The effects of chronic active and passive exercise in the aging rats

Abstract of PhD Thesis

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1 Introduction

1.1 The aging brain

It is known that the number of people (over 60 years of age) in the elderly population is increasing dramatically with the average age. As the age progresses, many physiological changes occur in the body and there is a higher risk of developing various diseases. The 60-year-old is generally characterized by impaired hearing, vision, movement, and heart and chronic respiratory disorders, including stroke, cancer, and dementia. However, contrary to popular belief, dementia is not an inevitable consequence of aging, with adequate mental and physical activity to prevent cognitive decline during illness: it primarily affects short- and long-term memory and movement. The most common form is Alzheimer's disease and vascular dementia. Current drug therapies do not know how to prevent and stop dementia, they can only delay the progress of the already established process. During aging, the brain mass and the number of synapses in each brain area are reduced. There is also a decrease in neuronal activity and the number of neurotransmitters. The number of cholinergic neurons decreases in Alzheimer's disease. At the center of my research is studying the aging of the hippocampus. This brain area is part of the limbic system and plays an important role in confirming long-term information from short-term and spatial memory. As the age progresses, the hippocampus also changes: its size, activation, nerve fiber, and the number of neurons.

1.2 Active exercise

It is well recognized that chronic physical exercise exerts a pro-cognitive action on the brain. It may help to attenuate progression of different neurodegenerative diseases and support long term sensorimotor functions. The intimate anatomical and functional connections between the motor and cognitive brain structures are clearly demonstrated in both rodents and human studies. The combining physical and mental activity has an additive effect on initiation and survival of newly born hippocampus cells. In another rodent study, it was found that exercise resulted in an improvement of spatial reference memory studied in Y-maze task. In human, physical activity has induced larger regional brain volumes in older adults also indicating that the function of motor and cognitive brain structures are closely cooperative.

1.3 Passive exercise

Advanced aging is accompanied by functional deteriorations in different organs including the neuronal and sensory-motor systems. Aging in rats alters learning and memory functions leading to cognitive disturbances and movement instability. Along this period of life for both active and passive exercise may be a significant therapeutic intervention to maintain optimal physical and mental conditions. The active types of exercise are common in practice, but their applicability is often hampered by motor and psychosocial constraints of the elderly. Passive types of exercise tend to play an additional or complementary role in maintaining the elderly's physical and mental conditions. On the other hand, translational experiments on animals may be a useful approach for detecting the underlying mechanisms of physiological action of passive exercise. In addition, animal experiments may more easily assist to uncover accidental unwanted side effects by outlining the physiological limits of interventions.

1.4 Cholinergic Neurotransmission

Cholinergic neurotransmission plays an important role in the skeletal muscle and in the autonomic and parasympathetic nervous system in addition to the central nervous system. The key molecule of transmission is acetylcholine, which is produced in cholinergic synapses from choline and acetyl-coA. The process is catalyzed by the choline-acetyltransferase (ChAT) enzyme. As the cholinergic neurons grow older, the expression of acetylcholine receptors changes, so since cholinergic pathways play a major role in cognitive functions, their degeneration leads to a decrease in memory and learning ability. In the case of neurodegenerative diseases, the outflow and recovery of choline from synapses is reduced.

1.5 The role of BDNF in the exercise

Determining the amount of brain-derived neurotrophic factors (BDNF) found in the brain and muscle is significant in these experiments. BDNF performs essential brain functions, including the process of learning and memory. BDNF is an important neurotrophic factor that plays an important role in brain synaptic relationships and synaptic plasticity. The signaling pathways are activated by active exercise. Alzheimer's model animals have already demonstrated that BDNF increases as a result of active exercise. It is known, therefore, that BDNF expression plays an important role in the beneficial effects of exercise.

2 Objective

2.1 Effects of chronic active exercise on brain aging in male rats of different ages

A1. Chronic active exercise can improve the short and long term memory of rats.

A2. Chronic active exercise can improve the spatial learning ability of rats.

A3. Regular exercise has a positive effect on the horizontal and vertical activity of older rats.

A4. In terms of the density of cholinergic fibers, active exercise reduces their death in some brain areas of older rats.

A5. Long-term exercise can have a positive effect on decreasing acetylcholine levels, thus affecting cholinergic neurotransmission in the elderly in certain areas of movement and memory of the brain.

2.2 Effects of chronic passive exercise on brain aging in elderly male rats

B1. Chronic ELF-EMF therapy improves the short and long-term memory of elderly rats.

B2. Regular ELF-EMF therapy affects the spatial learning ability of older rats by improving it.

B3. Chronic EMF therapy has a positive effect on activity in elderly rats (horizontal and vertical activity).

B4. Based on the studies detailed above, I suppose that ELF-EMF also has a positive effect on brain trophic processes, thus increasing the amount of BDNF in the hippocampus.

3 Materials and methods

3.1 Animal experiment design

Experiment A

In total, we used 55 male Wistar rats of different ages (12, 24 and 32 mo of ages) for this study. Animals were housed in a room maintained at 22 ± 1 °C with a 12:12-h light/dark cycle (light on at 7:00). The relative humidity of animal's house was 40–50 %. Food and tap water were available ad libitum.

The animals of each age were divided randomly into experimental or sedentary control groups. The experimental groups were subjected to exercise of moderate intensity walking on a rodent treadmill (see Fig. 1). Treadmill training went on for six months, three times a week and 40 minutes each daily session. The incline of running belt constantly stayed at 0 %. The walking speed was increased gradually along the first two weeks from 6 m/min until reached the velocity of 18 m/min which corresponded to an average VO2 max of 60 %. Animals from the sedentary groups were placed on the treadmill for the same period as the trained animals at each session without receiving any exercise training.

Experiment B

All experiments were carried out by using 30-32 months old male Harlan-Wistar rats (430- 500 g) from our own colony. Animals were housed under standard laboratory conditions (12:12 h light–dark cycle with lights on at 07.00 h, 23°C temperature, and 40–50% relative humidity), food and water was provided *ad libitum*.

The rats were randomly divided into four groups: control group (sham exposure, n=11), 45 μT group (with 45 μT EMF exposure, n=8), 95 μT group (with 95 μT EMF, n=11), and 1250 μT group (with 1250 μT EMF n=10). It may be added for comparison that the EMF potential of the Earth is varying from 30 to 60 μT. A single plastic animal cage was placed on the pillow in the case of applying the two lower intensities. The Helmholtz adapter of the instrument was applied for providing the high intensity of 1250 μT. From the age of 30 months the rats were treated with pulsed EMF stimulation for six weeks as it is indicated in Table 1. Each stimulation session lasted for 24 min. For the control animals of the same treatment condition was set up without EMF exposure (sham stimulation).

3.2 Behavioral testing

In the open field test (OF), we measured vertical and horizontal activity of rats principally as described by us earlier. Open field test box consisted of a circular arena with 80 cm in diameter, which was subdivided into 20 subsectors by concentric and radial lines, and surrounded by a 45 cm high aluminum wall. The arena was lighted with a bulb of 40 W which was positioned 60 cm above the floor of the apparatus. Each animal was placed in the center of the open field and the novelty-induced psychomotor activity was measured by direct visual observation for 5 min. The arena was cleaned with a wet sponge and a dry paper towel between testing each animal. We counted the intensities of horizontal and vertical exploratory activities, i.e. number of line crossings by walking and number of rearing activity, respectively. If the single rearing response lasted for more than 1 sec, another score was added at every further second to measure the intensity of vertical mobility.

Novel object recognition was evaluated essentially as described previously and was tested in the habituated OF arena two days after the OF motility measurement. Briefly, during the first session (sample trial) two identical objects were placed in the arena keeping equal distances from the wall in an asymmetric position regarding to the center. These objects became familiar objects during the free 5 min exploration period. After 120 min intersession intervals spent in the home cage, the rats were replaced into the open field arena for the second session for another 5 min (test trial). During the second session, one of the familiar objects was replaced by a novel object. Frequency (total number of visits) and duration (total time spent with visiting objects in sec) were recorded. If an animal was not exploring the objects at least five times during the test trials, it was not included in the statistical analysis because did not reach our behavioral criterion in this test. For evaluating behavioral performance in recognizing the novel object (NOR, recognition index in percent) against the familiar one the following calculation was applied:
recognition index (%) = [duration of visits to novel object / (duration of visits to novel + familiar objects)] x 100.

The Morris water maze spatial learning test (MWM) was performed basically according to the original description in a round black water tank (diameter 153 cm, height 63 cm) filled to a depth of 53 cm with water of 24°C. A black hidden platform (diameter 10.8 cm) that was located at a fixed position was submerged 1.5 cm below the surface of the water.

Four trials with different starting positions were spaced around the perimeter of the tank. The animals had to learn the place of the hidden platform guided by different cues in the surrounding of the experimental chamber. Being dependent on age 5 or 6 sessions was applied. Rats received four trials per each session. The order of starting positions varied randomly by trials, but remained stable during a session. Each trial lasted until rats had the platform. If the platform was not found in the course of 90 seconds, the experimenter led the animal to it. Rats spent 30 seconds on the platform at the end of each trial.

Time spent to find the platform was measured at each trial and registered as latency in seconds. During each session the latency time of the first trial served for reference memory (RM) recording and the mean latency time of the daily 4 trials for working memory (WM) counting. To evaluate the learning performance in the Morris water maze test, we performed two-way analysis of variance (ANOVA) with repeated measures as one of the factors and groups for the other including the entire acquisition period.

3.3 Immunohistochemistry analysis

Twenty four hours after the last exercise training the rats were sacrificed in deep sodium pentobarbital anesthesia by transcardial perfusion with 250 ml fixative composed of 4% paraformaldehyde and 0.05 % glutaraldehyde in phosphate buffer (PB, 0.1 M, pH = 7.4), which was preceded by a quick pre-rinse (60 ml) with heparinized physiological saline. After post fixation of the brains for 48 hours in the same fixative the brains were kept in 0.1 M PB containing 0.1% Na-azide until histological examinations. For histological processing the brains were dehydrated by storage in 30 % sucrose and sectioned on a Leica cryostat microtome at a thickness of 20 μm to obtain coronal sections at the level of dorsal hippocampus, primary motor cortex (M1) and somatosensory cortex (S1) according to the coordinates (Bregma - 3.30 ± 0.25 mm) of stereotaxic atlas of Paxinos and Watson.

ChAT immunoreactive swollen fiber varicosities and enlarged axon fragment were selected for quantification of fiber aberrations by means of computer-assisted image analysis (Quantimet 600HR, Leica, Germany) as described earlier. Briefly, using 20 x primary objective magnification and no. 600 emission filter, and following shading corrections and background subtraction, an optimal threshold level was selected, which was kept constant at every separate area measurement throughout the entire quantification procedure. Following unbiased manual delineation of areas around the clusters of swollen fibre varicosities (called

infiltrated area), the quantitative determination of the surface area of structural malformations (i.e. fiber aberrations) was performed. With the computer program the net area of aberrations was highlighted and computed in calibrated μm^2 while the normal thinner fibers remained unselected. Density of fiber aberrations were counted on three sections (within Bregma -3.30 ± 0.25 mm) and the average values were taken for further analysis regarding to three brain areas: the hippocampus CA1-subiculum, the dentate gyrus (DG) and the neocortical area (NC) covering the motor and sensory cortex and medially also the retrosplenial cortex (see Fig. 10 as well). The results were expressed as the averaged area of aberrations per single brain section.

3.4 Molecular biology

An immunostaining procedure on free floating coronal sections was applied to visualize choline acetyltransferase (ChAT) positive axon ramifications in the target brain areas, i.e. in the hippocampus, in motor and somatosensory neocortex. Briefly, the primary antibody was a goat anti-ChAT (AB144P, Chemicon) which was used at a dilution rate of 1:500 to visualize cholinergic fibers. Biotinylated rabbit anti-goat IgG and Vectastain ABC kit were available from Vector Laboratories (CA, USA). Staining was completed with nickel-enhanced diaminobenzidine (DAB) reaction in the presence of H_2O_2.

4 Results

4.1 Open field activity – active exercise

Number of crossing and the number of rearing are indicated at the three ages. Data were analyzed by two-way ANOVA followed by *post hoc t*-test. Eight to ten animals varied in the groups. Significant declines in activities against the 12 months old age are indicated by asterisks: *$p < 0.05$, **$p < 0.01$. Interestingly, exercised rats (Ex) maintained their horizontal walking activity (left panel) in the arena throughout all ages, which resulted in significant difference among groups at the age of 32 months (#$p < 0.05$ vs. sedentary controls). Further decline in crossing and rearing activities from the age of 24 mo to 32 mo was only significant in the untreated controls, see the horizontal lines above columns. No decline was found between the exercised groups along these ages (ns – not significant).

4.2 Open field activity – passive exercise

Number of line crossings as horizontal activity did not change significantly [$F(3.35) = 1.33$, $p = 0.28$] only a tendency of the increment in this behaviour could be found. Numbers of rearing as vertical activity, however, was influenced by the chronic pulsed EMF exposure [$F(3.35) = 3.18$, $p = 0.036$]. The higher doses increased this type of activity, i.e. standing up on two feet was more frequent in the middle and high dose treated groups ($p = 0.020$ and $p = 0.0090$, respectively).

4.3 Novel object recognition test – active exercise

Regarding general object exploration with two-way ANOVA only an age related effect could be revealed by *post hoc t*-test compared to 12 mo: *$p < 0.05$, **$p < 0.01$. On the other hand, in recognizing the novel object a group (treatment) difference could be found by two-way ANOVA. That was caused by the difference at the oldest age of 32 mo between sedentary (Sed) and exercised (Ex) rats (#$p < 0.05$ vs. sedentary group). All groups showed a clear NOR response against the chance level, the only exception was the 32 mo old control group ($_{x}p = 0.75$). Furthermore, NOR performance from the age of 24 mo to 32 mo declined in the control group indicated at the horizontal line above columns ($p < 0.05$), but in the

exercised group there was no age-related decline in the discrimination ability. Number of animals varied from 8 to 10 per group.

4.4 Novel object recognition – passive exercise

Pulsed EMF exposure improved novel object recognition [F (3.37) = 7.82, p = 0.00040] and both the middle (p = 0.00087) and the high (p = 0.0043) doses were effective on the ability to discriminate between novel *versus* familiar objects compared to Sham controls. Since basic recognition capability for discrimination is only present in the individual groups if the ratio of visiting novel vs. familiar objects is significantly above 50%. This criterion was met only by the middle and high intensity groups (p = 0.000009 and p = 0.00063, respectively, calculated by testing the mean performances against the reference constant of 50%). At the level of 45 μT intensity the difference only approached significance (p = 0.056). The Sham control group clearly did not show any sign of recognition which is usual at this very advanced old age. Evaluating the interaction between sensory-motor abilities in OF test vs. NOR cognitive capability was estimated by correlation analysis including all groups. It was found that both the vertical and horizontal activities positively correlated to NOR performance (n = 38; rearing: r = 0.626, p = 0.000; walking: r = 0.407, p = 0.021) suggesting the presence of a positive impact of motor abilities on cognition.

4.5 Morris water maze test – active exercise

Progression of learning slowed down at the two higher ages along the consecutive sessions. Based on calculations with two-way repeated measure ANOVA, there was an exercise induced difference only at 32 mo of age. The *post hoc t*-test results: #p < 0.05, ##p < 0.01 vs. sedentary control group. Number of animals in groups ranged from 8 to 11.

4.6 Morris water maze learning – passive exercise

Regarding all groups the reference memory (left panel) improved along the sessions (repeated measures ANOVA: F (6.204) = 7.21, p = 0.000001). However, there was no significant differences among the groups [F (3.34) = 0.40, p = 0.75]. In case of working memory there could also be found an improvement along the sessions (repeated measures ANOVA: F (6.204) = 5.11, p = 0.000065). However, in this memory parameter a difference

among the groups could be revealed: F (3.34) = 3.30, p = 0.032. Furthermore, the interaction between the two factors, i.e. session and group, approached significance: F (18.204) = 1.47, p = 0.10. Lastly, post hoc t-test disclosed that at the last two sessions only the group treated with the highest intensity performed better as compared to the sham control group (p = 0.012 at session 6, and p = 0.0090 at session 7).

The spatial learning performance in MWM test was also compared by correlation to the spontaneous sensory-motor activity levels in OF test, i.e. walking and rearing scores.
Regarding RM no correlations were found. Correlation analysis regarding WM, however, revealed that rearing correlated with the cumulative latency time counted throughout all the seven sessions (n = 38; rearing: r = -0.498, p = 0.002). This means that the higher rearing activity is correlated to a decreased latency time founding the hidden platform.

4.7 Immunohistochemistry analysis – active exercise

Significant decline in ChAT fiber density against the 12 months old age is indicated by asterisks: **p < 0.01. The only treatment effect of chronic exercise could be revealed at the age of 32 mo (#p < 0.05). Another difference between the exercise vs. sedentary groups was found by comparing decline in fiber density from the age of 24 to 32 mo (*post hoc t*-test). Each group contained 7 animals.

The age-related decline of ChAT positive fiber density in the hippocampus DG area ($F_{2,38}$ = 152.7, p = 0.0001) from the supragranular stratum moleculare. Compared to 12 mo of young control age older rats showed a reduced amount of fiber density in both sedentary control and exercised groups. In this hippocampus area, there were no significant differences between the exercised vs. sedentary animals.

Significant decline in ChAT fiber density against the 12 months old age is marked with asterisks: *p < 0.05, **p < 0.01. Treatment effect of chronic exercise was found at both 24 and 32 mo of age (#p < 0.05). Measuring ChAT positive fiber density in the primary somatosensory cortex layer V also revealed the age related decline in fiber density ($F_{2,38}$ = 14.5, p < 0.0001). Two-way ANOVA analysis confirmed the effect of exercise as another factor: $F_{1,38}$ = 4.42, p = 0.042. Exercise attenuated the decrement of ChAT positive fibers in the senescent age.

4.8 Molecular biology – passive exercise

EMF treatment significantly increased BDNF levels for all three doses ($p = 0.0002$; $p = 0.0035$; $p = 0.00017$) in the hippocampus compared to the control group animals. The relative density of BDNF was higher by 33% in the 45 μT group and by 45% in the 95 μT group and 55% in the highest dose compared to the control group.

5 Conclusions

A1 Chronic active exercise improved the short-term memory of the 32-month-old rats in the experiment in the NOR test. The results of this group of active exercise animals were almost identical to this group of 24 months old animals.

A2 In the MWM test for reference and work memory, 32-month-old animals performed better than the control group, thus improving spatial learning ability and memory as a result of chronic active exercise.

A3 Regular exercise had a positive effect on the horizontal activity of tempered groups in experimental animals, age-related decline did not occur in the tempered groups, and the activity of 32-month-old physical exercise was higher than that of the control group. In vertical activity, age-related decline was not affected by exercise.

A4 In terms of the density of cholinergic fibers, active exercise reduces their death in some brain areas of older rats.

A5 Long-term exercise can have a positive effect on decreasing acetylcholine levels, thereby affecting cholinergic neurotransmission in the elderly in certain areas of movement and memory of the brain.

B1 Chronic EMF therapy improves the short- and long-term memory of elderly rats at two higher doses (92 and 1024 uT).

B2 Regular EMF therapy affects the spatial learning ability of older rats.

B3 Chronic EMF therapy has a positive effect on activity in elderly rats (horizontal and vertical activity).

B4 Based on the studies detailed above, I suppose that ELF-EMF also has a positive effect on brain trophic processes, thus increasing the amount of BDNF in the hippocampus.

6 List of own publications

6.1 Publications related to the dissertation

1. Téglás T, Dörnyei G, Bretz K, Nyakas C. (2018) Whole-body pulsed EMF stimulation improves cognitive and psychomotor activity in senescent rats. Behav Brain Res, 349: 163–168. . https://doi.org/10.1016/j.bbr.2018.04.036 **IF: 3,1732.**

2. Téglás T, Németh Z, Koller Á, Van der Zee EA, Luiten PGM, Nyakas Cs. **(**2019) Effects of long-term moderate intensity exercise on cognitive behaviors and cholinergic forebrain in the aging rat. Neuroscience, 411: 65-75. https://doi.org/10.1016/j.neuroscience.2019.05.37 **IF: 3,382**

6.2 Independent publications

1. Berekméri E, Deák O, Téglás T, Sághy É, Horváth T, Aller M, Fekete Á, Köles L, Zelles T. (2019) Targeted single-cell electroporation loading of Ca2+ indicators in the mature hemicochlea preparation. Hear Res, 371: 75–86. https://doi.org/10.1016/j.heares.2018.11.004 **IF: 2,824**