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Published in:
Neuroscience

DOI:
10.1016/j.neuroscience.2019.05.037

Publication date:
2019

NEUROSCIENCE
RESEARCH ARTICLE

Tímea Téglás et al. / Neuroscience 411 (2019) 65–75

Effects of Long-Term Moderate Intensity Exercise on Cognitive Behaviors and Cholinergic Forebrain in the Aging Rat

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Abstract—Physical exercise is now generally considered as a strategy to maintain cognitive abilities and to prevent age-related cognitive decline. In the present study, Wistar rats were subjected to moderate intensity treadmill exercise for 6 months prior to sacrifice at 12-, 24- and 32-month of age. This chronic physical intervention was tested on motility in the Open field (OF). Cognitive functions were measured in the Morris water maze (MWM) for spatial learning and in the Novel object recognition (NOR) tests. Since learning and memory are closely associated with cholinergic forebrain function ChAT fiber density after exercise training was assessed in hippocampus, and motor- and somatosensory cortical areas. Furthermore, quantification of ChAT-positive fiber aberrations as a neuropathological marker was also carried out in these brain areas. Our results show that in OF chronic exercise maintained horizontal locomotor activity in all age groups. Rearing activity, MWM and notably NOR performance were improved only in the 32-months old animals. Regarding cholinergic neuronal innervation, apart from a general age-related decline, exercise increased ChAT fiber density in the hippocampus CA1 area and in the motor cortex notably in the 32-months group. Massive ChAT fiber aberrations in all investigated areas which developed in senescence were clearly attenuated by exercise. The results suggest that moderate intensity chronic exercise in the rat is especially beneficial in advanced age. In conclusion, chronic exercise attenuates the age-related decline in cognitive and motor behaviors as well as age-related cholinergic fiber reduction, reduces malformations of cholinergic forebrain innervation.

Key words: aging, treadmill exercise, cognitive and motor functions, cholinergic forebrain innervation.

INTRODUCTION

It is now commonly accepted that chronic physical exercise exerts a cognition improving action on the brain. It may help to attenuate progression of different neurodegenerative diseases (Sutoo and Akiyama, 2003; Leem et al., 2011; Zigmond et al., 2012) and support long-term sensorimotor functions (Voelcker-Rehage and Niemann, 2013). The intimate anatomical and functional connections between motor and cognitive brain structures have convincingly been demonstrated in both rodent and human studies (Buzsáki, 2005; Hillman et al., 2008; Erickson et al., 2011). In mice, Fabel et al. (2009) have shown that combining physical and mental activity has an additive effect on initiation and survival of newly born hippocampal neurons. In another rodent study it was found that exercise resulted in an improvement of spatial reference memory (Van der Borght et al., 2007). In human, physical activity has induced larger regional brain volumes in older adults (Colcombe et al., 2006; Erickson et al., 2011; Benedict et al., 2013) also indicating that the function of motor and cognitive brain structures are closely related.

The cholinergic system of the forebrain is involved in cognitive processes such as attention, learning and memory functions (Blokland, 1995; Myhrer, 2003; Steriade, 2004; Luiten et al., 2013). One of the oldest hypotheses for cognitive decline during aging is coupled to the function of cholinergic brain structures in both animals and humans

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Abbreviations: ACh, Acetylcholine; AChE, acetylcholinesterase; AD, Alzheimer's disease; CA1, Cornu Ammonis region 1; ChAT, choline acetyltransferase; DG, dentate gyrus; MC, motor cortex; mo, months; MWM, Morris water maze; NC, neocortex; NOR, novel object recognition; OF, open field; RM, reference memory; SSC, somatosensory cortex; WM, working memory.

https://doi.org/10.1016/j.neuroscience.2019.05.037
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(Dringenberg, 2000; Kotagal et al., 2012). Already during normal aging clear changes can be observed with respect to the anatomical integrity of cholinergic fiber pathways innervating the hippocampus and neocortical regions. In the aged brain axon malformations develop like fiber varicosities and swellings, suggesting impairment of normal axonal transport processes (Gaykema et al., 1992; Van der Zee et al., 1997). Lesioning the nucleus basalis medialis, which is the main source of cholinergic innervation of the neocortex (Nyakas et al., 2011), or the septal cholinergic innervation to the hippocampus (Ho et al., 2009) results in cognitive decline in rodents. Interestingly, lesion of the nucleus basalis medialis also leads to the development of ChAT fiber aberrations in aged rats (Gaykema et al., 1992). A functional link between locomotor activity and the cholinergic response in the hippocampus of aged rats was suggested by the impact of movement on enhancing blood flow and acetylcholine (ACh) release in this brain structure (Uchida et al., 2006).

A decrease in cognitive capacity including spatial learning performance in old rats was extensively reported in previous studies (Gage et al., 1988; Van der Staay, 2002; McQuail et al., 2011; Fuermaier et al., 2014; Hovens et al., 2015; Arias-Cavieres et al., 2017). One of the conclusions of these rodent studies is that age-related cognitive function in spatial learning declines progressively during aging (Van der Staay, 2006). In rodents, the spontaneous development of cognitive deficits in the course of aging is not considered as pathological (Foster, 2006), which makes rodents an appropriate model to study non-pathological cognitive decline during aging even in relation to humans. With respect to motor behavior, the aging induced decline in mobility appears to be partly caused by reduced variability of motor patterns, coordination abilities, and slowing of movement speed in both rodents and humans (Van der Zee et al., 1990; Krampe, 2002). Movement impairment is becoming prominent in older ages, especially under sedentary lifestyle conditions, which is also characteristic for laboratory rodents. In the present study we investigated a wider age period in rat to gain more relevant comparisons to human aging conditions including senescence.

To that aim, three age groups were selected of 12, 24, and 32 months (mo) of age. We investigated the age-related effect of 6 months of moderate intensity chronic exercise on psychomotor and cognitive behaviors, followed by postmortem measurement of cholinergic fiber density, and of cholinergic fiber aberrations in the hippocampus and neocortex. The treadmill exercise lasted for 6 months before testing the spontaneous psychomotor activity in open-field, and discriminative and spatial learning behaviors in novel object recognition and spatial learning paradigms, respectively.

EXPERIMENTAL PROCEDURES

Animals

In total, we used 55 male Wistar rats of different ages (12, 24 and 32 mo) 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) and in a relative humidity of 40–50%. Food and tap water were available ad libitum. All experimental procedures which were carried out on the animals had been approved by the Animal Examination Ethics Council of the Animal Protection Advisory Board at the Semmelweis University, Budapest; and comply with the European Community Council Directive of 24 November 1986 (86/609/EEC) for animal experiments.

Experimental design

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 6 months, three times a week 40 min per sessions. The incline of running belt constantly stayed at zero angle. The walking speed was increased gradually during the first 2 weeks from 6 m/min until a velocity of 18 m/min, which corresponded to an average VO_2 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.

Behavioral testing

All behavioral tests were performed blindly and the individual animals from the different groups and cages were selected randomly for each testing. The experimental animals could not see the experimenter, because the test arenas were observed through an optical camera positioned 1.5 m above the arenas. By the end of the 6 months exercise period the behavioral tests were carried out during the resting days while the training schedule was continued.

Open field test

In the open field test (OF), we measured vertical and horizontal activity of rats as described previously (Harkany et al., 1997). The open field test box consisted of a circular arena 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

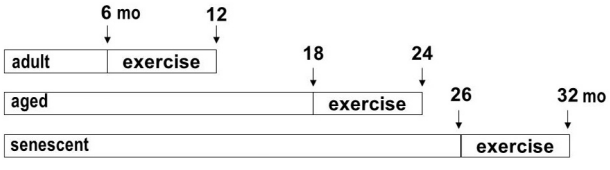

Fig. 1. Experimental design showing the time period (6 months) of treadmill exercise at three different ages. Exercise training started at 6, 18, and 26 months and finished at 12, 24, and 32 months of ages in adult, aged and senescent rats respectively.

horizontal movements as the number of line crossings by walking, and vertical activity as number of rearing. When a single rearing response lasted for more than 1 s, another score was added at every further second to measure the intensity of vertical mobility.

Novel object recognition test

Novel object recognition was evaluated as described previously (Ennaceur and Meliani, 1992; Nyakas et al., 2009) and was tested in the open field arena 2 days after the OF motility measurement. Briefly, during the first session (sample trial) two identical objects were placed in the arena at equal distances from the wall in an asymmetric position regarding to the center. These objects became familiar objects during the 5 min exploration period. After 120 min inter-session 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 it 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)] × 100.

Morris water maze test

The Morris water maze spatial learning test (MWM) was performed basically according to the original description (Morris, 1984) in a round black water tank (diameter 153 cm, height 63 cm) filled to a depth of 53 cm with water of 24 ± 1 °C. A black hidden platform (diameter 10.8 cm) at a fixed position was submerged 1.5 cm below the surface of the water. Animals were tested in four trials with different starting positions around the perimeter of the tank. The animals had to learn the place of the hidden platform guided by different visual cues in the surrounding of the experimental chamber. Dependent on the age 5 or 6 sessions were applied with four trials per session. The order of starting positions varied randomly by trials, but remained stable during a session. Each trial lasted until the rats located the platform. If the platform was not found within 90 s, the experimenter led the animal to it. Rats spent 30 s 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.

Brain tissue analysis

Twenty-four hours after the last exercise training the rats were sacrificed with deep sodium pentobarbital anesthesia and 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 h 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 (1997).

Cholinergic fiber staining and quantification

An immunostaining procedure (Högyes et al., 2003) on free floating coronal sections was applied to visualize choline acetyltransferase (ChAT) positive axon fiber patterns in the hippocampus, and 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. As next steps biotinylated rabbit anti-goat IgG and the Vectastain ABC kit from Vector Laboratories (CA, USA) were applied. Staining was completed with nickel-enhanced diaminobenzidine (DAB) reaction in the presence of H_2O_2.

The quantification procedure for cholinergic fiber density we previously described in greater detail (Harkany et al., 2000). Briefly, ChAT-positive fiber density was measured with the Quantimet 600HR (Leica, Germany) image analysis program. Three brain sections were analyzed per animal and the results averaged. Percent surface area of positively stained fibers against zero background (immunostaining in the Corpus Callosum) was calculated in the dorsal hippocampus and in the motor and somatosensory neocortex. In the dorsal hippocampus the stratum radiatum of the CA1 area and the molecular layer of the inner blade of the dentate gyrus were analyzed. In neocortical areas, ChAT-positive fiber density was measured in layer V of the respective regions.

Analysis of ChAT-immunoreactive fiber aberrations

ChAT immunoreactive swollen fiber varicosities and enlarged axon fragments were selected for quantification of fiber aberrations by means of computer-assisted image analysis (Quantimet 600HR, Leica, Germany) as described earlier (Nyakas et al., 2003). Briefly, using 20 x objective magnification, a no. 600 emission filter, and following shading corrections and background subtraction, an optimal threshold level was selected, which was kept constant for each measured area throughout the entire quantification procedure. Following unbiased manual delineation of areas around the clusters of swollen fiber varicosities the surface

area of these structural malformations was measured. With the image analysis program the net area of aberrations was quantified and expressed in calibrated μm^2. Density of fiber aberrations were counted in three sections (within Bregma −3.30 ± 0.25 mm) and the average values taken for further analysis of three brain areas: the hippocampus CA1-subiculum, the dentate gyrus (DG) and the neocortical areas (NC) covering the motor and sensory cortex (see Fig. 10). The results were expressed as the averaged area of aberrations per single brain section.

Statistics

The Statistica 13.2 program was used for evaluation of the numerical results by using two-way ANOVA, followed by paired comparisons between two groups by the Tukey's post hoc *t*-test. Novel object recognition performance against 50% chance level in individual groups was evaluated by the dependent samples *t*-test. Means ± SEMs are shown in the Figs. $P < .05$ was considered as statistically significant.

RESULTS

Open field activity

Two-way ANOVA showed that the open field locomotor behavior changed according to both factors, i.e. age and treatment (see Fig. 2). The horizontal ambulation (crossings) declined with age ($F_{2,49} = 8.22$, $P < .0001$), the overall effects of exercise (treatment) approached significance ($F_{2,49} = 3.26$, $P = .076$), and the interaction between age and treatment being highly significant ($F_{2,49} = 5.26$, $P < .01$). This remarkable interaction may be due to the finding that horizontal activity declined with age in sedentary controls but not in exercised rats (see asterisks indicating *t*-test results in Fig. 2). In addition, crossing further declined from age 24 to 32 mo ($P < .05$) in controls but not in exercised rats, showing that chronic exercise between ages of 26 and 32 mo can prevent further decline in horizontal locomotor activity. Furthermore, exercised senescent rats are more active compared to controls of the same 32 mo age ($P < .05$, post hoc *t*-test). Therefore, it became clear that the moderate-intensity exercise could maintain horizontal activity throughout all investigated ages at a level characteristic to 12 months of age, probably because walking was the main movement pattern repeatedly practiced in the treadmill test. Regarding frequency of rearing (right side panel of Fig. 2) an aging related decline was clearly present ($F_{2,49} = 19.8$, $P < .0001$), but exercise did not reveal any effect on vertical ambulation in the treated vs. control groups. Further analysis of the advanced ages of 24 mo and 32 mo with post hoc *t*-test in this motility parameter also resulted in a difference between the treated vs. control groups. Namely, regarding exercised groups there was no significant further decline in rearing between ages 24 vs. 32 months as compared to the sedentary controls, which showed an age-related decline between these two ages ($P < .01$).

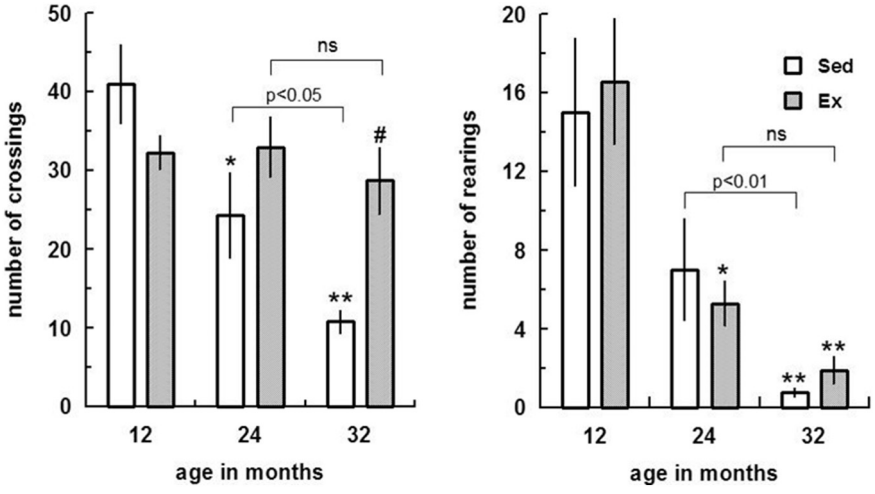

Fig. 2. Novelty-induced locomotion in the open field test. Number of crossing (left panel) and the number of rearing (right panel) are indicated at the three ages. Data were analyzed by two-way ANOVA followed by post hoc *t*-test. Eight to 10 animals varied in the groups. Significant decline in activities against the 12 months old age are indicated by asterisks: *$P < .05$, **$P < .01$. 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 (#$P < .05$ 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. No decline was found between the exercised groups along these ages (ns – not significant).

Morris water maze spatial learning

The reference and working memory (RM, WM) data of each age group are presented in Fig. 3. Repeated measure ANOVA with two factors (sessions and treatment) confirmed a highly significant increase in learning scores at the age of 12 mo in both RM and WM ($P < .0001$) without any group difference, showing that exercise was not effective. In the more advanced ages the rate of acquisition throughout the test was delayed. In the 24 mo age group, the progress of RM was significant (repeated measure ANOVA: $F_{5,100} = 2.42$, $P = .040$), and that of WM just approached significance level ($F_{5,100} = 2.23$, $P = .057$). Senescent rats also showed a statistically supported improvement in the learning processes: RM: $F_{5,80} = 2.42$, $P = .043$; WM: $F_{5,80} = 2.32$, $P = .051$. In addition, a treatment effect was also shown by the significant interaction between sessions and treatment groups in WM: $F_{5,80} = 2.57$, $P = .034$. The post hoc *t*-test showed differences in the last two sessions at the end of training between the two groups (see WM at Fig. 3). A similar tendency in performance could be revealed in RM in the senescent age, and also here a significant improvement was observed in the last period of training. In this advanced age (32 mo) the spatial and working memory scores fluctuated from session to session, which

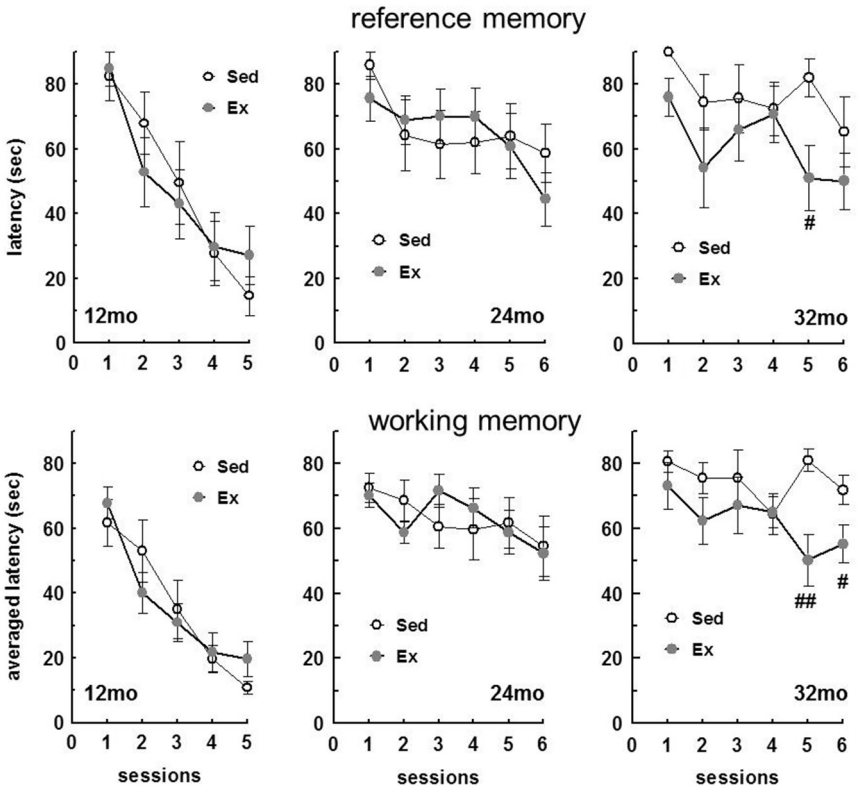

Fig. 3. Morris water maze performance as expressed in reference and working memories at three ages. Progression of learning slowed down at the two higher ages along the consecutive sessions. Based on calculations with two-way repeated measure ANOVA, there was an exercise induced difference only at 32 mo of age. The post hoc *t*-test results: #$P < .05$, ##$P < .01$ vs. sedentary control group. Number of animals in groups ranged from 8 to 11.

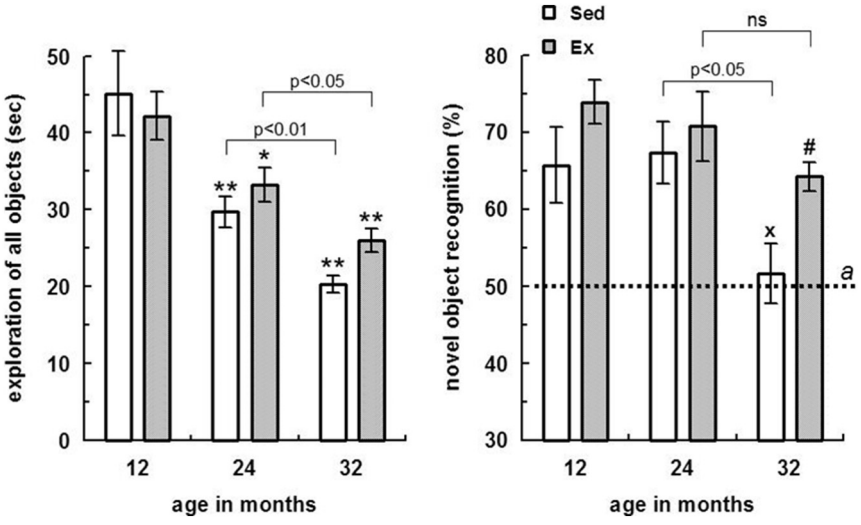

Fig. 4. Novel object recognition in three different ages after chronic exercise. In NOR the general level of exploration all objects is indicated in the left panel, and the recognition score of the novel object in the right panel. Regarding general object exploration (left panel) with two-way ANOVA only an age-related effect could be revealed by post hoc *t*-test compared to 12 mo: *$P < .05$, **$P < .01$. On the other hand, in recognizing the novel object a group (treatment) difference could be found by two-way ANOVA. This effect was caused by the difference at the oldest age of 32 mo between sedentary (Sed) and exercised (Ex) rats (#$P < .05$ vs. sedentary group). All groups showed a clear NOR response against the chance level (indicated by broken line 'a'), the only exception was the 32 mo old control group ($^{x}P = .75$). Furthermore, NOR performance from the age of 24 mo to 32 mo declined in the control group ($P < .05$), but not in the exercised group. Number of animals varied from 8 to 10 per group.

was notably less in the exercised animals in the end of training period.

Novel object recognition

Results are presented in Fig. 4. General exploration of all objects in both sessions of the test (left panel) declined with age ($F_{2,50} = 27.88$, $P < .0001$), without any effect of treatment ($P = .38$) or interaction between the factors ($P = .29$). Analyzing the two advanced ages, the overall exploratory activity declined from 24 mo to 32 mo in both the sedentary and exercise animals. Recognition of the novel object assessed during the second session (right panel, Fig. 4), however, revealed a treatment difference ($F_{2,46} = 6.19$, $P = .017$). The NOR behavior at 12 mo vs. 24 mo ages were comparable, the 24 mo old rats did not lose the ability to discriminate between the novel vs. the familiar objects. However, at the age of 32 mo sedentary animals did not show any discrimination since the recognition score was not different from the 50% chance level ($t = 0.32$, $P = .75$, $df = 8$, one group *t*-test). In this group, the recognition index was lower as compared to the 24 mo old control group ($P < .05$). Exercise, however, preserved recognition ability in the senescent age group as shown by the difference between the exercised and sedentary animals at this advanced age ($P < .05$, indicated by # at the Fig. 4).

Cholinergic fiber densities in hippocampus and neocortex

Fig. 5 shows the age-related decrease of cholinergic fiber density in the hippocampus CA1 region ($F_{2,37} = 111.4$, $P = .0001$, two-way ANOVA). Representative histological pictures visualize this decline in the suprapyramidal stratum radiatum. Compared to 12 mo of age, older rats showed a reduced of ChAT-positive fiber density in both sedentary control and exercise groups. Also, comparing the two advanced ages there was no further decline in fiber density from 24 to 32 mo old ages after exercise, while in the sedentary groups there was a significant decline ($P < .01$, post hoc *t*-test comparisons). Furthermore, a statistically significant difference was found between the two 32 mo groups, i.e. exercised rats showed a significantly higher ChAT fiber density compared to sedentary controls (#$P < .05$, see Fig. 5).

Fig. 6 shows the age-related reduction of ChAT positive fiber density in the hippocampus DG area ($F_{2,38}$ = 152.7, P = .0001) and representative histochemistry from the supragranular stratum moleculare. Compared to 12 mo controls, older rats showed a significant reduced density of cholinergic fiber innervation in both sedentary control and exercised groups. In this hippocampus area, there were no significant differences between the exercised vs. sedentary animals.

ChAT immunoreactive fiber density in the primary motor cortex is depicted in Fig. 7 including representative micrographs in the lower row covering supra-, pyramidal and infrapyramidal regions of layers V. Employing two-way ANOVA revealed an age-related decline ($F_{2,38}$ = 36.0, P < .0001) and a treatment effect: $F_{2,38}$ = 6.00, P = .018, indicating that exercise increased fiber density, in this case both in 24 and 32 mo of age.

Measuring ChAT positive fiber density in the primary somatosensory cortex layer V (Fig. 8) also revealed the

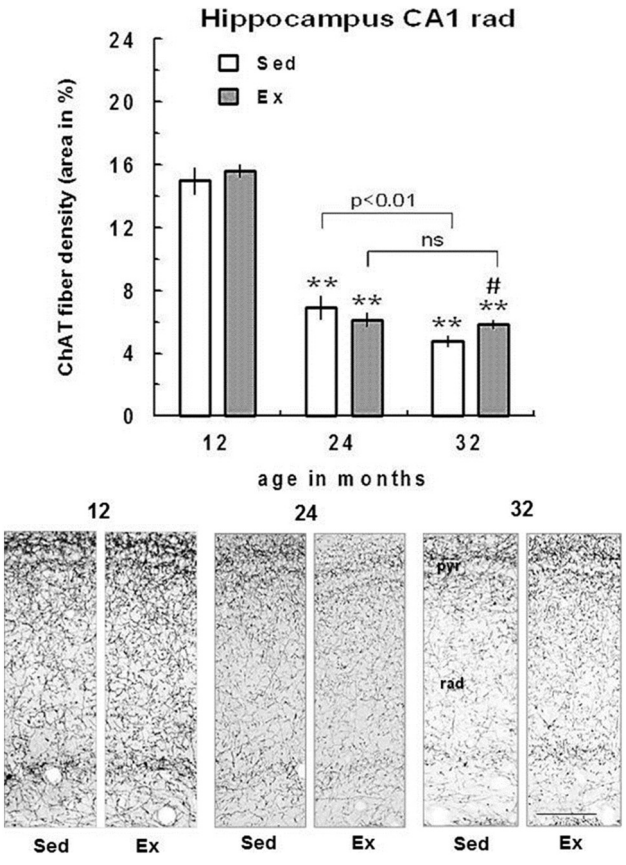

Fig. 5. Age-dependent reduction of cholinergic fiber density in the hippocampus CA1 area and the effect of exercise in senescent age. Significant decline in ChAT fiber density against the 12 months old age is indicated by asterisks: **P < .01. The only treatment effect of chronic exercise could be revealed at the age of 32 mo (#P < .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. Representative photomicrographs from the CA1 areas are presented below the column diagram at the three ages of both sedentary and exercise groups. Abbreviations: pyr – pyramidal layer, rad – stratum radiatum. Scale bar: 100 μm.

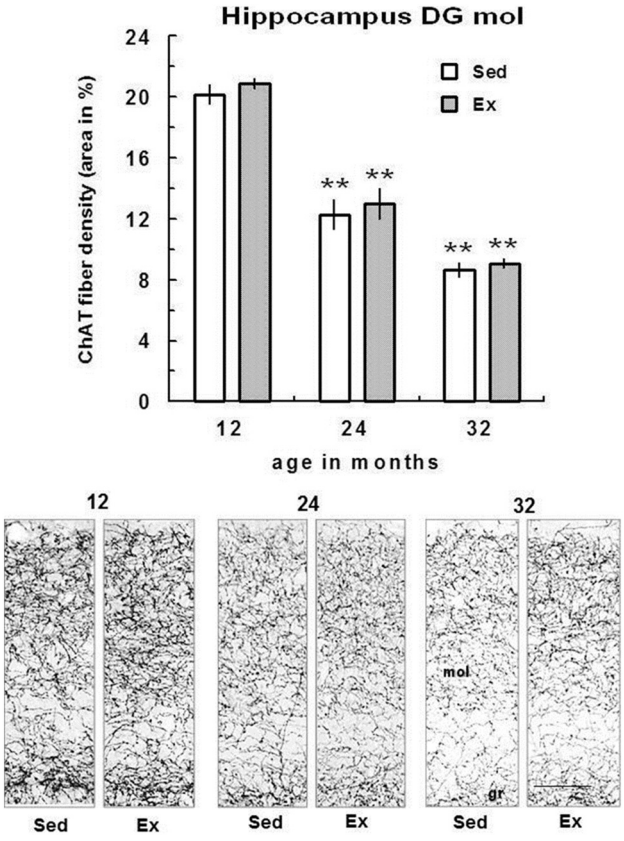

Fig. 6. Age-dependent decline of cholinergic fiber density in the hippocampal DG area of sedentary and exercise groups. Significant decline in ChAT fiber density against the 12 months old age is indicated by asterisks: **P < .01. Representative photomicrographs from the DG areas are shown below the three age group diagrams. Groups included 7–8 rats. Abbreviations: mol – molecular layer, gr – granular layer. Scale bar: 50 μm.

age related decline in fiber density ($F_{2,38}$ = 14.5, P < .0001). Two-way ANOVA analysis confirmed the effect of exercise as another factor: $F_{1,38}$ = 4.42, P = .042. Exercise attenuated the decrease of ChAT positive fibers in the senescent age.

Cholinergic fiber aberrations

ChAT positive fiber aberrations were quantified in 24 and 32 mo old rats in the same brain areas in which cholinergic fiber densities were measured (Figs. 9 and 10). Fig. 10 shows examples of characteristic axon aberrations in these forebrain areas. Axon malformations did not occur in the 12 mo old animals. Even at the age of 24 mo the incidence of fiber aberrations was moderate compared to the senescent age of 32 month (Fig. 9). Furthermore, as shown in Fig. 9, the density of fiber aberrations was lowest in DG compared to the other two cortical areas, indicating a region dependent difference in axon pathology. Two-way ANOVA analyzing the two factors, i.e. age and treatment effect revealed significant differences in the three brain areas. In hippocampus CA1 age factor: $F_{1,25}$ = 18.9, P = .0002; treatment factor: $F_{1,25}$ = 7.48, P = .011; age x treatment interaction: $F_{1,25}$ = 3,81, P = 0,062. In the hippocampus

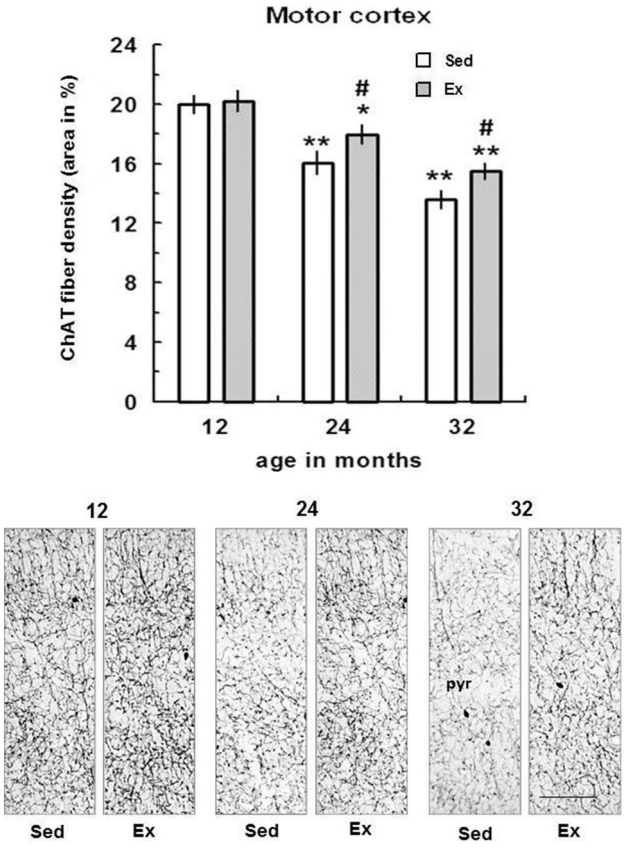

Fig. 7. Age-dependent decline of cholinergic fiber density in the motor cortex and the effect of exercise. Significant reduction in innervation density against the 12 mo age group is marked with asterisks: **P* < .05, ***P* < .01. Treatment effect of chronic exercise was found at both 24 and 32 mo of age (#*P* < .05). Representative photomicrographs from the motor neocortex areas are shown under the diagrams. Cortical areas selected for measurements covered supra-, pyramidal and infrapyramidal regions of layer V (pyr). Groups included 7–8 rats. Scale bar: 100 μm.

DG only the age factor showed a significant result: $F_{1,25}$ = 27.01, *P* = .00002. In the neocortex significant effects were found in both factors and interaction: age factor: $F_{1,25}$ = 26.75, *P* = .00002; treatment factor: $F_{1,25}$ = 6.61, *P* = .016; interaction between factors: $F_{1,25}$ = 5.13, *P* = .032. The Tukey's post hoc *t*-test evaluations are summarized in Fig. 9, which indicated a marked prevention of axonal degeneration in CA1 and neocortex by the chronic exercise (##*P* < .01).

DISCUSSION

The main findings of the present study are that long-term moderate intensity aerobic exercise supports neuromotor and cognitive performances especially during the age of senescence in rat from ages 26 to 32 mo. Physical exercise in the other two age groups indicate either no effect in the 6–12 mo age period and only a marginal impact on the 18–24 mo old animals. Notably, in this latter age group exercise significantly prevented age dependent loss of cholinergic fiber innervation in the motor cortex compared to sedentary control animals.

With respect to the senescent age period and the results of the OF test, we measured no age-dependent reduction of horizontal activity in exercised animals as compared with the substantial reduction in walking mobility of sedentary control rats. This marked preventive motility effect can be partly contributed to the type of physical training on treadmill, i.e. the walking-jogging exercise, but also to an improved physical condition of the trained animals. The other motility parameter in OF test, the vertical activity (rearing), was also influenced by the chronic exercise in the oldest age group compared to 24 mo old trained rats. In the 30 mo old animals there was no further decline in the rearing activity as a result of physical training.

In the present study, exercise led to minor improvements in working- and reference memory as tested in the water maze, and only in the 32 mo old senescent animals. With respect to the performance in the water maze we measured an obvious decline in learning improvement in the course of the subsequent sessions in the two older ages compared to the 12 mo old age. Since in the OF behavioral test exercise improved motor performance and coordination only in the senescent age group, we assume that this motor improvement may well contribute to the spatial learning capacity of senescent animals in the MWM test. The underlying

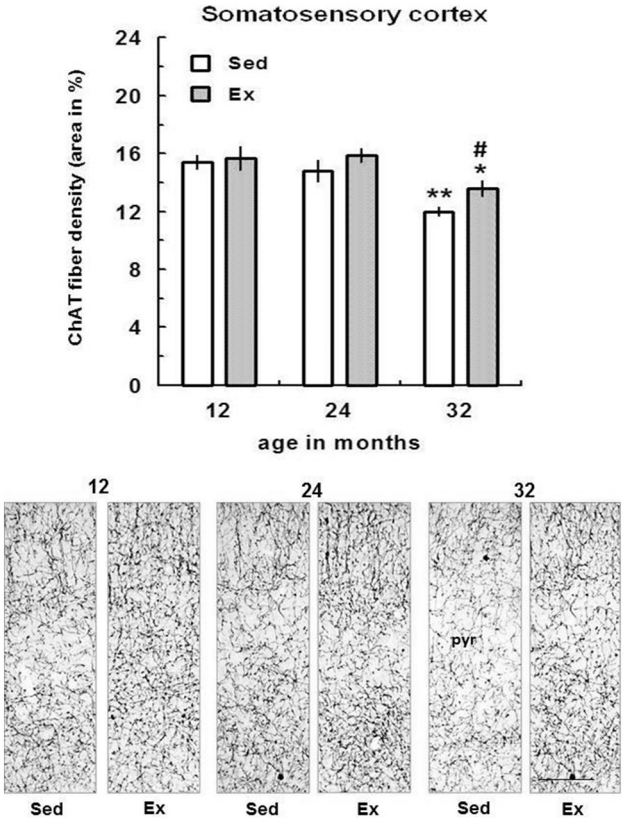

Fig. 8. Age-dependent decline of cholinergic fiber density in the somatosensory cortex and the effect of exercise. Significant decline in ChAT fiber density against the 12 months old age is indicated by asterisks: **P* < .05, ***P* < .01. Treatment effects of chronic exercise could be observed only at the age of 32 mo (#*P* < .05). Representative photomicrographs from the somatosensory neocortex areas are shown. Groups included 7–8 rats. Scale bar: 100 μm.

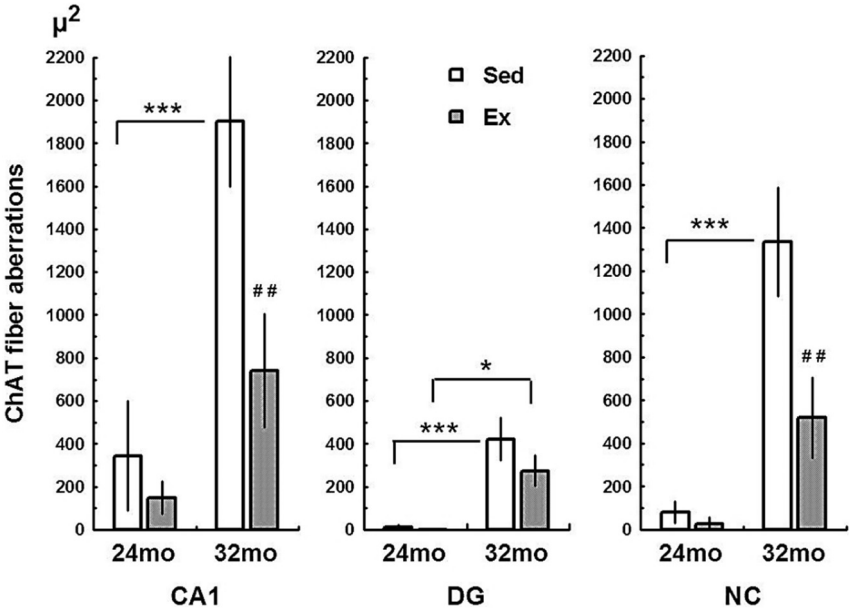

Fig. 9. Density of ChAT positive fiber aberrations are shown in some selected brain areas corresponding to those where the cholinergic fiber densities were evaluated: hippocampal CA1 and DG, as well as neocortical motor and sensory areas (NC). The figure compares 24 vs. 32 months old animals and clearly shows a marked increment in axonal aberrations as a result of exercise (*P < .05, ***P < .001). Chronic exercise considerably decreased the density of fiber aberrations in the senescent animals (##P < .01).

mechanism may be a close interaction between cognitive and motor regulatory functions of the brain in rodents during aging as proposed e.g. by Forster et al. (1996). In addition, D'Hooge and DeDeyn (2001) presented evidence for functionally integrated cognitive and motor neuronal networks involved in MWM performance. It was also reported previously that exploration and locomotion activity in an open-field and the MWM learning performance showed a correlation in aged rats (Gage et al., 1984).

In the NOR cognitive test we only measured exercise effects in the senescent age compared to the other two age periods. In this test the sedentary controls did not make any distinction between familiar vs. novel objects, whereas the exercised rats performed significantly well. The other behavioral paradigm, i.e. the exploratory activity towards all objects was also measured in the NOR test. Although an age-related decline was obvious, this type of behavior was not influenced significantly by the chronic exercise. In conclusion, only the selective attention towards the novel versus familiar objects could be influenced and improved by the chronic motor training. Looking at all behavioral results, obtained in this study it appears that the most obvious cognitive effect of chronic exercise in the sedentary age could be observed in the NOR test. Ample evidence confirms that the rodent hippocampus is essential for non-spatial object memory as well (Cohen et al., 2013; Cohen and Stackman, 2015). At the same time the cholinergic function of the hippocampus has been repeatedly connected to the performance of novel object recognition in rats (Melichercik et al., 2012; Vieira-Brock et al., 2015).

In the present study, analysis of cholinergic forebrain innervation was included to find neurobiological effects of chronic physical activity. The hippocampus and the primary

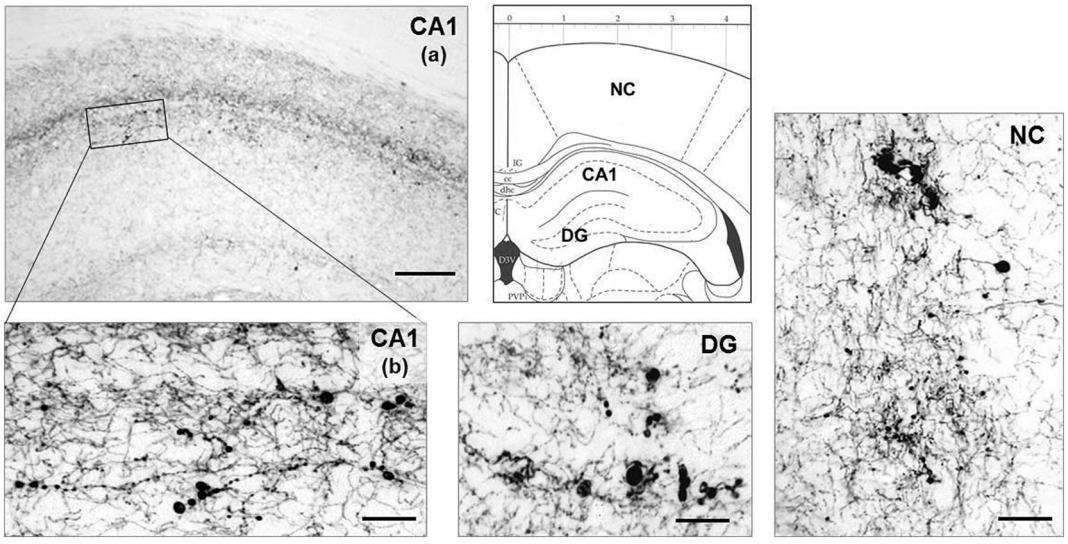

Fig. 10. Photomicrographs with ChAT-positive degenerative structures (fiber aberrations) in the hippocampal CA1 and DG and neocortical (NC) areas of rats aged 32 months. The investigated anatomical regions are indicated in a figure from the atlas of Paxinos and Watson (1997). CA1(a) represents some groups of fiber aberrations around the pyramidal cell layer (bar = 300 μm). At higher magnification (bar = 50 μm) single fibers show ChAT positive pearl-like immunoprecipitates. Fragments taken from the other investigated areas (DG and NC) provide examples for thickened axonal fragments and grape-like clusters of enlarged swellings (bar = 50 μm). Seven animals were taken from each group.

motor and somatosensory cortical areas were chosen to analyze the effect of moderate chronic treadmill exercise on the integrity of this neurotransmitter system. The results showed that the ChAT immunoreactive fiber density considerably declined with advancing age in the investigated hippocampus areas. Chronic exercise compensated for this neurodegenerative mechanism mainly in the senescent age of 32 mo in the CA1 region as well as in both neocortical areas. This effect shows that during senescent age moderate intensity exercise is quite effective for this forebrain transmitter system. The cholinergic innervation of the neocortex and the hippocampus in the mammalian brain originates from cholinergic cell groups in the basal forebrain and medial septal region respectively (Luiten et al., 1987; Nyakas et al., 1987). The anatomical organization of these pathways has been investigated on various cholinergic markers (Kása et al., 1997). It is now well established that the activity of the acetylcholine producing enzyme ChAT is markedly reduced both in cortex and hippocampus during aging in experimental animals and human (Perry, 1980; Gaykema et al., 1992; Nyakas et al., 2011; Luiten et al., 2013). Already Gottfries in 1990 suggested that the age-related cognitive and behavioral deficits partially arise from decreased levels of acetylcholine in the brain. Either the reduced activity of choline acetyltransferase or the increased activity of acetylcholine esterase may result in this condition (Gottfries, 1990).

In the present study, we assume that ChAT synthesis and/or transport was facilitated by the excess physical training. This assumption might be corroborated by our observation that the amount of ChAT positive axonal aberrations decreased markedly in the hippocampus but also in the neocortex of senescent rats after exercise. It is known that the intra-axonal transport processes are carried out by major families of cellular transport proteins (Hirokawa and Takemura, 2005). There is evidence showing that the anterograde transport of ChAT enzyme is maintained by the kinesin-2-motor complex which is dysfunctional e.g. in the amyotrophic lateral sclerosis motor disease (Tateno et al., 2009).

It is widely excepted and acknowledged that in Alzheimer's disease (AD) physical exercise can be a preventive lifestyle intervention slowing down the progression of the disease (Geda et al., 2010; Abe, 2012; Karceski, 2012; Luck et al., 2014; Norton et al., 2014), and defend neurons in amygdala, hippocampus and neocortex against AD-related degeneration (Voss et al., 2010; Lin et al., 2015). From both human (Abbott et al., 2004; Hillman et al., 2008; Erickson et al., 2011) and animal (Buzsáki, 2005) studies it can be concluded that cognitive and motor brain areas are functionally connected which lends support to the idea that increased physical activity positively affects cognitive performance.

We conclude from the present experimental findings that physical exercise training is most effective in the sedentary aging period in rats when both the motor and cognitive functions become largely impaired. This conclusion is consistent with previous observations that cognitive performance declines progressively with advancing age and senescence (Van der Staay, 2006). In our present study we applied treadmill exercise of moderate intensity which is adequate to maintain the physical condition of aged animals. It has been reported that during aging moderate intensity training is helpful to counteract aging or lesion-induced cognitive decline (Azimi et al., 2018; Tsai et al., 2018). It might be considered that this level of motor activity has little impact on the physical condition of younger or middle-aged rats as we also observed in the present experiment. In other words, the currently applied level of training intensity is effective during advanced aging. To yield exercise effects at younger ages probably higher intensity training is needed.

In conclusion, moderate intensity chronic exercise is beneficial in the senescent age in intact rats. Testing motor activity in novel surroundings (OF) and assessing cognitive capacities in the different learning-memory tests clear improvements could only be obtained in the senescent animals as opposed to other age-periods. The observed increased cholinergic innervation in the hippocampus and neocortical areas induced by chronic exercise may be part of the underlying mechanism to explain the improvements seen in cognitive performance and motor behavior.

ACKNOWLEDGMENTS

This work was supported by the National Research, Development and Innovation Office – NKFIH, K116511 in Hungary; and by the Higher Education Institutional Excellence Program at Semmelweis University, Budapest. Csaba Nyakas and Paul G.M. Luiten designed and monitored these experiments. Tímea Téglás and Zoltán Németh performed the experimental tests and analyzed the data. Eddy A. van der Zee and Ákos Koller contributed to the writing of manuscript. All authors have approved the final manuscript.

Declaration of interest: none.

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(Received 8 January 2019)
(Available online 28 May 2019)