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RESEARCH ARTICLE

Are the neuroprotective effects of estradiol and physical exercise comparable during ageing in female rats?

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Abstract Ageing of the brain is accompanied by variable degrees of cognitive decline. Estrogens have profound effects on brain ageing by exerting neurotrophic and neuroprotective types of action. Furthermore, exercise has also been claimed to play a role in the non-pharmacological prevention of psycho-neuronal decline with ageing. In the present study the question was asked whether chronic physical exercise might substitute the action of estrogens in aged rats. We compared the effects of 17β-estradiol (E2) treatment and long-term moderate physical exercise in ageing (15 months, early stage of ageing) and old (27 months) female rats, on cognitive functions and the relevant intracellular molecular signaling pathways in the hippocampus. Results showed that both treatments improved attention and memory functions of the 15 months old rats. Like E2, physical training enhanced the level of brain derived nerve growth factor and the activation of PKA/Akt/CREB and MAPK/CREB pathways. The treatments also enhanced the levels of synaptic molecules synaptophysin and synapsin I, which could explain the improved cognitive functions. In the 27 months old rats the behavioral and molecular effects of E2 were indistinguishable from those found in the 15 months old animals but the effects of physical exercise in most of the measures proved to be practically ineffective. It is concluded that the effectiveness of regular and moderate intensity physical exercise is age-dependent while the action of E2 treatment is comparable between the ageing and old female rats on maintaining cognition and its underlying molecular mechanisms.

Keywords Estradiol · Exercise · Ageing · Cognition · Hippocampus

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Introduction

Ageing of the brain is accompanied by structural and neurophysiological changes associated with various degrees of cognitive impairment (Baquer et al. 2009). 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. Postmenopausal estrogen replacement therapy can delay the decline in cognitive function (Phillips and Sherwin 1992) and reduce the risk of Alzheimer disease (Henderson et al. 1994; Kawas 1998). Estrogens affect neurotransmission functions (Luine et al. 1975), especially by enhancing the activity of cholinergic neurons (Abraham et al. 2009). Neurogenesis (Pawluski et al. 2009), neuronal survival (Jover et al. 2002), and synaptogenesis (Murphy and Segal 1997; Woolley and McEwen 1992) are also influenced by estrogen treatment in the hippocampus. In addition, estrogens can reverse the age-related oxidative stress in different tissues (Kumar et al. 2011a, b, c), indicating its potential role in the preventive type of modulation of the advanced ageing processes.

The neuroprotective and neurotrophic effects of estrogens are mediated by estrogen receptors through direct modulation of gene transcription (McEwen et al. 1990) by the activation of protein kinase B (PKB, Akt) (Honda et al. 2000) and mitogen activated protein kinase (MAPK) (Migliaccio et al. 1996) intracellular signaling molecules. The nuclear target of these molecular pathways is the c-AMP response element binding (CREB) transcriptional factor (Sharma et al. 2007), and its gene products are involved in synaptogenesis (Murphy and Segal 1997) and long-term memory formation (Miyamoto 2006). 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 (Rossouw 2010).

In addition to potential health benefits of pharmacological interventions numerous clinical (Colcombe and Kramer 2003; Heyn et al. 2004) and animal (Fordyce and Farrar 1991; van Praag et al. 2005) studies have supported the role of enhanced physical activity in the promotion of cognitive health during ageing. Exercise has been shown to upregulate the levels of brain derived nerve growth factor (BDNF) (Oliff et al. 1998), the intensity of neurogenesis (Lee and Son 2009; Choi et al. 2009) and the power of synaptic plasticity (Vaynman et al. 2006). BDNF activates PKA/Akt/CREB and MAPK/CREB pathways (Ying et al. 2002) indicating convergence in the molecular actions with estrogen. Moreover BDNF gene expression increases in vivo in response to estrogen (Singh et al. 1995) suggesting that estrogen may exert some of its beneficial effects through BDNF. 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.

Synapsin I and synaptophysin are presynaptic vesicle proteins which are considered to be markers of synaptic efficiency (Cesca et al. 2010). Moreover, synaptic proteins play an important role in learning and memory functions through their role in synaptic plasticity (Cesca et al. 2010). Decreases in synapsin and synaptophysin levels in the hippocampus correlate with cognitive decline and dementia (Schmitt et al. 2009; Sze et al. 2000). We have hypothesized that the protein levels of synaptic molecules might be increased via estradiol- and exercise-mediated signaling pathways as well. In the current study we have also hypothesized that the actions of exercise and estrogen are convergent to modulate intracellular signal transduction-related biochemical markers associated with improved cognitive performance. The action of combined exposure of aged rats to physical activity and estradiol treatments on cognition and neurotrophic intracellular pathways has not been explored yet. Therefore, we examined whether exercise, combined with estrogen administration might exert different actions as compared to the individual treatment effects alone. Since the sensitivity of the brain to different stimuli may change with age, the efficacy of the treatments in ageing and old female animals, i.e. 15 and 27 months old rats, respectively, was investigated. These two ages were selected and compared to each other in the evaluation of ageing related treatment effects. It is known that the normal cyclic female rats show a gradual decrease in serum estradiol level starting at the age of 12 months (Lapolt et al. 1988; Moorthy et al. 2005). Therefore, in the selected first age group treatments started at the age of 12 months and lasted for 15 weeks, i.e. in the course of early phase of 'postmenopausal' period attempting to couple this age period to human conditions. This group was named as 15 months old ageing group since the effects of different treatments were assayed at the age of 15 months. In the advanced age group—called old age group (27 months)—the treatments started at the age of 24 months and also lasted for 15 weeks.

Materials and methods

Animals and treatments

Thirty-two middle-aged (12 months old) and 32 aged (24 months old) female Wistar rats were selected for the study. Animals were housed in a temperature controlled room (22 ± 1 °C) equipped with a 12:12 h light/dark cycle starting the light period at 7:00 am. Food and tap water were available ad libitum. The ageing and old animals were divided into four experimental groups that were subjected to the following treatments: (1) 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), (2) exercise treatment (EX) included 30 min of moderate intensity running (speed: 18 m/minutes) on a rodent treadmill for 15 weeks, five times a week, (3) estradiol treatment combined with exercise (E2 + EX) and (4) sham-injected controls: only the sesame oil vehicle was injected twice weekly. After the 15 weeks period, the cognitive functions of the animals were tested. In the course of these tests the animals continued the daily exercise routine except at those experimental days when the Morris maze test was utilized. Estradiol treatment continued without cessation. Cognitive testing was followed by eight days of exercise before sacrifice which occurred 24 h after the last exercise treatment session. Animals were decapitated under light CO_2 anesthesia and the brains were quickly dissected. The two hemispheres were rapidly separated along the midline on an ice-cooled glass plate. The hippocampus was quickly excised bilaterally and immediately frozen on dry ice. The hippocampal samples were stored at −80 °C until processing. Uterus, pituitary gland, adrenals, and adipose tissue pads were collected, cleaned, and weighed. All the experimental procedures which were carried out on animals had been approved by the Animal Examination Ethical Council of the Animal Protection Advisory Board at the Semmelweis University, Budapest.

Cognitive testing

Novel object recognition (NOR)

In all behavioral tests the animals' behavior was video-recorded and the records were analyzed by manual scoring techniques. The NOR test was performed in a habituated open field arena as described earlier (Nyakas et al. 2009). This test allows to asses attention and memory based on differentiation. The diameter of the arena was 80 cm and it was surrounded by a wall of 35 cm high. During the first session rats were allowed to freely explore two identical objects, which were at an equal distance from the wall in an asymmetric position with respect to the center of arena. These objects became familiar to the animals during the 5 min of this session. After a 120 min delay period, spent in the home cage, one object from the first session was replaced by a novel object and the animals were tested for another 5 min (2nd session). The duration of visiting the novel and familiar objects was recorded in seconds. The recognition index was calculated as the ratio of duration of visits to the novel object, divided by the duration of visits to the novel plus familiar objects, and the ratio was multiplied by 100. Visiting of an object was defined as sniffing or touching the object with nose or forepaws. Any rats not exploring each object at least five times per session were excluded from the test. Lack of recognition of the novel object corresponded to the 50 % chance level, in which case novel and familiar objects were visited for the same duration. Numbers of animals satisfying the test criterion were: 15 months-C ($n = 7$), EX ($n = 8$), E2 ($n = 8$), E2 + EX ($n = 6$); 27 months-C ($n = 7$), EX ($n = 6$), E2 ($n = 6$), E2 + EX ($n = 6$).

Spontaneous alternation

Attention and working memory can be assessed by measuring spontaneous alternation in an Y-maze (Lalonde 2002). The test was performed in a black plastic Y-maze: 30 cm high, 10 cm wide, the arms were 50 cm long and converged at an angle of 120°. Each animal was placed into the centre of the maze and then allowed to move freely for 5 min. The number of arm entries was counted. Alternation was defined if the animal entered the arm different from the two previously visited arms. The relative alternation was calculated as the ratio of the number of alternations divided by the number of total arm entries minus one. The chance level was set as 33.3 %, which indicates lack of alternations. Any rats not entering at least 4 times to the arms of the box were excluded. Numbers of animals satisfying the test criterion were:

15 months-C ($n = 7$), EX ($n = 7$), E2 ($n = 7$), E2 + EX ($n = 6$); 27 months-C ($n = 7$), EX ($n = 8$), E2 ($n = 7$), E2 + EX ($n = 8$).

Morris water maze test

Spatial learning was assessed in the Morris water maze test (Morris 1984). The parameters of the maze were: 100 cm in diameter, 80 cm high, filled to a depth of 53 cm with water at 26 ± 1 °C. The surface of a platform (11 cm in diameter) was fixed 1.5 cm below the water surface and its position was not altered during the test (fixed platform test). The ageing 15 months old groups were tested for 5 days while the aged 27 months old groups were tested for seven consecutive days, due to the inferior learning capability of aged rats in this test. The animals were placed into the pool facing the wall from one of the equally spaced four start points, which was randomly changed in every trial. In each daily session, four trials were given, one from each quadrant of the pool. The order of starting position varied randomly between sessions, but was constant within the daily session. The trials lasted until each animal found the hidden platform or for a maximum of 90 s in case the platform was not found. It was followed by a 30 s inter-trial interval spent by sitting on the platform. The latency time to reach the platform was recorded at each trial. The exclusion criterion in the Morris water maze test was the lack of swimming ability by obvious somatic or healthy reasons like skin erosion for example. Numbers of animals satisfying the test criterion were: 15 months-C ($n = 7$), EX ($n = 8$), E2 ($n = 7$), E2 + EX ($n = 8$); 27 months-C ($n = 7$), EX ($n = 7$), E2 ($n = 6$), E2 + EX ($n = 7$).

Measurement of plasma 17β-estradiol and corticosterone levels

Trunk blood samples obtained with decapitation were heparinized and collected into glass tubes. The plasma was separated by centrifugation at 15,300×g for 20 min at room temperature. The samples were stored at −80 °C until use. The plasma 17β-estradiol and corticosterone levels were measured using the 96-well enzyme immunoassay kits (#5882251 Estradiol EIA kit, #500651 Corticosterone EIA kit, Cayman, USA). The assays were carried out according to the manufacturer's instructions.

Western blots

The hippocampi of animals ($n = 6$ in each group) were homogenized in lysis buffer containing 137 mM NaCl, 20 mM Tris–HCl pH 8.0, 2 % Nonidet P-40, 10 % glycerol and protease inhibitors. The homogenate was sonicated for 30 s in a cold pack. Lysates were centrifuged for 15 min at 15,300×g at 4 °C. Supernatants were collected and stored at −20 °C until use. The concentration of protein was determined using the Bradford assay (Bradford and Williams 1976). Twenty μg of protein were electrophoresed on 8–15 % (v/v) polyacrylamide SDS-PAGE gels. Proteins were electro-transferred onto PVDF membranes (Amersham, Piscataway, NJ). The nonspecific binding of immunoproteins was blocked with 5 % non-fat dry powdered milk for 2 hours at room temperature (RT). After blocking, the membranes were incubated with primary antibodies overnight at 4 °C. Antibodies were dissolved in Tris-Buffered Saline Tween-20 (TBS-T) containing 5 % non-fat powdered milk. The primary antibodies were: anti ERα 1:1000, sc-542, Santa Cruz; anti CREB 1:1000, #9197 Cell Signaling; anti p-CREB ser133 1:1000, #9198 Cell Signaling; anti MAPK 1:1000, #9215 Cell Signaling; anti p-MAPK 1:2000, #4631 Cell Signaling; anti Akt 1:2000, #9272 Cell Signaling, anti p-Akt 1:1000, #9611 Cell Signaling, anti-synapsin I 1:2000, #2315 Cell signaling; anti-synaptophysin 1:10000, ab23754 Abcam; and anti BDNF 1:1000, sc-546, Santa Cruz. 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 chemiluminescence reagent (ECL plus, RPN 2132, Amersham). The protein bands were visualized on X-ray films. The bands were quantified by Image J software, and standardized to β-actin (1:2000, sc-47778; Santa Cruz). With this software the optical density of the protein bands was measured. Results were expressed in relative density units compared to β-actin. The phosphorylation of the phosphoproteins was evaluated by dividing the phospho-specific form with the total form.

Statistical analyses

Comparing treatments at different ages, two-factorial ANOVA was used followed by the Fisher post hoc

t test. Results of ANOVA (F values, degrees of freedom and p values) as well as results of interaction between age effect and treatment effect were indicated in the text. Statistical significance was set at $p < 0.05$. Results of post hoc t test comparing the independent groups in both ages were indicated on the figures as followings: $*p < 0.05$; $**p < 0.01$ vs. 15 months old control; $^{\#}p < 0.05$; $^{\#\#}p < 0.01$ vs. 27 months old control. Both in NOR and spontaneous alternation tests the results of experimental groups were compared to chance level by paired t test in each group separately. The results of the Morris water maze test were analyzed by repeated measures of ANOVA. Means and standard errors of means (SEM) were presented to demonstrate the results. All statistical analyses were done applying the Statistica 8.8 program.

Results

Cognitive tests

The novel object recognition test (Fig. 1) showed that ageing itself caused a decrement in visiting novel objects as the old age groups compared to the ageing groups (two-way ANOVA, overall age effect: $F[1,45] = 6.43, p < 0.01$). Significant treatment effects were also confirmed by two-way ANOVA, $F[3,45] = 5.14$, $p < 0.05$, without an interaction with age. The Fischer post hoc t test revealed that exercise, 17β-estradiol and the combined treatment resulted in significant enhancement in attention of the ageing animals (see Fig. 1). Both E2 and the combined treatment enhanced NOR performance in the old rats. Each ageing (15 months) group visited novel objects significantly above the chance level of 50 % ($p < 0.01$), while the aged (27 months) controls and the physically trained group failed to do so. Aged animals treated with E2 and E2 + EX performed above the chance level ($p < 0.01$).

Spontaneous alternation in the Y-maze did not show significant changes in the course of further ageing (Fig. 2). The treatment effect proved to be significant with two-way ANOVA ($F[3,49] = 6.90$, $p < 0.01$). According to the post hoc multiple comparisons E2 and combined treatments increased the number of alternations in both age groups. Exercise improved performance in the 15 months old animals. Each group alternated significantly above the 33 % chance level based on the paired t test ($p < 0.01$). Using two-way ANOVA only for control and exercise groups revealed a significant treatment effect ($F[3,49] = 3.43$, $p < 0.05$). Post hoc t test between the old C and EX groups showed a significant difference indicating that exercise was effective in improving alternation in the old groups ($p < 0.05$, see also in the figure legend).

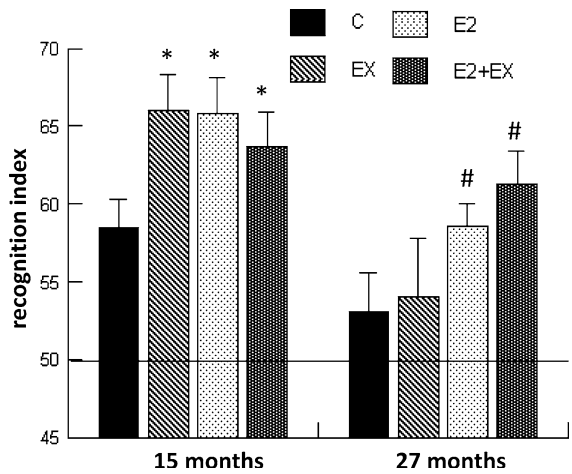

Fig. 1 Recognition index representing differentiation ability based on attention in the novel object recognition test is shown. The effects of exercise (EX), 17β-estradiol (E2) and combined treatments (E2 + EX) on the preference toward novel object are depicted at different ages (15 and 27 months). Fifty percent represents the chance level visiting both familiar and novel objects equally. Fifteen months old EX, E2 and E2 + EX groups showed better performance compared to control group ($*p < 0.05$ vs. 15 months old C).Twenty-seven months old E2 and EX groups spent more time with visiting novel object than the aged control animals ($^{\#}p < 0.05$ vs. 27 months old C). Columns represent mean ± SEM. Numbers of animals: 15 months—C ($n = 7$), EX ($n = 8$), E2 ($n = 8$), E2 + EX ($n = 6$); 27 months—C ($n = 7$), EX ($n = 6$), E2 ($n = 6$), E2 + EX ($n = 6$)

In the Morris water maze test (Fig. 3) two-way ANOVA showed a significant age effect ($F[1,49] = 9.78, p < 0.01$) and also a treatment effect ($F[3,49] = 2.78$, $p < 0.05$). The escape latency to reach the platform was significantly shorter in the ageing groups (15 months old), compared to the 27 months old C, EX and E2 + EX groups. E2 treatment in the old animals shortened the escape latency to the platform against controls revealed by post hoc comparisons at sessions 4–7. The performance of this group (E2) did not differ from those of the ageing groups. It is unique that the combined E + EX treatment was not effective

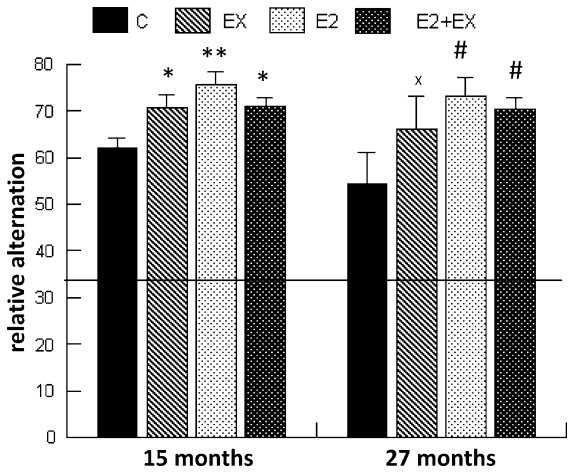

Fig. 2 Spontaneous alternation expressed as relative alternation of ageing (15 months) and old (27 months) rats in Y-maze is shown following EX, E2 and E2 + EX treatments compared to controls (C). Chance level (33.3 %, *shown by line*) indicates arm entering without alternations. All treatments increased the number of alternations of 15 months old animals ($^{*}p < 0.05$, $^{**}p < 0.01$ vs. 15 months old C). E2 and E2 + EX treatments resulted in better performance at the age of 27 months ($^{\#}p < 0.05$ vs. 27 months old C). Based on two-way ANOVA including only the groups of C and EX from both ages revealed significant difference between the two 27 months old groups ($^{x}p < 0.05$ vs. 27 months old C). Columns represent mean ± SEM. Numbers of animals: 15 months—C ($n = 7$), EX ($n = 7$), E2 ($n = 7$), E2 + EX ($n = 6$); 27 months—C ($n = 7$), EX ($n = 8$), E2 ($n = 7$), E2 + EX ($n = 8$)

in the 27 months old age. The spatial learning of the ageing animals was not affected by the treatments, i.e. probably the treated animals could not surpass the performance of controls in this test.

Plasma 17β-estradiol and corticosterone levels

Table 1 shows that the treatment of 15 and 27 months old rats with 17β-estradiol markedly elevated plasma estradiol levels (overall treatment effect: $F[3,52] = 31.62$, $p < 0.01$). In addition there was a significant interaction between the treatment and age factors ($F[3,52] = 10.16$, $p < 0.05$), i.e. at the younger age E2 treatment increased the plasma estradiol level more than in the 27 months old aged animals. No effect of exercise could be obtained in either age on the level of estradiol. Ageing from 15 to 27 months tended to enhance the plasma corticosterone level as revealed by two-way ANOVA ($F[3,52] = 2.38$, $p = 0.13$), but it was not considered significant based on the statistics. No treatment effect was found in either age on the corticosterone levels.

Organ and tissue weights

Table 1 summarized also these results. No group differences could be found in the body and adrenal weights (not shown in the table). Exercise tended to enhance the adrenal mass in both age groups, however, the enhancement did not reach significance. Therefore, no solid sign could be found for a remarkable stress condition by treatments including exercise. Two-way ANOVA showed significant treatment effect on the weight of pituitary gland ($F[3,53] = 7.48$, $p < 0.01$). The gland mass increased in both ages exposed to 17β-estradiol administration (E2, E2 + EX)

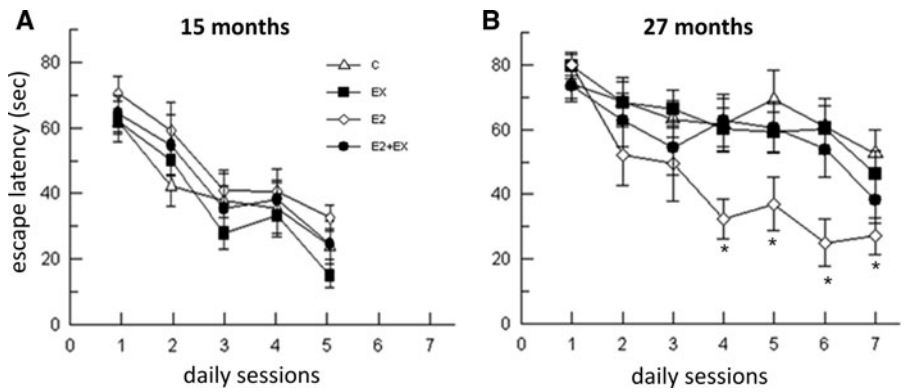

Fig. 3 Spatial learning in the Morris water maze test after EX, E2 and E2 + EX treatments at different ages (*panel A* and *B* indicate 15- and 27-months of age, respectively). Ageing animals were trained for 5 while old animals for 7 days. E2 treatment improved the spatial learning of 27 months old group ($^{*}p < 0.05$ vs. 27 months old C). Numbers of animals: 15 months—C ($n = 7$), EX ($n = 8$), E2 ($n = 7$), E2 + EX ($n = 8$); 27 months—C ($n = 7$), EX ($n = 7$), E2 ($n = 6$), E2 + EX ($n = 7$)

Table 1 Effects of EX, E2 and E2 + EX treatments on plasma corticosterone and plasma estradiol levels at 15 and 27 months of ages

Age	Treatment	Corticosterone (ng/ml)	Estradiol (pg/ml)	Pituitary gland (mg)	Uterus (g)	Adipose tissue (retroperitoneal) (g)	Adipose tissue (uterinal) (g)
15 months	C	42.10 ± 16.49	40.97 ± 12.17	15.65 ± 0.75	0.71 ± 0.04	3.51 ± 0.71	7.65 ± 0.38
	EX	101.10 ± 35.60	46.14 ± 7.21	16.58 ± 0.67	0.50 ± 0.06	**2.76 ± 0.60***	**5.54 ± 0.46***
	E2	65.93 ± 24.62	**816.91 ± 145.00****	**25.81 ± 1.32****	**0.79 ± 0.02***	2.85 ± 0.83	6.18 ± 0.39
	E2 + EX	65.61 ± 24.11	**892.35 ± 135.04****	**26.12 ± 2.13****	**0.82 ± 0.07***	**2.61 ± 0.15***	**5.65 ± 0.59***
27 months	C	112.53 ± 19.57	14.30 ± 3.20	16.48 ± 2.42	0.53 ± 0.07	9.88 ± 1.74	17.43 ± 1.75
	EX	101.54 ± 23.56	16.72 ± 2.70	17.80 ± 3.12	0.45 ± 0.08	**5.84 ± 1.09**$^{\#}$	**10.4 ± 01.19**$^{\#}$
	E2	111.57 ± 33.45	**333.33 ± 55.36**$^{\#\#}$	**26.62 ± 4.16**$^{\#\#}$	**0.92 ± 0.09**$^{\#}$	7.44 ± 1.56	14.26 ± 1.66
	E2 + EX	142.81 ± 33.73	**255.71 ± 350.58**	**31.87 ± 5.99**$^{\#\#}$	**1.09 ± 0.09**$^{\#}$	**4.03 ± 0.61**$^{\#}$	**8.07 ± 1.11**$^{\#}$

Table also shows the weights of hypophysis, uterus and adipose tissues

* $p < 0.05$, ** $p < 0.01$ vs. 15 months old C

# $p < 0.05$, ## $p < 0.01$ vs. 27 months old C

as indicated by post hoc analysis. 17β-estradiol administration (E2, E2 + EX) enhanced the uterine weight in both ages ($F[1,53] = 2.79$, $p < 0.05$). Uterine weight showed a decrement with age (overall age effect: $F[1,53] = 3.79, p < 0.05$). The results confirmed that in the advanced old age the different organs maintained their responsiveness to exogenous estradiol, which may well be compared to the effectiveness on the brain (see the figures).

Fat accumulation was significantly higher in the 27 months old groups compared to the 15 month old groups (age effect: retroperitoneal fat pad: $F[1,53] = 4.01$, $p < 0.05$, uterine fat pad: $F[1,53] = 6.42$, $p < 0.01$). EX and E2 + EX treatments resulted in reduction of adipose tissue in both ages compared to those of controls (treatment effect: retroperitoneal fat pad: $F[3,53] = 3.55$, $p < 0.05$, uterine fat pad: $F[1,53] = 7.65$, $p < 0.01$), therefore, the exercise was effective in metabolic level even in the old age.

Western blot analysis

Significant decline in the density of ERα immunoreactive bands was measured in the old animals compared to the younger 15 months old groups ($F[1,40] = 21.78$, $p < 0.01$, Fig. 4, panel A), in the hippocampus. Two-way ANOVA revealed a significant treatment effect ($F[3,40] = 8.00, p < 0.01$) without an interaction with age. ERα proteins were increased in the hippocampus in both ages exposed to E2 and combined treatments ($p < 0.05$). Interestingly, exercise also enhanced the ERα protein level but only in the ageing (15 months) animals as confirmed by post hoc multiple comparisons. There was no effect of exercise in the old rats on the expression of ERα protein.

Similarly to ERα, BDNF immunoreactivity (Fig. 4, panel B) showed a significant decline in the course of ageing (age effect: $F[1,40] = 15.76$, $p < 0.01$). The two-way ANOVA revealed a significant treatment effect: $F[3,40] = 8.57$, $p < 0.01$. The EX, E2 and E2 + EX treatments resulted in upregulation of BDNF levels in the ageing groups. BDNF levels also increased in the estradiol treated (E2, E2 + EX) old animals compared to their control group. Exercise slightly increased the levels of BDNF in old rats, although this enhancement did not reach statistical significance under the present conditions ($p = 0.063$).

No age-related differences in the amounts of intracellular signaling molecules were observed. The

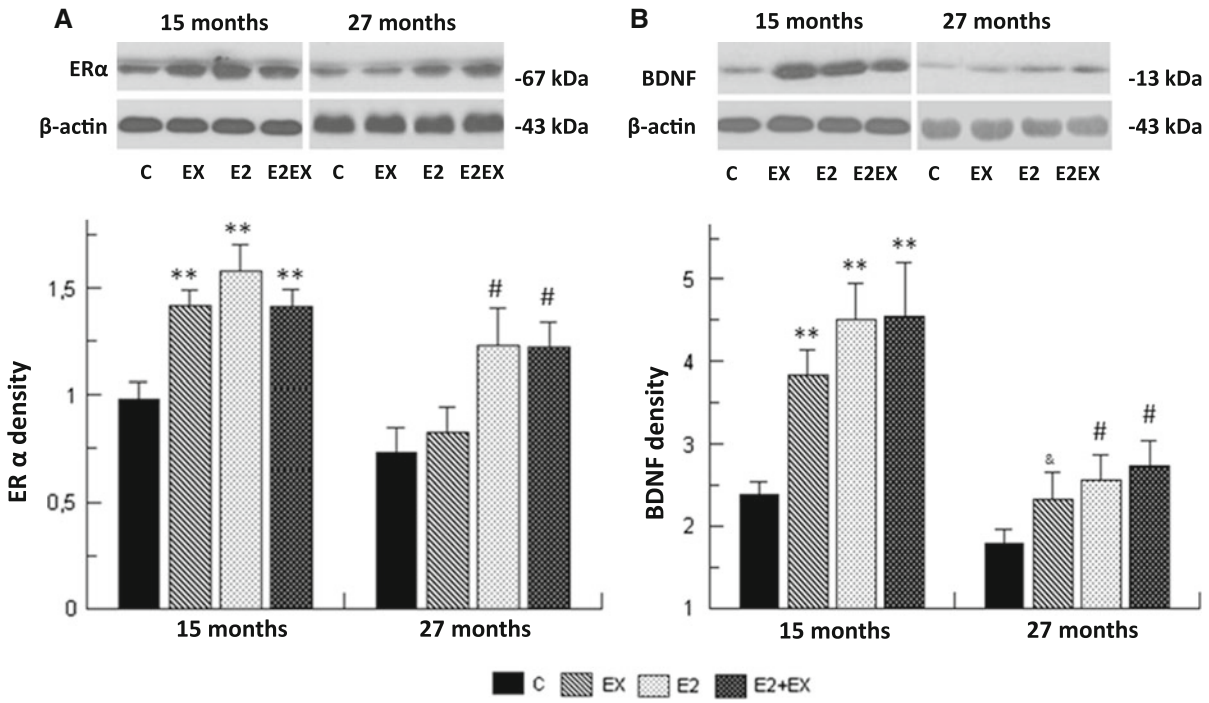

Fig. 4 ERα (*panel A*) and BDNF (*panel B*) expressions in the hippocampus of 15 and 27 months old rats following EX, E2 and E2 + EX treatments compared to controls (C). The *upper* part of figures illustrates expression of ERα and BDNF by representative Western blots from each group compared to *β*-actin. The *columns* show the mean ± SEM from each group. Mean ERα expression increased following interventions of EX, E2 and E2 + EX treatments in 15 months rats (**$p < 0.01$ vs. 15 months old C). E2 and E2 + EX increased the immunoreactivity of ERα at the age of 27 months ($^{\#}p < 0.05$ vs. 27 months old C). BDNF expression increased following EX, E2 and E2 + EX treatments in 15 months rats (**$p < 0.01$, vs. 15 months old C). E2 and E2 + EX increased the immunoreactivity of BDNF at the age of 27 months ($^{\#}p < 0.05$, $^{\&}p = 0.063$ vs. 27 months old C). Number of animals in each group $n = 6$

phosphorylation of MAPK and CREB (Fig. 5) significantly increased in both ages after 17*β*-estradiol treatments (E2, E2 + EX; treatment effects: p-MAPK: $F[3,40] = 15.16, p < 0.01$; p-CREB: $F[3,40] = 17.48$, $p < 0.01$). Exercise enhanced immunoreactive p-MAPK and p-CREB concentrations in the 15 months old ageing animals, as assessed by post hoc *t* test regarding both parameters, but was not effective in the old age groups.

All treatments increased the phosphorylation of Akt in the younger groups (treatment effect: $F[3,40] = 8.56$, $p < 0.01$) but not in the old groups (Fig. 6). No significant differences in the p-Akt protein levels were found among any of the aged groups, indicating that the probability of increasing Akt phosphorylation seems to be the least functional causative factor among the selected signaling parameters.

Synapsin I immunreactivity was not influenced by ageing (Fig. 7, panel A). A treatment effect was confirmed by two-way ANOVA ($F[3,40] = 3.62$, $p < 0.05$). Synapsin I was significantly increased in the ageing groups that received E2, EX and E2 + EX treatments. Estradiol enhanced the synapsin I expression in the hippocampus of 27 months old rats. The concentration of synaptophysin declined with age (age effect: $F[1,40] = 22.21$, $p < 0.01$, Fig 7, panel B). Two-way ANOVA detected a significant effect for the treatment factor as well ($F[3,40] = 9.15$, $p < 0.01$). 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.

Discussion

In this study we investigated the possibility that estrogen and exercise may affect in a similar way the cognitive functions and those molecular signaling

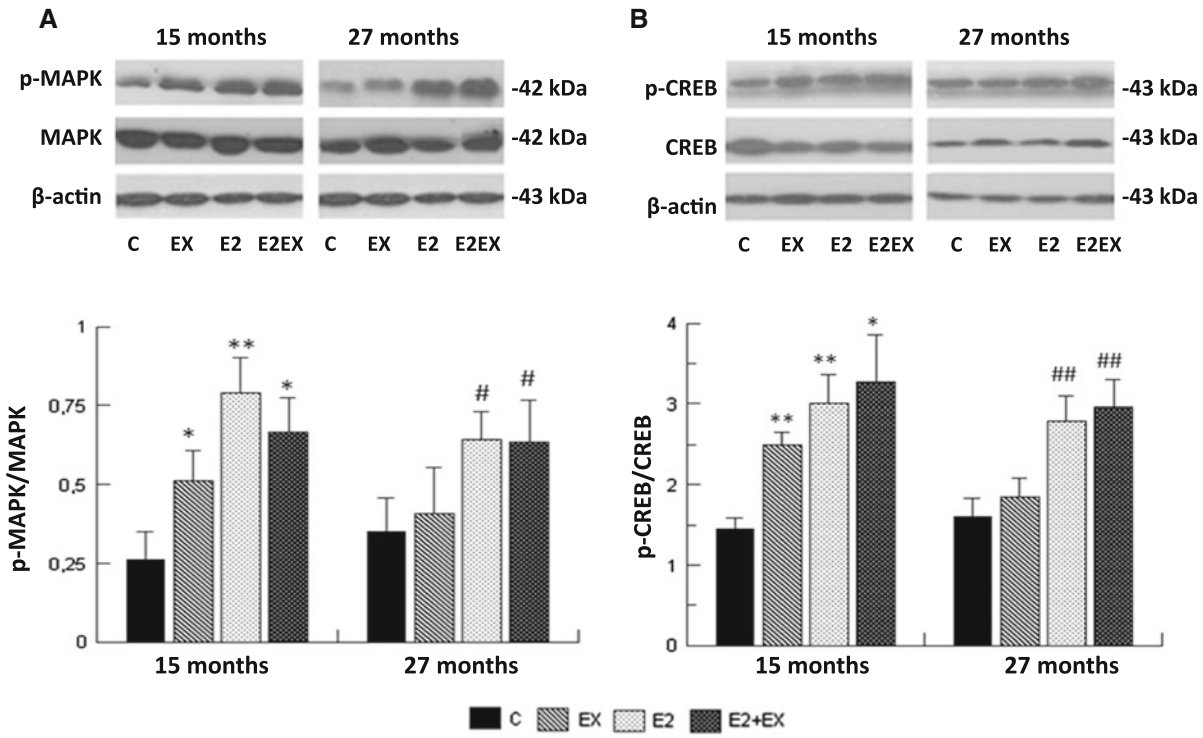

Fig. 5 Intensities of MAPK-(*panel A*) and CREB (*panel B*) phosphorylation in the hippocampus of 15 and 27 months old rats following EX, E2 and E2 + EX treatments compared to controls (C) are presented. The intensity of phosphorylation is expressed by the ratio of p-MAPK/MAPK and p-CREB/CREB (see also the representative Western blots above the columns). MAPK phosphorylation increased following EX, E2 and E2 + EX treatments in 15 months old rats ($^{*}p < 0.05$, $^{**}p < 0.01$ vs. 15 months old C). E2 and E2 + EX increased the immunoreactivity of p-MAPK at the age of 27 months old rats ($^{\#}p < 0.05$ vs. 27 months old C). CREB phosphorylation increased following interventions of EX, E2 and E2 + EX treatments in 15 months of age ($^{*}p < 0.05$, $^{**}p < 0.01$ vs. 15 months old C). E2 and E2 + EX increased the immunoreactivity of p-CREB at the age of 27 months ($^{\#\#}p < 0.01$ vs. 27 months old C). *Columns* represent mean ± SEM. Number of animals in each group $n = 6$

pathways which may underlie cognition and neural plasticity during ageing in female rats. This assumption received confirmation in the early phase of ageing, which may be specified as the early phase of postmenopausal period if we try to put the global ageing lifespan of the rat into a human perspective. Opposed to that, in the old animals with long-term constant anestrus only the estrogen action was detectable clearly, but a similar kind of action of exercise was largely invisible.

Starting with summarizing the behavioral effects of estradiol it was clearly detectable that it enhanced cognitive functions in both investigated ages in a well comparable manner with some variations mentioned below in more details. The effectiveness of estradiol treatment was confirmed by the marked increase in plasma 17β-estradiol levels of E2-treated groups in both ages. Since uterine tissue, but also the hypophysis are responsive to estrogen, the uterine and pituitary weights could also be used to confirm the biological efficacy of 17β-estradiol treatment in both ages (Kelner et al. 1982).

Exercise was clearly affective in the 15 months old ageing rats in cognitive behavioral tests assessing attention and memory since it exerted beneficial effects in NOR and Y-maze behaviors. These findings are on the line with several previous studies (O'Callaghan et al. 2007; Van der Borght et al. 2007) carried out in middle-aged and ageing rats. The present behavioral results showed that the moderate intensity and regular physical exercise was far less effective in aged compared to ageing female rats, i.e. in the advanced vs. early ageing periods, respectively. It should be added, however, that in old age, exercise

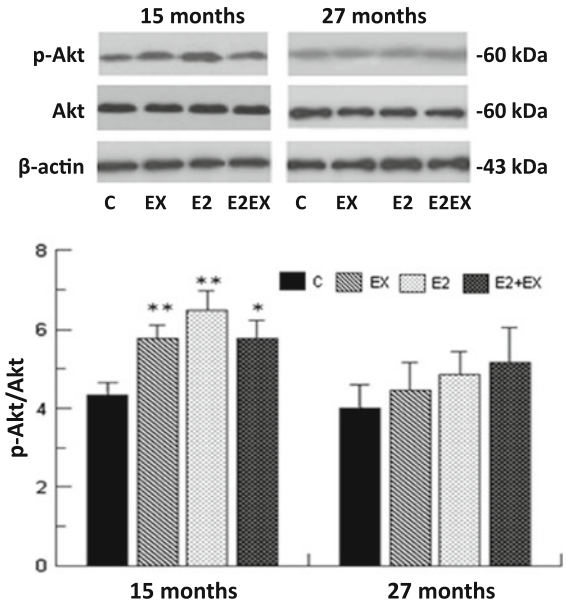

Fig. 6 Intensitiy of Akt phosphorylation in the hippocampus of 15 and 27 months old rats following EX, E2 and E2 + EX treatments compared to controls (C) are presented. The intensity of phosphorylation is expressed by the ratio of p-Akt/Akt (see also the representative Western blots above the columns). Akt phosphorylation increased following interventions of EX, E2 and E2 + EX treatments in the age of 15 months (*$p < 0.05$, **$p < 0.01$ vs. 15 months old C). *Columns* represent mean ± SEM. Number of animals in each group $n = 6$

slightly but significantly increased alternation in the Y-maze in the present study. Exercise was reported to improve learning in aged rodents (Albeck et al. 2006), whereas there is observation showing that spatial learning in Morris maze was unaffected by enhanced physical activity (Barnes et al. 1991) similarly to our present result. These behavioral results might mean that during advanced ageing the neuronal targets are becoming less sensitive to the beneficial effects of exercise or the behavioral response to physical activity might depend on the type and intensity of exercise interventions. This is in contrast to the effectiveness of estradiol treatment in aged animals. Corresponding to the results of Frick (2009), estradiol treatment was proved here to be effective in attenuating the age related memory impairments in the 27 months old rats.

For the regular, moderate intensity exercise treatment a treadmill paradigm was used which belongs to the forced exercise regimen. Forced exercise might evoke a chronic stress with elevated blood corticosterone levels. If it is extensive, memory processing may be impaired as it is known from neuroendocrine studies dealing with elevated corticosterone (McEwen and Sapolsky 1995; Brown et al. 2007; Schaaf et al. 1998). However, we observed tendencies but not a significant enhancement in the adrenal weight and plasma corticosterone levels of exercising rats of both ages. Therefore, the chronic stress-response to forced running probably did not interfere with the behavior or molecular signaling in this experiment. Furthermore, it may be mentioned here that the mass of abdominal fat pads decreased after exercise in both ages indicating that the presently applied regular physical activity paradigm exerted beneficial metabolic effects in not only the ageing but also in the aged animals, which might be a counterbalancing metabolic factor against stress.

The combined treatment also resulted in improvement in certain behavioral tasks in both ages although the changes mainly followed those of estradiol treatment. The results of combined treatments did not reveal additive type of action. For explanation it might also be possible to assume that estradiol treatment reached already a ceiling effect which could not be further enhanced by exercise. In certain cases the effect of combined treatment started to decline from the E2 level. For example, the combined treatment did not affect the spatial learning in the Morris water maze test in aged rats although the E2 treatment alone was effective, which might suggest an inverted U-shape action curve after introducing an additional stimulation by exercise. Similarly, Koltai et al. (2011) also found that both exercise and IGF-1 supplementation alone caused neurotrophic actions in the hippocampus of aged animals, but the combined treatment eliminated the beneficial effects of the single treatments. To unravel the real aspects of the effects of combined treatment, however, needs further studies with applying more graded doses of both estradiol and/or the intensity of exercise.

The molecular changes in the hippocampus may underlie the improved behavioral performance in response to treatments and may put light on the mechanisms of treatment effects. BDNF, but also estrogen receptors are known as neuronal trophic targets to activate downstream intracellular pathways involved in memory formation (Vaynman et al. 2004). In the present study an age related decline in BDNF protein level was found in the hippocampus which might explain the declining learning functions of old

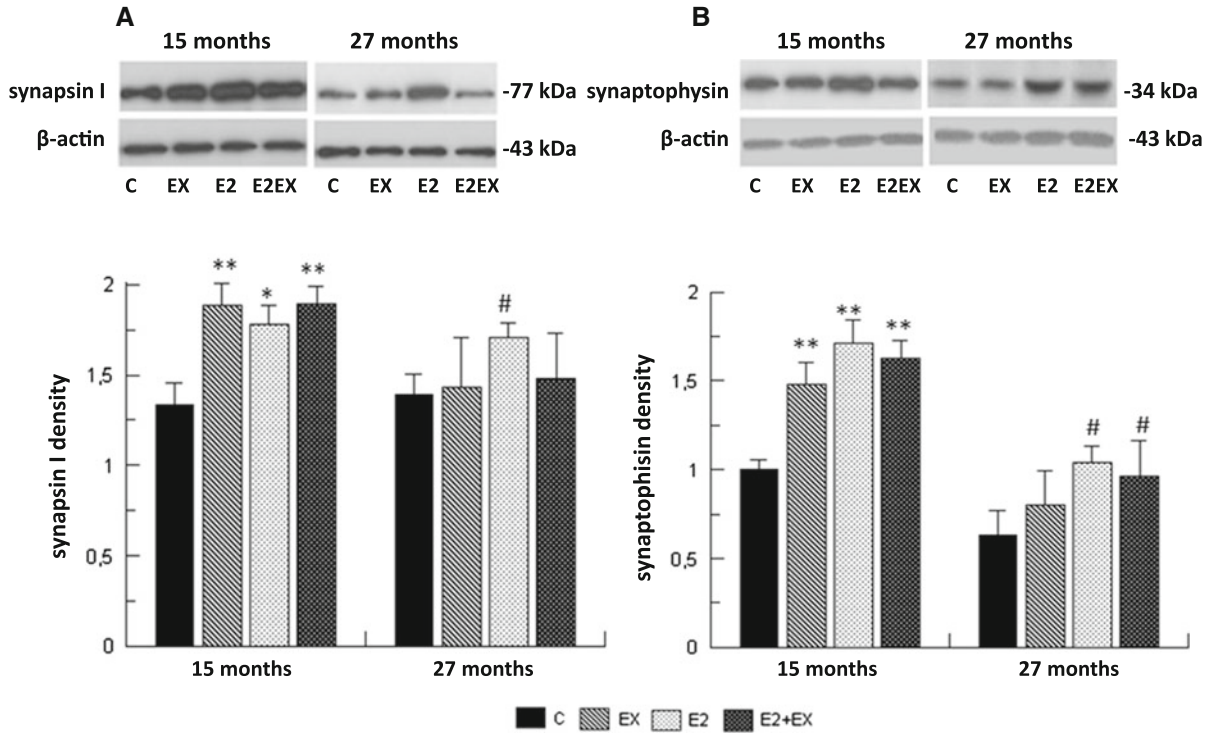

Fig. 7 Synapsin I (*panel A*) and synaptophisin (*panel B*) expressions in the hippocampus of 15 and 27 months old rats following EX, E2 and E2 + EX treatments compared to controls (C). Synapsin I expressions increased following treatments of EX, E2 and E2 + EX treatments in the age of 15 months ($^{*}p < 0.05$, $^{**}p < 0.01$ vs. 15 months old C). E2 increased the immunoreactivity of synapsin I at the age of 27 months ($^{\#}p < 0.05$ vs. 27 months old C). Synaptophysin expressions increased following interventions EX, E2 and E2 + EX treatments in the 15 months of age ($^{**}p < 0.01$ vs. 15 months old C). E2 and E2 + EX increased the immunoreactivity of synaptophysin at the age of 27 months ($^{\#}p < 0.05$ vs. 27 months old control). *Columns* represent mean ± SEM. Number of animals in each group $n = 6$

rats compared to the ageing animals in the attention and spatial learning tests. In addition to BDNF, the level of ERα and that of synaptophysin also declined with ageing. In agreement with other studies (Berchtold et al. 2005; Soya et al. 2007), we showed that BDNF protein levels were significantly increased in response to exercise in the ageing animals, i.e. at the beginning of neuronal ageing in rats. However this type of action was absent in the 27 months old animals. It is possible that the threshold of aged brain to respond normally to moderate intensity exercise stimuli is elevated. BDNF gene regulation was reported to depend on the intensity of treadmill exercise in young rats (Lou et al. 2008). Physical activity might result in neurotrophic actions only under specific conditions at late old ages, for example by combining exercise with social-environmental interactions or by simply increasing the intensity of exercise, although this later ways may include a risk for overtraining or exhaustion. Furthermore, Berchtold et al. (Berchtold et al. 2001) suggested that the regulation of BDNF level in the brain by physical activity may depend on the presence of estrogens in female rats. Thus, this explains that the exercise induced enhancement in BDNF level may be attenuated if the endogenous gonadal steroid production declines as it happens during ageing found also in the present study. Further studies are needed to accurately determine the relationship between the declining estradiol level during ageing and the sensitivity of different molecular targets in the ageing neurons.

In contrast to exercise, BDNF level in the hippocampus was increased by estrogen in the E2 and E2 + EX groups in both ages, indicating that estrogen may exert its trophic effect at least in part through BDNF. Estrogen-mediated regulation of BDNF could involve direct regulation of BDNF expression by nuclear ERα or activation of estrogen-mediated

messenger cascades (Berchtold et al. 2001). As regards the other gender, O'Callaghan et al. (2009) found that exercise can compensate the age-related decline in the expression of growth factors (i.e. BDNF), indicating that the BDNF response to exercise can be increased during ageing also in male rats. It may also be noted that the BDNF response to testosterone has been proved to be less pronounced compared to estrogens in females (Bakos et al. 2009).

The age related decline of ERα expressions and the estrogen receptor enhancement by E2 administration were demonstrated here confirming earlier studies by other authors (Bohacek and Daniel 2009; Mehra et al. 2005; Waters et al. 2011). It must be noted, that the expression of estrogen receptors are differentially modulated by age in specific brain areas (Wilson et al. 2002). The relation between the exercise and estrogen receptor expression in the hippocampus have not yet been studied according to our knowledge. Important finding of this study is that not only the estradiol treatment but also the exercise enhanced the protein level of ERα in the hippocampus of 15 months old ageing rats, i.e. at the early phase of ageing. It should be recalled here that the plasma estradiol concentration was not changed after exercise in this study. At that finding it is important to stress that neuronal ERα can be stimulated independently of estrogen ligand binding (Kato et al. 1995). Furthermore, it was proposed that ERα signaling could be activated by IGF-I protein (Mendez et al. 2006). IGF-1 is known to be increased by physical activity (Llorens-Martin et al. 2008), thus it can be assumed that exercise might modulate estrogen signaling through IGF-1. In addition, the expression of ERα has been shown to be increased in muscle (Cartoni et al. 2005) and liver (Hao et al. 2010) by enhanced physical activity. Due to the exercise-induced enhancement in ERα protein level in the present study, we claim that exercise may affect the brain ERα concentration in the ageing rats (15 months old) and it may compensate the natural aged-dependent decline of ERα expression during ageing, at least until a critical decline in estrogen level and its secondary functional consequences. This assumption may serve also for explanation that in old age—as shown here—the regular exercise of the given intensity was not effective to increase the amount of ERα receptor protein. Further studies are necessary to show that is there any other mode of action to increase the expression of ERα in the ageing brain by exercise in the advanced ageing stages.

In the present study exercise as well as estradiol and combined treatments stimulated the phosphorylation of MAPK, CREB and Akt signaling intermediaries in the hippocampus of ageing rats. One of the important endpoints of MAPK and PKA/Akt cascades is CREB which can regulate the expression of synaptic proteins (Meyer et al. 1993). The phosphorylation of CREB was found to be increased by all treatments in our study in ageing 15 months old rats, i.e. by both estradiol and exercise. Others also found an enhancement in the MAPK/CREB or Akt/CREB pathways after exercise (Shen et al. 2001) or after estrogen administration (Fan et al. 2008). We observed that exercise and estradiol induced an enhancement of synapsin I and synaptophysin protein levels that may be partially responsible for the improved learning and memory performance in ageing rats since they are end-stage proteins in synaptic plasticity. This confirms earlier results by other laboratories (Molteni et al. 2002; Hescham et al. 2009).

At the age of 27 months the exercise intervention used in this study did not affect the signaling molecules or the synaptic markers in the hippocampus of aged rats. It can be assumed that since BDNF is located upstream in the signaling chain pathways its enhancement should be marked enough to evoke intracellular molecular responses including plastic changes in synaptic protein levels. The results of this study call for attention to consider old age as a distinctive condition regarding the effects of exercise used to obtain in younger ages including early stage of ageing.

Estrogen and the combined treatment of 27 months old rats resulted in similar actions on MAPK/CREB signaling as in the 15 months old animals. E2 and E2 + EX enhanced the level of synaptophysin but did not activate Akt according to the present findings. Earlier reports presented findings that estradiol activates the CREB pathway and the synthesis of synaptophysin in ovarectomized aged rats (Sharma et al. 2007). Interestingly, Yildirim et al. (2011) reported that the Akt activation during ageing in response to estrogen is less pronounced as compared to young age, which might serve a possible explanation on the absence of the activation of Akt by estrogen interventions in aged animals found here.

In conclusion, 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. During the well advanced stage of ageing, estradiol was still effective like in younger age but the behavioral and molecular effectiveness of regular physical exercise proved to be far less pronounced, better to conclude as nearly ineffective. 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.

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