanced by exercise in

the 15 months old animals. No significant differences in the p-Akt and p-AMPK protein levels were found among any of the aged groups.

Effects on synaptic plasticity markers

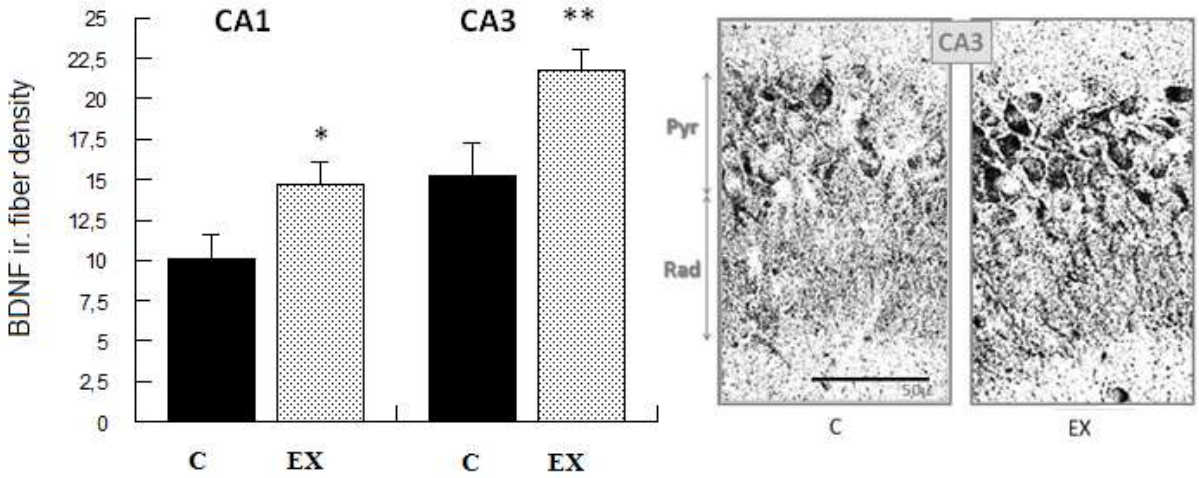
P-synapsin I was significantly increased in the ageing groups that received E2, EX and E2+EX treatments. Estradiol enhanced the p-synapsin I expression in the hippocampus of 27 months old rats. Higher values of synaptophysin proteins were measured in the hippocampus of EX, E2 and E2+EX groups of the ageing rats. With regard to the old animals, only the E2 and the combined treatments resulted in enhanced synaptophysin levels.

Regulation of redox balance

The amount of reactive oxygen species (ROS) was significantly decreased in the ageing groups that received E2, EX and E2+EX treatments. Estradiol and combined treatments lowered the ROS content in the hippocampus of 27 months old rats. The amount of protein carbonyls was lower in the hippocampus in response to E2, EX and combined treatments. No significant difference in the levels of protein carbonyls were observed in the aged animals. PGC-1α was increased by EX and E2 in the hippocampus of ageing animals. At the age of 27 months, no significant difference was found in the PGC-1α levels among the groups. Superoxide dismutase-1 (SOD-1) expression was increased by physical exercise as well as by estradiol treatment at the age of 15 months. SOD-2 was elevated by E2, and glutathione peroxidase (GPx) was unregulated by physical activity in the hippocampus of 15 months old animals.

Experiment B. The effects of lifelong exercise on brain ageing in male rats

Lifelong exercise improved the memory functions in the NOR test compared to the sedentary controls. Physically trained animals performed better in the Morris Water Maze test on the 3rd test day. As regards the molecular changes in the hippocampus, the immunoreactivity of BDNF positive fibers were higher both in the CA1 and the CA3 areas in the hippocampus in response to physical activity. Doublecortin fiber density was also higher in the physically trained group. Glucose-1 transporter and choline-acetyltransferase levels were also elevated by exercise. The phosphorilation of Akt and AMPK was higher in the animals subjected to lifelong exercise compared to controls. Significant increment in the levels of synaptophysin was also found in the hippocampus of physically trained group compared to the sedentary control group.



BDNF immunopositive fiber density in the CA1 and CA3 areas of the hippocampus

*BDNF positive fiber densities were higher both in the CA1 and the CA3 areas in the hippocampus of physically trained animals compared to the sedentary controls. Figures represent means +/- SEM, *p<0,05 , **p<0,01 vs. control.*

Conclusions

In summary exercise can emerge as a potential non-pharmacological intervention that can improve the cognitive vitality around the onset of menopause or the early stage of postmenopausal period, because it exerted rather comparable behavioral and molecular neurotrophic effects with estradiol treatment in female rats in the early ageing period found in this study. The data from this study demonstrate that long term physical exercise results in lower levels of ROS and protein carbonyls as well as elevated levels of antioxidant enzymes in the hippocampal neurons of older rats. Physical training, thus, can be an effective option to regulate the oxidative balance and thus delay the onset of oxidative stress-related neurodegenerative processes. During the well advanced stage of female ageing effectiveness of regular physical exercise proved to be far less pronounced. However, it is not excluded that under other more optimized conditions exercise might also be effective in aged female rats and the movement therapy may compensate at least partly the natural decline of estrogens including their dose-dependent beneficial actions on the ageing brain.

Life-long exercise has also resulted in beneficial effects on brain ageing in male rats. Physical activity during the whole life-span had neurotrophic actions; it influenced the signal molecules regulating the energy metabolisms of the neurons. In addition life-long exercise had beneficial effects on cognition as well. The redox balance and the related signaling molecules were not affected by exercise neither in the female nor in the aged male rats.

In conclusion physical activity has preventive role in terms of brain ageing, however there are gender and age differences regarding its effectiveness. Further studies are necessary to establish the effective training protocols in different age groups and genders.

Publications

Publications closely related to the thesis:

Marosi K, Felszeghy K, Mehra RD, Radák Z, Nyakas C. Are the neuroprotective effects of estradiol and physical exercise comparable during ageing in female rats? Biogerontology. 2012 Jun 22. PMID: 22722983
IF:3.339

Marosi K., Bori Z., Hart N., Sárga L., Koltai E,, Radák Z., Nyakas C. Long-term exercise treatment reduces oxidative stress in the hippocampus of ageing rats. Neuroscience. 2012 Sep 12. pii: S0306-4522(12)00902-5.PMID:22982624
IF:3.380

Publications not related to thesis:

Marosi K, Horváth E, Nagy P, Köles B, Nagy ZB. Review of genetic research and testing in sport. Orv Hetil. 2012 Aug 12;153(32):1247-55. Hungarian.

Marosi K, Agota A, Végh V, Joó JG, Langmár Z, Kriszbacher I, Nagy ZB. The role of homocysteine and methylenetetrahydrofolate reductase, methioninesynthase, methioninesynthase reductase polymorphisms in the development of cardiovascular diseases and hypertension. Orv Hetil. 2012 Mar 25;153(12):445- 53. Review. Hungarian. PMID:22411217

Börzsönyi B, Demendi C, Pajor A, Rigó J Jr, **Marosi K**, Agota A, Nagy ZB, Joó JG. Geneexpressionpatterns of the 11β-hydroxysteroid dehydrogenase 2 enzymein human placenta from intrauterine growth restriction: therole of impaired feto-maternalglucocorticoid metabolism. Eur J Obstet Gynecol Reprod Biol. 2012 Mar;161(1):12-7. Epub 2012 Jan 11. PMID:22239940
IF:1,974

Hornyák I, **Marosi K**, Kiss L, Gróf P, Lacza Z. Increased stability of S-nitrosothiol solutions via pH modulations.Free Radic Res. 2012 Feb;46(2):214-25. Epub 2012 Jan 12.
PMID:22149535
IF:2,878