ALZHEIMER'S DISEASE: THE ROLE OF EXERCISE AND MICROBIOME IN A TRANSGENIC MICE MODEL

Abstract of PhD Thesis

Dóra Ábrahám

Doctoral School of Sport Sciences
University of Physical Education

Supervisor: Dr. Zsolt Radák professor, DSc

Official reviewers: Dr. László Tretter professor, DSc
Dr. László Virág professor, DSc

Head of the Final Examination Committee:
Dr. József Tihanyi rector emeritus, DSc

Members of the Final Examination Committee:
Dr. Levente Rácz senior research fellow, PhD
Dr. Andor Molnár college associate professor, PhD

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1. Introduction

Alzheimer's disease (AD) is the most common form of dementia worldwide, it was first described by Alois Alzheimer back in 1907. AD is responsible for 50-75% of the dementia cases and it affects mainly the elderly, on top of that it rises to be the second largest cause of death in western countries and yet without an effective medical treatment. The disease has several clinical and pathological symptoms. Clinical symptoms are ranging from mild to severe in accordance with the disease progression. In the beginning patients have impairments in short term memory, they have verbal difficulties and they feel lonely and depressed. Later on these problems are growing to difficulties in everyday tasks such as grocery shopping, breakfast preparation and results in social separation. At late stages patients have navigation skill problems like after a short walk cannot remember how to go home, they have long term memory problems they cannot remember to their family. AD has 6 stages and diagnosis happens usually at stage V. when it is already in a progressive state. In the last stage patients need continuous surveillance. AD has two incidences, sporadic and familial. Sporadic is the late onset form when a spontaneous gene mutation happens meanwhile familial is hereditary and comes with early onset. Sporadic AD is more common, it is responsible for 99% of the cases.

There are numerous molecules responsible for this disease. Most frequently affected molecules are: amyloid precursor protein (APP), Presenilin 1 and presenilin 2 (PS1, PS2), Neprilysin and neprilysin 2 (NEP, NEP2), Apolipoprotein E (ApoE) and Tau protein.

Several transgenic mouse models are available to mimic the disease progression where at least one of the above mentioned molecules are present. Models are representing the familial form of the disease because AD is naturally not present in mice. However numerous models are available, no one is able to present the disease as it happens in humans. It is only possible to get a better view on specific pathways of the disease.

Oxidative stress may play a major role in disease development and progression. It is believed that damage caused by oxidative stress happens a long before AD can be detected. Main factor in AD can be the DNA damage which is most commonly repaired

with 8-oxoguanine DNA glycosylase 1 (OGG1). The level of OGG1 is usually significantly lower in AD patients than in healthy individuals.

Microglial activation has a critical role in β-amyloid (Aβ) degradation. Microglia is usually in an activated state around the plaques. Unfortunately uncontrolled microglial activation may lead to serious inflammatory response causing neurodegenerative diseases such as AD.

There is no effective cure for the disease but there are several preventive methods available. Regular exercise is proven to be beneficial in several health conditions. Physical activity can induce neurogenesis in the hippocampus on top of that it can elevate the levels of OGG1 in the brain thus it can help neutralize the damage made by reactive oxygen species and it can be protective against central nervous system diseases like AD. Marathon type activity was investigated by several research groups while interval type training got in the scope of science lately. Interval type training may have similar effects on cognition as marathon type training, and may be also effective as a preventive method.

The role of microbiome in prevention is not proven yet, but there are some promising results. The microbiome is a continuously changing environment where several bacterial species are present. The composition of the microbiome depends on age, environment, eating habits, physical activity, and diseases. While physical activity, a healthy diet and a stable environment favors the enrichment of beneficial bacterial strains, aging and diseases are in favor of injurious bacteria. It was observed that in early stages of AD probiotic usage can be beneficial, it can help maintain cognitive function and it can be in favor of antioxidants. Also, it can help the appearance of butyrate and vitamin producing bacteria in the gut and promote the colonization of beneficial bacterial strains such as *Lactobacillus spp.* Since AD occurs at old age the presence of leaky gut cannot be ignored. Leaky gut is a collective nomenclature for insufficient mucus layer in the gut paired with epithelial inflammation. Leaky gut is more common when a patient suffers from persistent stress, is at an old age and has a chronic disease. Taking into consideration the above mentioned factors there is a huge chance for AD patients that they have a leaky gut and their microbiome is in an interrupted state.

2. Objectives of the study

The main objective of the study was to investigate if interval treadmill running and specific probiotic lysate supplementation can delay the onset of dementia in APP/PS1 transgenic mice.

We have found several publications with the positive effects of marathon type exercise on the development of AD and we were interested if high-intensity interval exercise can have similar positive effects on the cognitive functions. Moreover, it was proved that higher running speed results in greater metabolic demand for the brain. Because we wanted to test interval training effects, which relies on continuous speed control we could not use voluntary exercise model.

We also wanted to reveal that a specific probiotic lysate supplementation can significantly modify the composition of the gut microbiome. And if yes, can these changes make such a big difference in cognition? Is it possible that changes in microbiome can enlarge the positive effects of exercise?

It was at the center of our interest that these two types of treatments can be effective alone, or they can have more beneficial effects if they are applied together. Is it possible, that these preventive methods can postpone the development of AD?

3. Materials and methods

I. Animals, experimental groups

Thirty-two male APP/PS1 transgenic mice (B6C3-Tg (APPswe, PSEN1dE9)85Dbo/Mmjax) were used in our experiment. Mice were randomly assigned to four groups (n=8 per group) control APP/PS1^{TG}-C, exercise APP/PS1^{TG}-Ex, nutrition APP/PS1^{TG}-Pr and combined (exercise and nutrition) APP/PS1^{TG}-ExPr group. An additional wild type control group was added to our experiment (n=10).

We have transferred the mice to our animal house when they have reached age 100days. After the transportation has been made, animals had a week of adaptation period. All animals were caged individually and as mice usually lives in colonies we needed to use environmental enrichment. The animals were provided water ad libitum, and they have got 5 gramms of classic rodent chow daily. We kept a 12:12 hour light-

dark cycle where the light cycle coincided with daytime. All experiments were carried out during the light phase. We have used interval treadmill running and specific probiotic lysate supplementation to test their effects on AD. In our study we have used APP/PS1 transgenic mice in which Abeta plaques are developing from as soon as 6 months, showing cognitive impairment as well. Tau aggregates are not present in our model. Animals were housed individually, because we needed to measure the probiotic intake per animal day by day. Hence for the well being of the animals we needed to use environmental enrichment which consisted of a big cage equipped with a plastic tunnel. Further than this water was given from a bottle where a rolling ball was in the way of water providing other stimuli to the mice. Running wheels were not implemented because that would interrupt the study design.

II. Experimental design, exercise protocol

All treatments were carried out for 20 weeks. Interval treadmill running was applied for the exercise and combined group. Previously all exercising animals were habituated with the motor-driven treadmill (Columbus Inst. Columbus Ohio) and the running speed for 2 weeks. Training was performed four times a week, for 60 minutes. Each training session lasted 10 cycles, each cycle consisted of 4 minutes of high intensity and 2 minutes of low intensity running. Low intensity running speed was permanent during the experiment meaning a speed of 10m/min. While high intensity running speed started at 16m/min and was elevated every third week with 1 m/min until 20m/min was reached. Control and Nutrition group were also habituated with the treadmill and stayed there for 5 minutes/day on a standing treadmill.

Nutrition supplement called Framelim® were given 5 times a week 120mg/day for 20 weeks along with the rodent chow. Framelim® contains Bifidobacterium longum and Lactobacillus acidophilus lysates among with B1, B3, B6, B9, B12 vitamins resolved in cod-liver oil. The positive effects of this exact supplement in irritable bowel syndrome and improvements in bowel- and neuropsychiatric symptoms have been reported. We have monitored daily food and probiotic lysate uptake, probiotic lysate supplementation did not influence the eating and drinking habits of the animals.

After the 20 week long treatment, animals were exposed to 2 weeks of cognitive testing. We have performed Morris water maze test, Y maze test, open field test and

novel object recognition test. After all the cognitive tests were finished, animals were anaesthetized with an intraperitoneal injection of ketamine (Richter, concentration: 100mg/ml) /xylazine (Produlab Pharma, concentration: 20mg/ml) cocktail in a dose of 0.1 ml/ 10g bodyweight and transcardially perfused with heparinized ice-cold saline.

Brain was removed and measured rapidly, afterward dissected in half along corpus callosum. One hemibrain was postfixed in 4% paraformaldehyde (PFA) for immunohistological staining, the other hemibrain was dissected into 3 parts (frontal, parietal and occipital), and furthermore hippocampus was taken out. All parts were collected, frozen in liquid nitrogen and stored in -80°C until further biochemical analysis.

Fecal samples were also collected for microbiome analysis.

III. Cognitive tests

Morris water maze test

The test was performed on four consecutive days, each day consisted of four trials. A circular pool with 60 cm in height and 100 cm in diameter were filled with water, and then a six cm wide circular platform was placed in the center of the northwest quadrant of the pool, just one cm below the water surface. Water temperature was maintained between 22° and 23° C throughout all sessions. The test was conducted in darkness so there was no need of water opaquisition.

Four starting points were used (north, south, west, or east) and mice were allowed to find the platform for a 60 second period. Each day the order of starting points were mixed. After the 60 second trial animals were allowed to rest on the platform for 30 seconds. This protocol was used for every animal in the experiment. Animal order was randomly assigned. The platform was in the same place on every trial. The latency time to find the hidden platforms was recorded.

Open field test

With this general test it is possible to measure locomotor and exploration activity as well as anxiety. A circular field is used, where outer and inner areas were set in order to measure these spontaneous activities. The mouse was placed in the middle of the circle as a starting point, afterwards for a 5-minute time period inner and outer area crossing, rearing, grooming, and latency time was measured and then analyzed.

Novel Object Recognition test

This test is performed in the same arena as the Open field test. It can be used the day after the open field test was performed, thus open field test can serve as a habituation test as well. Here the recognition capacity of the mice is evaluated as well as their tendency to restart exploring when they are presented to a novel environment. This trail consists of 2 consecutive test days. On the first day 2 items which are the same size, color and material are placed in the arena. Mice can observe these 2 objects for a certain period of time, and time spent with object observation is recorded. On the second test day 1 item is changed to another one which differs in size and material a so called new item. Observation time within old and new object is measured, than analyzed.

Spontaneous alternation test

With this test it is possible to investigate the natural behavior of mice, the willingness to explore new areas. In healthy animals it is observed that they are exploring new arms of the Y shaped maze instead of previously visited ones. Maze arms are set 120 angles apart, starting point is in the middle. Animals are placed individually in the starting point of the maze then they could freely explore the arms. We have used a 5-minute trial with a check point at 3 minutes, where latency time and correct alteration was measured. It is counted as a correct alteration when the current entry of the mice differs from the two previous ones, and the 2 previous ones are also different. High rate in alteration refers to sustained cognition. Entry is counted when all 4 limbs are inside on of the arms.

IV. Immunohistochemistry

After conservation in 4% PFA for a day brain hemispheres were washed in 0,1% phosphate buffer (PB) in room temperature. Then hemispheres were put into 15% saccharose solution for 4 hours and then placed in 30% saccharose solution overnight in 4° Celsius. Tissue was than embedded in cryoprotectant (Tissue Tek, Sakura Finetek Europe Ref. 4583) over liquid nitrogen.

Brains were than sectioned in a Leica Sliding Microtome (Model SM2000R) to 40 μm coronal sections and stored in PB with sodium-azide.

β- amyloid and OGG1 staining

Every sixth free- floating brain section of groups was immunostained for amyloid plaques (6E10, Anti-β- amyloid, 1-16 antibody, BioLegend #803015).
DNA repair enzyme (Anti-OGG1 Abcam #ab22766) was also visualised with immunostaining.

Double labeling 6E10 and Iba1

Brain sections of animals were processed for double labeling with 6E10 and Iba1. Every sixth free-floating section was first stained for Iba1 (AIF/Iba1 Novus biologicals # NB100-1028) by using FITC-tyramide amplification fluorescent immunocytochemistry. Sections were then incubated overnight in 6E10 (1:7000; BioLegend #803015) at room temperature.

Microscopy and image processing

An Olympus BX60 light microscope equipped with fluorescent epi-illumination and a dark-field condenser were used to examine the sections. Images were captured at 2048 × 2048 pixel resolution with a SPOT Xplorer digital CCD camera (Diagnostic Instruments, Sterling Heights, MI) using 10-20 × objectives. Images were adjusted using the "levels" and "sharpness" commands in Adobe Photoshop CS5.1. Full resolution of the images was maintained until the final versions, which were adjusted to a resolution of 300 dpi. Images were analyzed with ImageJ Software version 1.48v.

Library preparation and identification of prokaryotic species

The DNA from stool samples was isolated by QIAmp Fast DNA stool mini kit (Quiagen). Fragment libraries were constructed from purified DNA using NEBNext Fast DNA Fragmentation & Library Prep Set for Ion Torrent (New England Biolabs) according to manufacturer's instructions. The library templates were prepared for sequencing using the Life Technologies Ion OneTouch protocols and reagents. Finally, sequencing was performed with the Ion PGM Hi-Q view OT2 Kit (Life Technologies).

V. Statistics

In the beginning of the experiment transgenic animals were randomly assigned to the following groups: Control-APP/PS1^{TG}-C, exercise- APP/PS1^{TG}-Ex, nutrition-APP/PS1^{TG}-Pr and combined (exercise and nutrition) group- APP/PS1^{TG}-Ex-Pr.

An additional wild type control group WT was added to the experiment. Each transgenic group consisted of 8 animals while WT group consisted of 10 animals.

For statistical analysis, except for microbiome samples, we used GraphPad Prism 5 Software. For the evaluation of physiological and biochemical data, we first performed Shapiro-Wilk normality test of all dependent variables. Based on the result we performed one-way analysis of variance (ANOVA) followed by Tukey's post hoc test or Kruskal-Wallis ANOVA with Dunn's post hoc test. Significance level was set at $p < 0.05$.

Microbial analytical methods

Bacterial genome annotation was carried out by uploading the FASTQ data files to the automated web-based metagenomics analysis server-MG-RAST version 3.6. MG-RAST takes FASTQ data files as input, identifies open reading frames that are likely to be genes, and uses a series of subsystem techniques (the 'ST' in RAST) to compare these with a sophisticated database of genes and RNA sequences, producing a high-quality annotation of the assembly. The data files, annotated based on the RefSeq database were downloaded for further analysis. The MG-RAST ids of the datasets are the following: Wt (healthy control): mgm4672670.3; APP/PS1^{TG}: mgm4673335.3; APP/PS1^{TG} -Ex: mgm4682164.3; APP/PS1^{TG} -ExPr: mgm4682165.3; APP/PS1^{TG} -Pr: mgm4682167.3.

Bioinformatics analysis of the microbiome

The data were filtered based on the following criteria of annotation quality: minimum alignment length: 30 base pairs; minimum percentage of identity: 60%; maximal E-value: 10^{-5}. Then, the annotated reads of each sample were permutated in random order, and were divided into 10 non-overlapping subsets, containing 10% of the original data. This process was performed via Python script. The generated populations were used for calculating the relative abundances and standard deviations of selected microbial groups. The results were visualised in bar graphs. The significance of differences between the groups was tested with two-sample Kolmogorov-Smirnov test with $p<0.001$ significance threshold. These analyses were carried out in MATLAB.

4. Results

I. Cognitive test results

In cognitive test we used wild type (WT) mice (N= 10) to evaluate the performance of transgenic mice compared to this group. Also, we have analyzed the results only between the transgenic groups.

Spatial memory was tested with MWM test, where wild type mice did not outperform all transgenic groups. Interestingly APP/PS1^{TG}-Ex-Pr group performed the best among all study groups, indicating a well-preserved brain function.

If we investigate only the performance of transgenic groups, we will find significant differences among the groups. APP/PS1^{TG}-Ex-Pr group outperformed the other transgenic groups in the second third and fourth day.

On the second day APP/PS1^{TG}-Ex-Pr group outperformed the APP/PS1^{TG}-C group.

On the third day APP/PS1^{TG}-Ex-Pr group outperformed the APP/PS1^{TG}-Ex group.

On the fourth day APP/PS1^{TG}-Ex-Pr group outperformed the APP/PS1^{TG}-Ex and APP/PS1^{TG}-Pr group.

On the open field testing, we have found no significant differences in exploratory activity among the study groups. Animals from all groups entered in the inner and in the outer areas as well, which means that none of the animals suffered from anxiety, they were all ready to explore. There were no significant differences between inner nor in outer crossing.

Novel object recognition task. Open field test needs to be performed previous to the NOR test, because animals should explore the objects in the arena instead of the new environment. Mice recognize the old object as a familiar one, thus spending more time discovering the new one. APP/PS1^{TG}-Ex mice significantly spent less time with the new object compared to the group APP/PS1^{TG}-C.

Spontaneous alternation is a good method to measure cognitive deficits in transgenic mouse models. As data with WT mice shows well, AD has serious effects on cognition. APP/PS1^{TG}-C animals performed significantly poorer than WT animals if we measure the % of good entries.

II. Results from brain tissue investigation

Amyloid plaque staining with 6E10 antibody shows that amyloid plaque deposition was most abundant in the hippocampal region as it was expected in this mouse strain. Most plaques were observed in the APP/PS1^{TG}-C group. Compared to APP/PS1^{TG}-C group (31.7 ±9.0) and APP/PS1^{TG}-ExPr group (29.1 ± 6.7) in the APP/PS1^{TG}-Ex group (13.9 ± 6.7) significantly lower level of plaques were counted. Interestingly we can notice that all treated groups had a significantly lower level of area covered by plaques compared to APP/PS1^{TG}-C group.

We have measured the microglia number in the hippocampal area as well. In one hand we did not find any differences in the total number of microglia among the groups. On the other hand area covered by microglia was significantly higher in APP/PS1^{TG}-ExPr group (8.11 ± 1.3) than in the APP/PS1^{TG}-C group (5.36 ± 2.4)

OGG1 levels are decreased in AD. Exercise can raise the OGG1 levels in the brain. We have measured if exercise can modify the levels of OGG1. It turned out that OGG1 levels were the highest in the APP/PS1^{TG}-Ex group (7951 ± 4085), it was significantly higher than in other treated groups APP/PS1^{TG}-Pr (2947 ± 2222) and APP/PS1^{TG}-ExPr (3358 ± 1127). Average area demonstrates the same pattern.

III. Results from microbiome analysis

Differences in microbiome composition between WT mice and APP/PS1^{TG}-C mice are outstanding if we investigate them at genus level. *Bacteroides spp*. was significantly lower while *Prevotella spp*. was significantly higher in WT group compared to APP/PS1^{TG}-C group. *Bacteroides spp*. is a known succinate producer which can negatively modify mucin production, also low levels of *Prevotella spp*. suggest problems with mucin production in APP/PS1^{TG}-C group.

Butyrate-producing genuses such as *Clostridium spp. Eubacterium spp*.and *Roseburia spp*. were found in elevated levels in WT group also suggesting a more preserved mucin layer in the gut.

If genus differences are compared between the transgenic groups, it can be seen that exercise had a positive effect on butyrate producing genuses while probiotic lysate receiving group had the lowest levels of these bacteria.
The probiotic lysate supplement contained Omega 3 fatty acids which should elevate the levels of Lactobacillus spp and should decrease the levels of Clostridium spp. Our data shows evidence for this fact.

At the species level we can observe other interesting differences.
Comparing WT and APP/PS1^{TG}-C groups we can observe that Bacteroides species *Bacteroides thetaiotaomicron* and *Bacteroides fragilis* are present in a significantly lower level in WT group, again suggesting a presence of a better conserved epithelial layer. Elevated leves of butyrate producer *Butyrivibrio proteoclastus* is in accordance with the previous suggestion.
Levels of *Bacteroides thetaiotaomicron* were significantly higher in APP/PS1^{TG}-Pr compared to APP/PS1^{TG}-ExPr group, this result correlates well with results from MWM test. Elevated levels of this bacteria can suggest the presence of leaky gut syndrome because they produce acetate and succinate as the end product of glucose and lactose fermentation, Both products are responsible for decreased mucin production. Probiotic lysate elevated the levels of *Bacteroides fragilis*, interestingly exercise could modify this effect. Exercise receiving groups APP/PS1^{TG}-Ex and APP/PS1^{TG}-ExPr have a significantly lower number from this bacteria than APP/PS1^{TG}-Pr group. *Lactobacillus reuteri* is a well known B12 vitamin producer, which vitamin is essential for optimal brain function and it has an elevated level in APP/PS1^{TG}-Pr and APP/PS1^{TG}-ExPr group. *Lactobacillus johnsoni* which is found to generate excessive H_2O_2 has significantly lower levels in APP/PS1^{TG}-Ex group compared to APP/PS1^{TG}-C, proving an evidence for the positive role of exercise against oxidative stress. Butyrate producing bacteria had different abundance levels among the groups. These bacteria are *Butyrivibrio proteoclastus, Marvinbryantia formatexigens, Roseburia intestinalis* and *Roseburia inulinivorans* all of this had an increased level in exercise receiving groups APP/PS1^{TG}-Ex and APP/PS1^{TG}-ExPr compared to only probiotic lysate receiving group APP/PS1^{TG}-Pr.

5. Conclusions

There was an emerging interest lately for the function of the gut-brain axis and for the possible preventive methods for AD. For this reason, we investigated the effects of interval training and probiotic lysate supplementation on transgenic AD mice.

Exercise seems to be a preventive method for AD because it not only preserved the spatial memory of the mice but decreased the amyloid levels in the hippocampus. Moreover, OGG1 levels were elevated in the brain. In the microbiome of exercised animals, we could measure elevated levels of butyrate-producing bacteria, while on the contrary *Prevotella spp* was present only at a lower level. Further investigation is needed to decide which changes have a major effect on brain function. Also, we should investigate that leaky gut syndrome can underlie AD.

In our study we have used a probiotic lysate (heat-killed bacteria) which of course do not mimic exactly the role of living bacteria, still we observed several positive effects on the microbiome. B12 vitamin-producing bacteria were present at an elevated level in APP/PS1^{TG}-Pr group. Levels of *Lactobacillus spp* increased while *Clostridium* levels decreased which can be the effect of Omega 3 fatty acids. Surprisingly probiotic lysate decreased the level of butyrate-producing bacteria. Unfortunately, on cognition, we could not measure significant improvements thanks to probiotic lysate usage.

In summary: both treatments had beneficial effects on the course of AD, mostly in cognition and microbial composition. We could prove the advantages of exercise with better cognition, decreased plaque number, elevated OGG1 level and with and elevated number in butyrate-producing bacteria. Probiotic lysate did not have as many positive effects as exercise, but an elevation in *Lactobacillus spp* could be observed which can serve as a substrate for other beneficial bacteria. Future direction is to find the exact mechanism on the microbial-brain communication and to find all the products secreted by bacteria which can be in connection with AD either positively or negatively hence creating the opportunity to find an effective but non-invasive treatment for patients suffering from this disease.

6. List of own publications

Abraham D, Feher J, Scuderi GL, Szabo D, Dobolyi A, Cservenak M, Juhasz J, Ligeti B, Pongor S, Gomez-Cabrera MC, Vina J, Higuchi M, Suzuki K, Boldogh I, Radak Z. (2019) Exercise and probiotics attenuate the development of Alzheimer's disease in transgenic mice: Role of microbiome. Exp Gerontol, 115: 122-131.
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IF: 3.262

Not in connection with the thesis:
Perényi H, Szegeczki V, Horváth G, Hinnah B, Tamás A, Radák Z, Ábrahám D, Zákány R, Reglodi D, Juhász T. (2020) Physical Activity Protects the Pathological Alterations of Alzheimer’s Disease Kidneys via the Activation of PACAP and BMP Signaling Pathways. Frontiers in Cellular Neuroscience, 14:
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Szegeczki V, Horvath G, Perenyi H, Tamas A, Radak Z, Abraham D, Zakany R, Reglodi D, Juhasz T. (2020) Alzheimer's Disease Mouse as a Model of Testis Degeneration. Int J Mol Sci, 21:
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