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Lifelong Aerobic Exercise Reduces the Stress Response in Rats ☆

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Abstract—The aim of this study was to analyze the effects of lifelong aerobic exercise (AE) on the adaptive response of the stress system in rats. It is well known that hypothalamic–pituitary–adrenal axis (HPA) activity differs when triggered by voluntary or forced exercise models. Male Wistar rats belonging to exercise (E) or control (C) groups were subjected to chronic AE, and two cutoff points were established at 8 (middle age) and 18 months (old age). Behavioral, biochemical and histopathological studies were performed on the main components/targets of the stress system. AE increased adrenal sensitivity (AS), brain corticosterone (CORT) and corticotropin-releasing factor (CRF), but had no effect on the thymus, adrenal glands (AGs) weight or plasma CORT. In addition, AE exerted no effect on the sympathetic tone, but significantly reduced anxiety-related behavior and emotionality. Aging decreased AS and deregulated neuroendocrine feedback, leading to an anxiogenic state which was mitigated by AE. Histopathological and morphometric analysis of AGs showed no alterations in middle-aged rats but adrenal vacuolization in approximately 20% old rats. In conclusion, lifelong AE did not produce adverse effects related to a chronic stress state. On the contrary, while AE upregulated some components of the HPA axis, it generated an adaptive response to cumulative changes, possibly through different compensatory and/or super compensatory mechanisms, modulated by age. The long-term practice of AE had a strong positive impact on stress resilience so that it could be recommended as a complementary therapy in stress and depression disease.

Key words: aging, anxiety, exercise, HPA axis, stress, treadmill running.

INTRODUCTION

A proper response to stress depends on the coordinate actions of two main systems: the hypothalamic–pituitary–adrenal (HPA) axis and the autonomic nervous system (ANS) (Fulford and Harbuz, 2005, Stanford, 2013). Additional factors such as: natural genetic variation, sex, age, health, vulnerability or dissimilar environmental exposure (Ulrich-Lai and Engeland, 2005; Beery and Kaufer, 2015) also contribute to shaping responses according to opportunity, relevance, duration and intensity of different stressors (Nicolaides et al., 2015). The activation of the stress response triggers a cascade of events that begins with the hypothalamic secretion of the corticotropin-releasing factor (CRF), which in turn stimulates the pituitary gland to release the adrenocorticotropic hormone (ACTH). ACTH then promotes the adrenal release of glucocorticoids (GCs), which mobilize energy sources to meet the increased metabolic demand. On

☆ Roles for all authors: Adriana Pietrelli (*first author*) was in charge of study design, training protocol, behavioral experiments, morphometric analysis, data interpretation, and manuscript writing and submission for publication. Adriana Pietrelli, Laura Matkovic and Matias Di Nardo were in charge of biochemical protocols. Alejandro Masucci was in charge of histological procedures and histopathological analysis. Alicia H. Brusco and Nidia Basso collaborated with manuscript proofreading.

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Abbreviations: ACTH, adrenocorticotropic hormone; AE, aerobic exercise; AGs, adrenal glands; AS, adrenal sensitivity; AT, aerobic training; BW, body weight; CORT, corticosterone; CRF, corticotropin-releasing factor; ELISA, Enzyme-Linked Immuno-Sorbent Assay; EPM, elevated plus-maze; GCs, glucocorticoids; H-E, Harris hematoxylin-eosin; HPA, hypothalamic–pituitary–adrenal axis; HPLC, high-pressure liquid chromatography; LDB, light–dark box; NA, noradrenalin; RABs, Risk Assessment Behaviors; RIA, radioimmunoassay.

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the other hand, the sympathoadrenal response mediates many well-known physiological responses to acute stress such as increased heart rate and blood pressure, glucose levels, muscle tension and sweating (Stanford, 2013).

The brain plays a key role in stress response (McEwen, 2007; Myers et al., 2012) as it identifies stressful events, coordinates and regulates the initiation and termination of the behavioral, physiological and autonomic responses. The HPA axis is subject to dynamic regulation involving various levels of control, both central and peripheral (Ulrich-Lai and Engeland, 2005). If the stress response is dysregulated in terms of magnitude and/or duration, homeostasis becomes cacostasis, with adverse effects on many vital physiological functions (Nicolaides et al., 2015).

Unlike the traditional notion, the current views on stress hold that certain interventions such as exercise lead to the amplification of the cellular function (Stranahan and Mattson, 2014) and psychobiologic resilience (McEwen et al., 2015). Exercise is a physical stressor which produces hormetic, i.e. beneficial, effects (Mattson, 2007; Stranahan et al., 2010; Gradari et al., 2016) at moderate-low doses, and has been shown to reverse chronic stress in rats (Haak et al., 2008). This type of paradoxical stress has been defined as eustress, to distinguish it from distress or harmful stress (Sanchis-Gomar et al., 2012). The knowledge of the nature of the relationship between stress and exercise and the response of the HPA axis to chronic exercise is still limited and controversial (Kaliman et al., 2011; Heijnen et al., 2016). It is well known that the pattern of physiological responses varies according to the model of exercise: voluntary (Droste et al., 2003, 2007; Campeau et al., 2010) or forced (Yanagita et al., 2007; Leasure and Jones, 2008; Costa et al., 2012), short or long term. Previous work from this lab with rats subjected to lifelong aerobic training (AT) showed that regular moderate treadmill running had an anxiolytic effect, especially in old animals (Pietrelli et al., 2011, 2012). These studies focused on the behavioral response as the first approximation to the complex relationship between eustress, exercise and stress resilience.

In the present manuscript, biochemical, histopathological and behavioral assays were performed in order to understand the possible adaptive changes that occur in adrenal glands (AGs) and brain in response to chronic aerobic exercise (AE). Studies included (1) CRF and corticosterone (CORT) concentration in plasma, brain and AGs; (2) urinary catecholamines adrenalin (A) and noradrenalin (NA); (3) adrenal structural and morphologic integrity; and (4) emotionality and anxiety-related behavior in response to a new stressor.

MATERIALS AND METHODS

Animal care and experimental design

Animals. Male weaning Wistar rats weighing 70–100 g, WKAH/Hok strain (n = 48), were obtained from the Animal Facilities of the School of Veterinary Science, University of La Plata (UNLP), housed in groups of two in standard laboratory cages, and randomly assigned to each of the following groups: Control (C, n = 24) or Exercise (E, n = 24). Animals were maintained at a temperature of 22 ± 2 °C, relative humidity 55–65%, and reverse photoperiod of 12:12 h light–dark (lights off at 6:00 am). Rats were clinically evaluated every week and subjected to microbiological monitoring every 6 months. Food and water were supplied *ad libitum.* Body weight (BW), food, and water intake were also evaluated every week. *Experimental design:* Two cut-off points were established which are representative of the physiological status at 8 months (middle age) and 18 months (old age) (Fig. 1, Panel A). The circadian and pulsatile pattern of stress hormones was taken into account so that all experiments were conducted during the dark cycle (6:00 am–18:00 pm). All efforts were made to minimize pain or discomfort and the number of animals used. This work was approved by the Institutional Ethics Committee for the Care and Use of Laboratory Animals at the School of Health Sciences (UCES) and complies with national and international standards on animal welfare.

AT protocol

The exercise model was based on two key concepts: hormesis and supercompensation. The hormetic model was proposed by Calabrese (2004) claims that low doses of an exogenous or intrinsic factor (for example, exercise) may enhance response, while high doses may produce inhibitory or adverse effects (Calabrese, 2004; Mattson, 2007). On the other hand, supercompensation is an event consisting in a metabolic rebound produced by the appropriate relationship between workload and regeneration (Bompa and Haff, 2009). Training has immediate, delayed and cumulative effects. Every time supercompensation occurs, the subject establishes a new, increased homeostatic level and higher neuropsychological adaptation (Bompa and Haff, 2009; Kenney et al., 2012). On the basis of these ideas, the aim of the training protocol was to progressively increase aerobic power (Fig. 1, Panel B) to acquire new levels of hormesis and maximize brain homeostatic adaptation.

As described in previous papers (Pietrelli et al., 2011, 2012), E animals were trained three times a week from 2 to 18 months of age at the same relative overload (60–70% max VO_2) in a motorized treadmill (Fig. 2A). Data on running speed (m/min), cumulative meters (m), total length (min), slope (degrees), internal temperature (°C), airflow pumped (ml/min) and oxygen consumption (VO_2, ppm) were acquired in real time using LabView v.8.6 software [National Instruments, USA]. Parameters were adjusted on the basis of data obtained from monthly tests of max VO_2 (Fig. 2B, C). At 45 days of age and before the beginning of the protocol, both E and C animals were submitted to a habituation period of 2 weeks. At 2 months of age, C rats were placed on a motionless treadmill, while E rats received an initial workload of 15-min duration; 6 m/min speed, and 0 degrees' slope. At 6–8 months, workload was increased to 60-min duration, 14 m/min speed, and 5 degrees' slope. Finally, at 18 months, workload

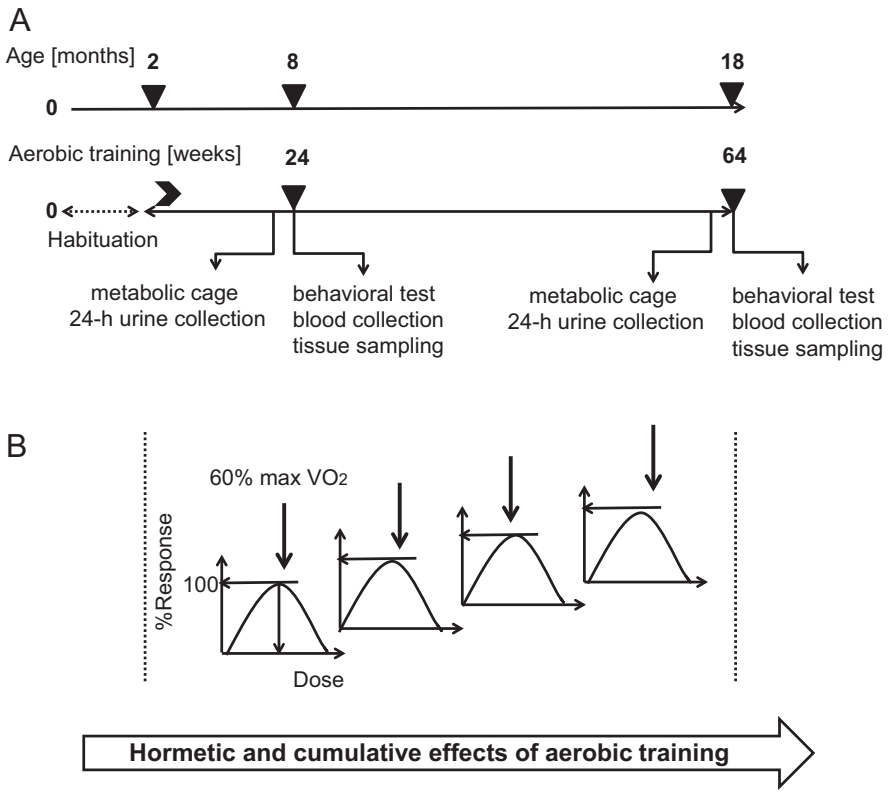

Fig. 1. Experimental design and protocol training. (A) Scheme of the experimental design. The animals were exercised regularly from adolescence (2 months) up to old age (18 months). Two cut off points (8–18 months) were established to study the cognitive function and obtain brain samples. A week before ending physical training, 24-h urine samples were collected with metabolic cages. Subsequently, the behavioral response to a new stressor (EPM and LDB) was studied, followed by sacrifices to obtain tissue and blood samples. (B) The training protocol was designed to stimulate the aerobic energy system (60% max.VO_2) and amplify the homeostatic capacity through successive cumulative, hormetic effects. Profiles represent multiple states of equilibrium (steady-state) of the aerobic power.

was 30-min duration, 4–6 m/min speed, and 0 degrees' slope. Middle-aged E rats underwent 24 weeks and old E rats underwent 64 weeks of AT. In order to prevent and/or reduce the possible effects of chronic stress as reported in certain forced exercise models, the following criteria were applied: (1) animals were extensively handled in the habituation period; (2) rats were allowed to freely explore the treadmill and establish social contact for 5 min before the beginning of AT; (3) rats were able to see their peers through the transparent walls of the treadmill; (4) E and C rats were positively reinforced with a ring of fruity cereal.

Behavioral testing

Behavioral testing was carried out 24 h post-training, first through the elevated plus-maze (EPM) test and, 24 h later, through the light–dark box (LDB) test with the same groups of rats (C, $n = 12 \times 2$; E, $n = 12 \times 2$). The goal was to study anxiety-related spontaneous behavior, activity levels and emotionality when rats are exposed to new stressors. EPM and LDB were placed in a soundproof room, with no objects or signals. All behavioral tests were scored by an experimenter who was blind to the experimental design and filmed with a camera fixed on the ceiling. Rats were excluded from the analysis if they fell off the EPM or if there were unexpected loud noises in the facilities during behavioral testing. Apparatuses were thoroughly cleaned with 70% ethanol between trials.

EPM

The EPM consisted of a central 12 × 12 cm square with four 12 × 60 cm arms elevated 50 cm above the floor. Two of the arms had black 40-cm-high walls and were closed at their ends. A 1-cm-high edge was placed around the open arms to prevent rats from falling. Bright lighting was provided by a 60-watt table lamp located 50 cm above each open arm. The task involved conflict between a tendency to avoid well-lit, exposed and elevated areas, or to explore a new environment. *Procedure:* Rats were initially placed in the central square of the apparatus, facing an open arm, and were allowed to explore the apparatus for 5 min. Parameters evaluated included (A) Latency (sec): time to leave the central platform; (B) Closed arms: number of entries in closed arms; (C) Time spent in closed arms (sec): total time spent in closed arms; (D) Fecal boli: number of fecal pellets; (E) Open arms: number of entries in open arms; and (F) Time spent in open arms (sec): total time spent in open arms. Entries were recorded when the animal crossed the central platform with its four paws. Variables (A), (C), (E) and (F) were considered the key indicators of anxiety-related behavior. Variable (B) was indicative of exploration and locomotor activity. Increased latency, fecal pellets, time spent in closed arms or decreased open arms activities without concomitant changes in general locomotion and exploration indicated increased anxiety in the EPM. Variable (D) was a sign of emotionality.

LDB

The 80 × 40 × 40 cm LDB consisted of two closed chambers – a black 30 × 40 cm chamber and a white 5 0 × 40 cm one – communicated by a 15 × 15 cm opening. Bright lighting was provided by a 60-watt table lamp located 50 cm above the center of the white chamber. As rodents show an innate preference for the small dark chamber over the larger bright one, the task involved conflict between a tendency to avoid open brightly lit areas or to explore a new environment. *Procedure*: Rats were placed into the brightly lit compartment facing the opening and allowed to freely

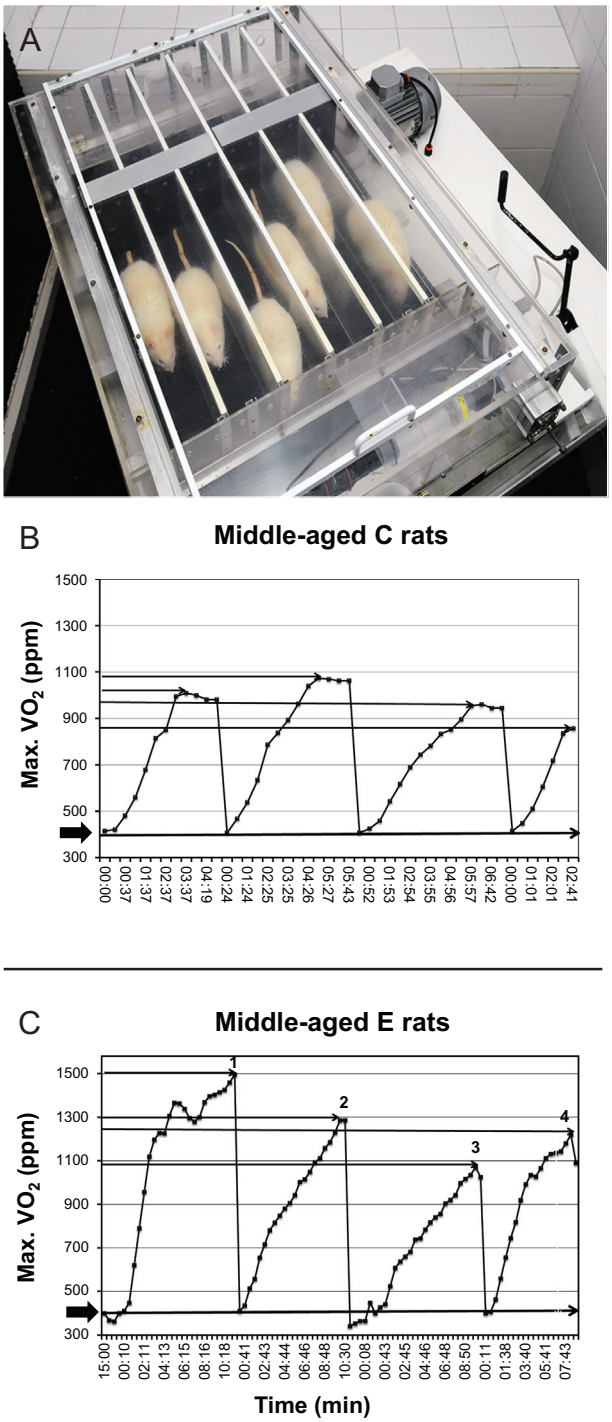

Fig. 2. Treadmill running. (A) General view. (B, C): Records obtained from single tests of max.VO_2, performed with middle-aged rats, C (n = 4) and E (n = 4). Notice that the C rats (B) reached lower maximum values, and in less time than E rats. By contrast, AE increased the aerobic power as evidenced by the higher records obtained in longer times (C).

explore the apparatus for 5 min. Data collected included (A) Latency (sec): time before first leaving the brightly lit compartment; (B) Number of light–dark transitions; (C) Stretch-attend posture; (D) Number of fecal pellets; (E) Time spent in the light zone (sec); and (F) Time spent in the dark zone (sec). Accordingly, variables (A), (B), and (E) were considered the key indicators of anxiety. Variable (C): The stretch-attend postures (the trunk extending and then flexing back to original position) are ethological indicators considered Risk Assessment Behaviors (RABs) (Kulesskaya and Voikar, 2014). These indicators represent a more sensitive index of anxiety-related behaviors than conventional measures obtained from the EPM alone. Under a potential hazard, rodents cease ongoing exploratory behaviors and display RABs as a mechanism to get threat-related sensory information and to optimize the most adaptive behavioral strategy. Variable (F) was related to aversion to bright light and variable (D) was a sign of emotionality.

Blood and tissue sampling

Twenty-four hours after the LDB test, C ($n = 12 \times 2$) and E ($n = 12 \times 2$) rats were sacrificed by decapitation (Harvard-Apparatus, South Natick, MA). Blood samples were obtained from the trunk and collected into EDTA-coated tubes (BD Vacutainer, K2 EDTA, 5,4 mg, 3 ml). Subsequently, samples were centrifuged at 3500g 15 min at 4 °C, and the plasma stored at −20 °C until analyzed. Brains, the hypothalamus containing the paraventricular nucleus (Bregma points: −0.22 mm to −0.58 mm, plates 33–36) and the pituitary gland (anterior, medial and posterior lobes, plate 107; Franklin and Paxinos, 2007) were dissected, weighed and homogenized at a ratio of 1 g brain/10 mL in a medium consisting of 0.32 M sucrose, 20 mM Tris–HCl, pH = 7,4, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 1 mM sodium orthovanadate, 0,1 mM ammonium molybdate, 1 mM PMSF, and protease inhibitors (Sigma Chemical Co., St. Louis, Missouri). Homogenates were centrifuged at 30,000g for 30 min to discard pellets. Then, supernatants were either analyzed immediately or kept at −80 °C before analysis. The thymus of middle-aged C (n = 6) and E (n = 6) rats were dissected out and weighed, while those of the old rats was discarded as atrophic, residual tissue. The C ($n = 6 \times 2$) and E ($n = 6 \times 2$) AGs were removed, placed in ice-cold saline solution, and trimmed free of fat and surrounding tissue according to the modified Gómez–Sánchez method (Gomez-Sanchez et al., 1975). Operations were carried out at 0–2 °C. Each AG (right/left) was weighed and cut into two pieces. The four pieces were divided as follows: two of the pieces (right/left) were incubated in the Krebs–Ringer glucose medium containing 2 mL of 10 mM Hepes, pH 7.4, and (+) 1 nM ACTH; the other two (right/left) were incubated without (−) ACTH. Incubations were carried out at 37 °C with continuous shaking and stopped after 1 h by chilling the samples. Media were quickly separated and stored at −70 °C until CORT assessment by radioimmunoassay (RIA). Protein concentration was measured by Bradford's method using bovine serum albumin as standard.

CORT

Levels of CORT were measured in C ($n = 6 \times 2$) and E ($n = 6 \times 2$) AGs, plasma and C ($n = 12 \times 2$) and E brains ($n = 12 \times 2$) by direct RIA. The cross reactivity

with aldosterone was less than 0.1% and assay sensitivity was 50 pg/mL. Drugs and chemicals were purchased from Sigma Chemical Co. (St. Louis, Missouri). The tritiated CORT (3H1,2,6,7), specific activity = 70 μCi/μm oles was purchased from New England Nuclear, Life Sciences Products (Boston, MA., USA). Other reagents were of analytical grade.

CRF

The levels of CRF were measured by a commercially available Enzyme-Linked Immuno-Sorbent Assay (ELISA) kit (EIA-ALPCO, CRFMS-E0148). The kit protocol was followed for quantitative determination of total CRF in extract samples of brain, hypothalamus, pituitary gland and plasma, which were previously separated. Samples from C ($n = 6 \times 2$) and E ($n = 6 \times 2$) groups were assayed together in duplicate. Assay sensitivity was within the range of 0.078–2.5 ng/mL. Its intra-assay CV (%) was 3–10 (plasma), and inter-assay CV (%) was 2–5. This ELISA kit showed no cross reactivity to urocortin.

Catecholamines: A and NA

A week before finishing AT, C ($n = 6 \times 2$) and E ($n = 6 \times 2$) rats were progressively habituated to a metabolic cage (Nalgene Metabolic Cages, Nalge Company, N.Y.) with food and drink *ad libitum*: rats were exposed 1 h the first day, 4 h the second day, and the experiment began on the third day (lights off, 8.00 am). On the fourth day, 24-h urine samples ($n = 24$) were collected in photoprotected tubes containing 6 N HCl. Aliquots were stored at −20 °C and then assessed for determination of A and NA (Crawford and Law, 1958) by high-pressure liquid chromatography (HPLC) with electrochemical detection (Westermann et al., 2002). HPLC catecholamines were extracted by aluminum oxide columns separated by reversed-phase HPLC. The separation was controlled and corrected with internal standard 3,4-dihydroxybenzylamine. The detection limit was found to be 10 pg/mL for both A and NA. The chromatographic analysis was carried out in a Spectra Physics high-performance liquid chromatography system equipped with an amperometric detector (LC4b/CC5), an isocratic pump, and a Thermo Scientific-Part N° 31605254630-ODS-2 HYPERSIL 250 × 4.6 mm column. A manual system was used for injection, Rheodyne (7125, Cotati, CA) with 10 μl sample. The data were processed on a Clarity Chromatography Station.

The standard solution of catecholamines was prepared by dissolving 1 mg of each catecholamine in 10 mL of 0.1 M HCl. Dilutions were made from the mother solution reaching concentrations ranging from 0.625 $mg.mL^{-1}$ to 5.0 $mg.mL^{-1}$. Retention times were 5.6 ± 0.1 min for NA, and 8.2 ± 0.1 min for A.

Histochemistry and digital imaging

C ($n = 6 \times 2$) and E ($n = 6 \times 2$) AGs were processed according to routine histological techniques in an automatic Microm STP 120 processor and embedded in paraffin blocks, with a melting point of 56–59 °C. Serial 3.5-μm sections were stained with Harris hematoxylin-eosin (H-E) and Masson trichrome and then examined with a clear light Nikon Eclipse E200 microscope. Thirty fields were finally digitalized per group.

Statistical analysis

Data were expressed as the mean ± SEM. Significance was established at $p < 0.05$. All data were tested for outliers, normality by Shapiro–Wilk's test and homoscedasticity by Levene's test, and shown to conform to the requirements for parametric statistics before ANOVAs were carried out. A two-way ANOVA (age × exercise) was used to analyze data from behavioral tests, biochemical determinations (CRF, CORT, A, NA, water consumption and urine), and physical parameters (body and adrenal weights, adrenal cortex, and medullary area), followed by *post hoc* comparisons with Bonferroni's or Tukey's tests when appropriate. Absolute and relative thymus weights of the middle-aged rats were evaluated by Student's *t* test for two independent groups. A three-way ANOVA (age × exercise × layer) was used to analyze morphometric data of the adrenal cortical layers (glomerular, fascicular and reticular), followed by *post hoc* Bonferroni's tests to determine specific differences and to test two-way interactions through a third factor. AGs' morphometric analysis was conducted with NIH Image J software (Bethesda, Maryland, http://rsb.info.nih.gov/nih-image/). All the variables were analyzed with SPSS v. 19.0 IBM (Armonk, NY) software 2012 and plotted with Graph Pad Prism v. 5.03 2010 (La Jolla, CA).

RESULTS

Training protocol and clinical status

Weekly physical examinations and biochemical routines indicated general good health along the study, with 100% survival at 18 months of age. E rats responded positively to AT and improved their physical performance, showing great tolerance for effort and progressive adaptation to increased workload, as indicated by subsequent increases of approximately 80% in max.VO_2 relative to C (Fig. 2B, C). None of the rats had to be withdrawn from the study for refusing to run or signs of pain, fatigue or disease. Animals showed a remarkable disposition to run, even at advanced ages. It is known that endurance exercise may cause inflammation by the eccentric overload on joints and muscles during the support phase (Kenney et al., 2012). Also, and contrary to expectations, some old C rats, but not E rats, showed a significant inflammatory state of their hind paws (10–15%).

Behavioral tests

EPM. A two-way ANOVA (age × exercise) was used to analyze data from EPM followed by *post hoc* Tukey's multiple comparisons test. Seven rats were excluded from the analysis [8C (2); 8E (1); 18C (2); 18E (2)] in

accordance with the exclusion criteria. Latency was significantly affected by age [$F(1,37) = 5.91$, $p = 0.020$], and some old C rats exhibited freezing behavior and difficulty to move. Significant differences ($p < 0.05$) were detected between 8E and 18C (Fig. 3A). The number of entries to closed arms was also significantly affected by age [$F(1,37) = 32.10$, $p < 0.0001$]. Significant differences ($p < 0.001$) were detected between middle-aged and old rats. As expected, middle-aged rats showed a higher level of locomotor and exploratory activity in relation to old animals (Fig. 3B). AE significantly affected the time spent in closed arms [$F(1,37) = 10.41$, $p = 0.0026$], with old E rats spending less time ($p < 0.01$) than age-matched controls and a higher level of activity (Fig. 3C). The number of fecal pellets was affected by AE [$F(1,37) = 6.017$, $p = 0.019$], as C rats showed a very strong and heterogeneous emotional response relative to E rats ($p < 0.05$), (Fig. 3D). The number of entries into open arms showed main effects due to AE [$F(1,37) = 11.17$, $p = 0.0019$] and age [$F(1,37) = 25.60$, $p < 0.0001$]. Significant differences ($p < 0.05$) by AE effect were detected between 8C and 8E. Middle-aged animals had increased exploratory and locomotor activity and less anxiety than old ones, and differences ($p < 0.001$) between middle-aged and old C rats were substantially larger than those observed among E rats (Fig. 3E). Time spent in open arms showed main effects due to age [$F(1,37) = 11.04$, $p = 0.002$] and AE [$F(1,37) = 18.42$, $p = 0.0001$], with E rats spending more time in the open arms than their sedentary counterparts ($p < 0.05$). Significant differences ($p < 0.001$) by age effect were detected between 8E and 18C (Fig. 3F). This response indicated an important anxiolytic effect of AE.

LDB. A two-way ANOVA (age × exercise) was used to analyze data from LDB followed by *post hoc* comparisons with Tukey's tests. Five rats were excluded from the analysis [8C (2); 8E (1); 18C (1); 18E (1)] in accordance with the exclusion criteria. Latency was significantly affected by the main effect of age [$F(1,39) = 10.55$, $p = 0.0024$]. Significant differences ($p < 0.01$) by age effect were detected between 8E and 18C. Middle-aged E animals were faster than old C ones. A pattern of freezing and motor difficulties to move quickly from one camera to another were again detected in many old C rats, (Fig. 4A). Transitions were significantly affected by the main effects of age [$F(1,39) = 47.19$, $p < 0.0001$] and AE [$F(1,39) = 21.93$, $p < 0.0001$]. Significant differences by AE effect were detected between 8E and 8C ($p < 0.0002$), 18C and 18E ($p < 0.05$). E rats were observed to go through more transitions than their respective controls. Significant differences ($p < 0.001$) by age effect were

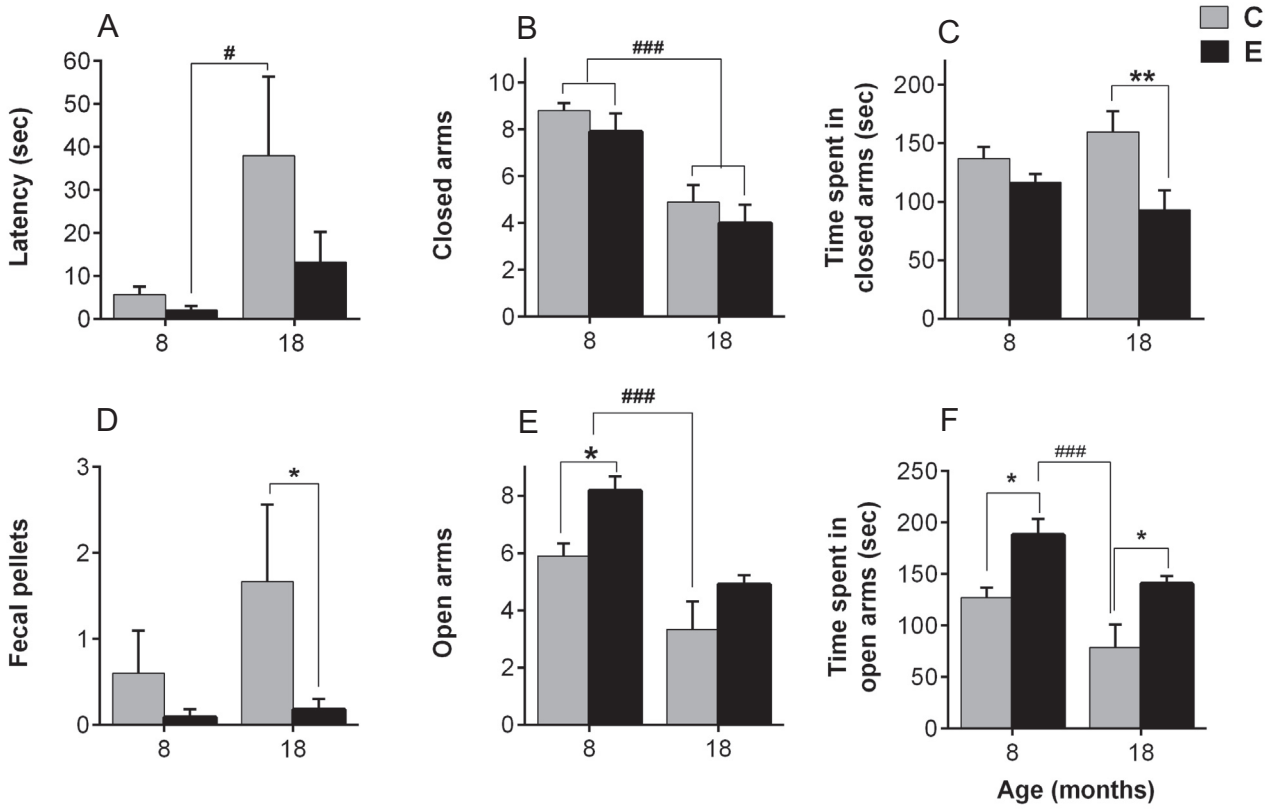

Fig. 3. Effects of AE and age on anxiety-related behaviors in the EPM. Bars represent the mean ± SEM. Symbols: age effects ($^{\#}$); exercise effects (*). Data were analyzed by a two-way ANOVA followed by *post hoc* Tukey's test. (A) Latency, $^{\#}p < 0.05$ 8E vs. 18C. (B) Closed arms, $^{\#\#\#}p < 0.001$ 8C-E vs. 18C-E. (C) Time spent in closed arms, $^{**}p < 0.01$ 18C vs.18E. (D) Fecal pellets, $^{*}p < 0.05$ 18C vs.18E. (E) Open arms, $^{*}p < 0.05$ 8C vs. 8E, $^{\#\#\#}p < 0.001$ 8 C-E vs. 18C. (F) Time spent in open arms, $^{*}p < 0.05$ 8C vs. 8E and 18C vs. 18E, $^{\#\#\#}p < 0.001$ 8E vs. 18C.

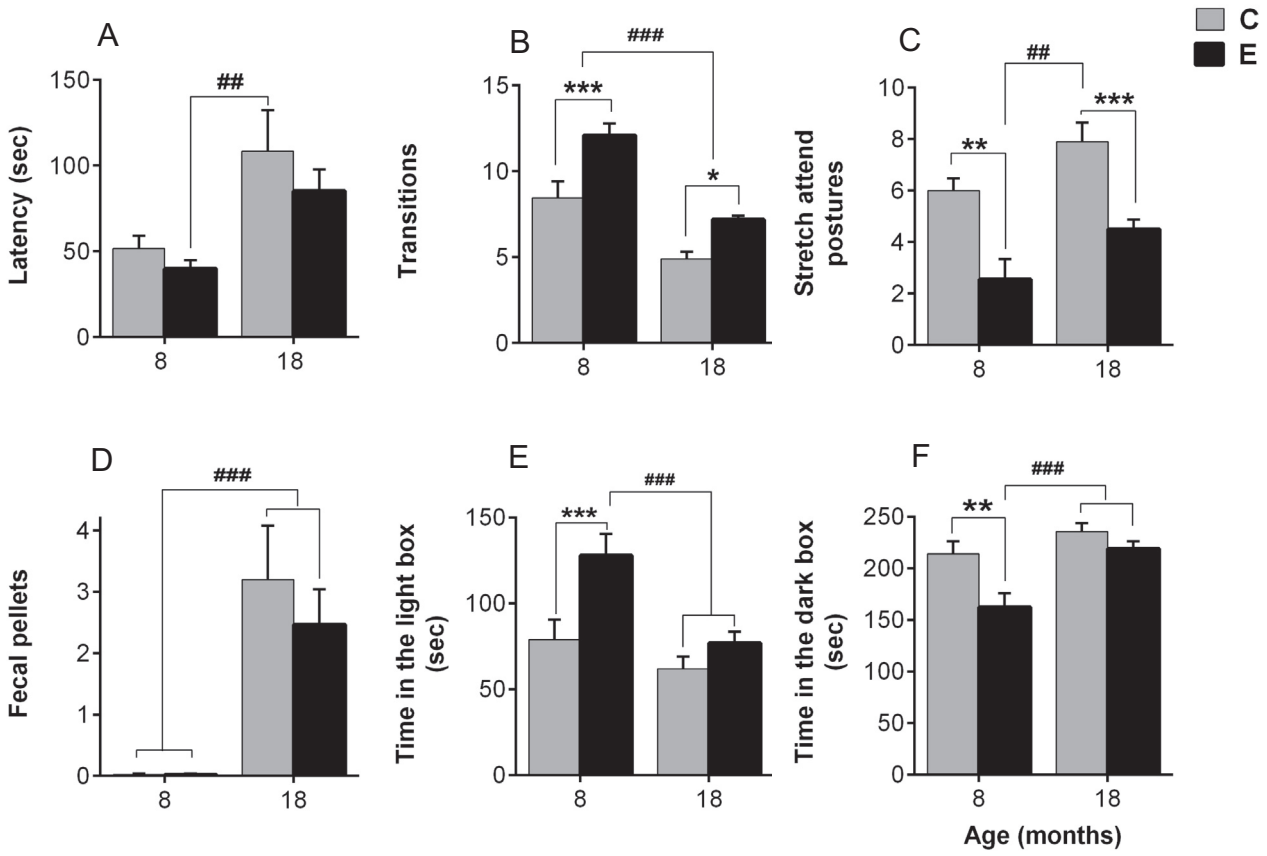

Fig. 4. Effects of AE and age on anxiety-related behaviors in the LDB. Bars represent the mean ± SEM. Symbols: age effects ($^{\#}$); exercise effects (*). Data were analyzed by a two-way ANOVA followed by *post hoc* Tukey's test. (A) Latency, $^{\#\#}p < 0.01$ 8E vs. 18C. (B) Transitions, $^{***}p < 0.001$ 8C vs. 8E and $^{*}p < 0.05$ 18C vs.18E; $^{\#\#\#}p < 0.001$ 8C-E vs. 18C-E. (C) Stretch attend postures, $^{**}p < 0.01$ 8C vs. 8E and $^{***}p < 0.001$ 18C vs. 18E; $^{\#\#}p < 0.01$ 8E vs. 18C. (D) Fecal pellets, $^{\#\#\#}p < 0.001$ 8 C-E vs. 18C-E. (E) Time in the light box, $^{***}p < 0.001$ 8C vs. 8E; $^{\#\#\#}p < 0.001$ 8E vs. 18C-E. (F) Time in the dark box, $^{**}p < 0.01$ 8C vs.8E; $^{\#\#\#}p < 0.001$ 8E vs. 18C-E.

detected between middle-aged and old rats which can be attributed to greater motor efficiency (Fig. 4B). AE [$F(1,39) = 34$, $p < 0.0001$] and age [$F(1,39) = 11$, $p = 0.0022$] significantly affected stretch-attend postures. Significant differences by AE effect were detected between 8E and 8C ($p < 0.01$), 18C and 18E ($p < 0.0001$). Significant differences by age effect were detected between 8E and 18C ($p < 0.01$). AE had an anxiolytic effect, while age increased RABs, particularly in old C rats (Fig. 4C). In turn, defecation was affected by age [$F(1,39) = 24.18$, $p < 0.0001$], as old animals showed a very strong emotional response compared to middle-aged rats ($p < 0.0001$), (Fig. 4D). The trend observed in this test was contrary to that observed in the EPM, a difference which can be interpreted in the context of different anxiogenic constructs. Both AE [$F(1,39) = 18.17$, $p = 0.0001$] and age [$F(1,39) = 13.25$, $p = 0.0008$] significantly altered the time spent in the light box. Significant differences by AE effect were detected between 8E and 8C ($p < 0.001$). Significant differences by age effect were detected between 8E and old rats ($p < 0.001$), as middle-aged E animals showed less aversion to intense lighting (Fig. 4E). Time in the dark compartment was directly correlated to the perceived aversion to bright light. Both AE [$F(1,39) = 11.74$, $p = 0.001$] and age [$F(1,39) = 12.53$, $p = 0.001$] exerted main effects on this parameter. Significant differences by AE effect were detected between 8E and 8C ($p < 0.01$). Significant differences by age effect were detected between 8E and old rats ($p < 0.001$), as middle-aged C rats and aged rats had a greater preference to stay in the dark box (Fig. 4F).

Biochemical determinations

CRF and CORT. A two-way ANOVA (age × exercise) was used to analyze data from biochemical determinations followed by *post hoc* comparisons with Tukey's tests. Pituitary CRF concentrations were affected by the main effects of age [$F(1,18) = 14.09$, $p = 0.006$], and AE [$F(1,18) = 4.707$, $p = 0.043$]. Significant differences by age effect were detected between middle-aged rats and old C rats ($p < 0.01$). Significant differences by AE effect were detected between 18E and 18C ($p < 0.05$), (Fig. 5A). Total hypothalamus CRF levels showed a significant main effect due to age [$F(1,18) = 7.271$, $p < 0.0148$] as

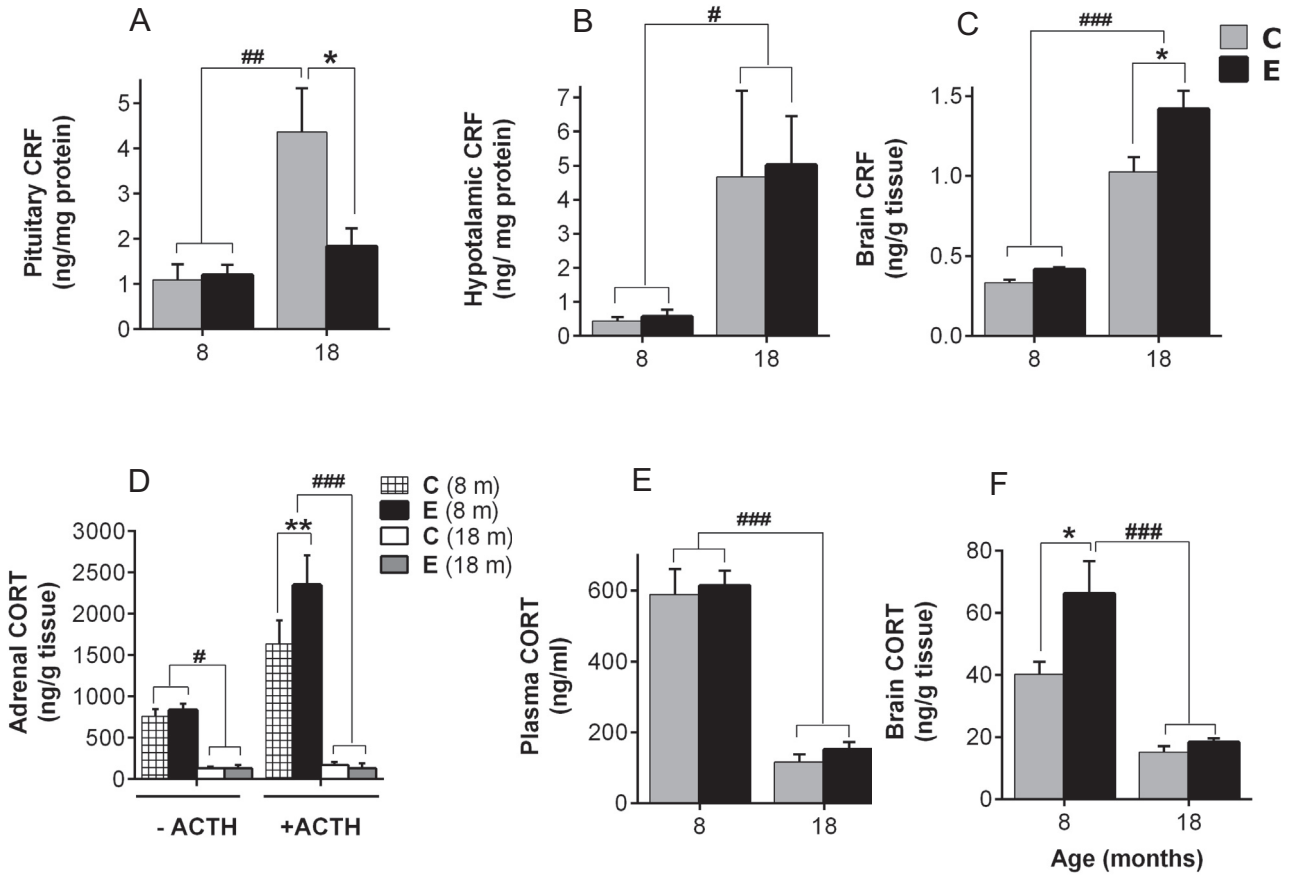

Fig. 5. Effects of AE and age on central CRF levels, and brain, plasma and adrenal CORT. Bars represent the mean ± SEM. Symbols: age effects ($^{\#}$); exercise effects (*). Data were analyzed by a two-way ANOVA followed by *post hoc* Bonferroni's or Tukey's test. Graphs a-c correspond to brain CRF and selected areas. (A) Pituitary CRF, $^{*}p < 0.05$ 18C vs. 18E; $^{\#\#}p < 0.01$ 8C-E vs. 18C. (B) Hypothalamic CRF, $^{\#}p < 0.05$ 8C-E vs. 18C-E. (C) Total brain CRF, $^{*}p < 0.05$ 18C vs. 18E; $^{\#\#\#}p < 0.001$ 8 C–E vs. 18C-E. (D) Adrenal CORT. AE had no effect ($^{*}p > 0.05$) in the basal state [(-) ACTH] but significant effects under treatment with ACTH [(+) ACTH] ($^{**}p < 0.01$ 8C vs.8E). The adrenal CORT levels were strongly age-dependent, in the presence ($^{\#\#\#}p < 0.001$ 8 C-E vs. 18C-E) or absence of ACTH ($^{\#}p < 0.05$ 8 C-E vs. 18C-E. (E) Plasma CORT, $^{\#\#\#}p < 0.001$ 8C-E vs. 18C-E. (F) Total brain CORT, $^{*}p < 0.05$ 8C vs. 8E; $^{\#\#\#}p < 0.001$ 8 C-E vs. 18C-E.

middle-aged rats presented lower CRF levels than old rats ($p < 0.05$), (Fig. 5B). Brain CRF levels showed significant main effects of both AE [$F(1,29) = 5.02$, $p = 0.033$] and age [$F(1,29) = 62.56$, $p < 0.0001$]. *Post-hoc* analysis determined that middle-aged rats showed lower CRF levels than old rats ($p < 0.001$). Significant differences by AE effect were detected between 18E and 18C ($p < 0.05$), with old E rats exhibiting higher CRF concentrations than age-matched controls (Fig. 5C).

In both the basal status without (−) ACTH and with (+) ACTH, CORT levels showed a strong dependence on age [$F(1,20) = 122.44$, $p < 0.0001$]. Exposure to ACTH produced a significant effect in middle-aged rats [$F(1,20) = 62.25$, $p < 0.0001$] but no response in old rats, showing a possible loss of functionality and/or structural impairment secondary to aging. Middle-aged E rats had higher CORT than age-matched C ($p = 0.042$) and old rats ($p < 0.0001$), which showed a significant increase in the adrenal sensitivity (AS) in response to ACTH (Fig. 5D). In addition, plasma CORT was unaltered by AE but affected by age [$F(1,20) = 113.87$, $p < 0.0001$]. Middle-aged C-E rats had higher CORT than age-matched C-E ($p < 0.001$), (Fig. 5E). The brain CORT was significantly affected by the main effects of AE [$F(1,20) = 6.37$, $p = 0.02$] and age [$F(1,20) = 39.87$, $p < 0.0001$], as middle-aged E rats showed higher levels than aged rats, C and E ($p < 0.001$). Significant differences by AE effect were detected between 8E and 8C ($p < 0.05$), (Fig. 5F). This result correlated with that observed in Fig. 5C, indicating a deterioration in the negative feedback exerted by CORT at brain level in aged rats.

Catecholamines A and NA

No significant differences were observed between C and E rats in either the drinking water (Fig. 6A) or 24-h urine (Fig. 6B). A (Fig. 6C) and NA (Fig. 6D) levels were not affected either by the AE or age. Although aging increases the variability in the response probably due to individual differences, all rats showed reduced levels of total catecholamines and relative percentages of NA (85–88%) and A (11–14%) similar to those previously reported for the same strain (Crawford and Law, 1958; Lepschy et al., 2008).

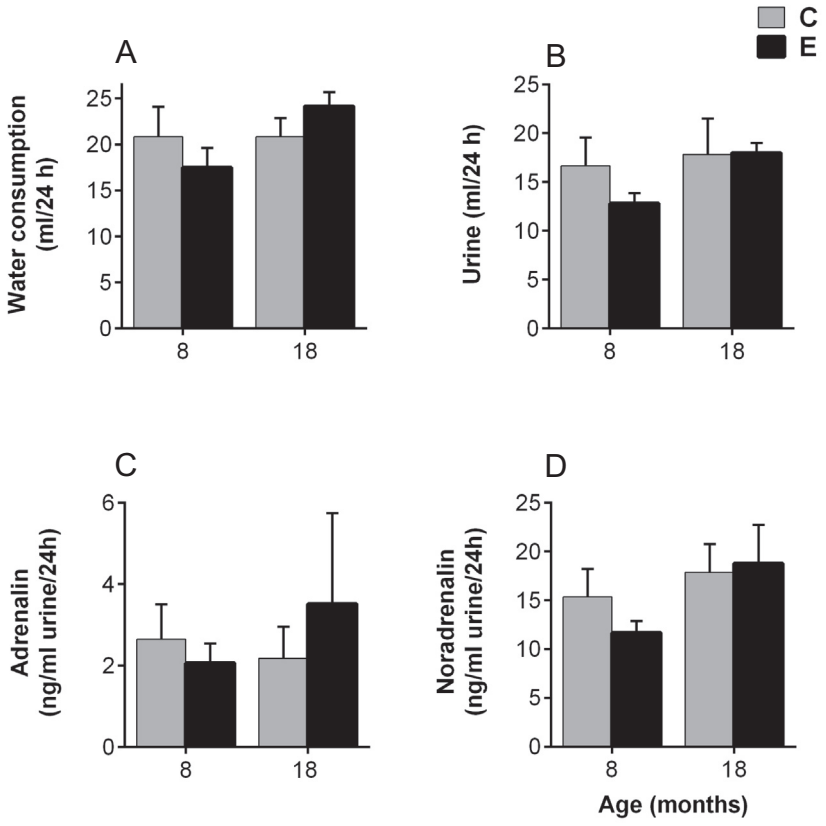

Fig. 6. Effects of AE and age on urinary catecholamine levels. Bars represent the mean ± SEM. AE and age had no effect on (A) water consumption, (B) the volume of urine in 24 h, or (C-D) catecholamine levels. Note the increased variability in old E animals.

Histopathology and morphometry of AGs

Histopathology: Sections stained with H-E and Masson trichrome showed typical histoarchitecture, preserved in both the C and E groups (Fig. 7). No epithelial or interstitial changes were detected in middle-aged rats, as evidence of inflammation, such as lymphocyte filtration or bleeding, or structural abnormalities in the cortex or medulla. However, approximately 20% old rats (Fig. 8, A-B) showed interstitial and/or intracellular macro/microvacuolization located in the cortex (Fig. 8C, D) as well as in the medulla (Fig. 8E), which can be attributed to the deleterious effects of aging. In addition, significant hypotrophism was observed in old C rats in relation to the remaining rats, which correlated with morphometric parameters (Fig. 7, C1; see Table 1).

Morphometry and physical parameters (BW, AGs and thymus): BW was unaltered ($p > 0.05$) by AE or age. However, absolute adrenal weights showed significant main effects of AE [$F(1,53) = 10.9$, $p = 0.002$] and age [$F(1,53) = 6.67$, $p = 0.012$]. No interaction was detected. Tukey's multiple comparisons test showed differences between 8C vs 18C ($p = 0.038$), 8E vs 18C ($p < 0.001$), and 18C vs 18E ($p = 0.010$). Relative adrenal weights were affected by AE [$F(1,53) = 22.7$, $p = 0.010$]. Tukey's multiple comparison test showed differences between old rats C vs E ($p < 0.05$), probably due to lower BW in the E group. Absolute and relative thymus weights presented no significant effects ($p > 0.05$) exerted by AE or age. In turn, adrenal cortex thickness correlated with AGs size and weight, mainly as a consequence of AE [$F(1,44) = 5.18$, $p = 0.027$], and *post hoc* Tukey's test revealed significant differences $^{*}p < 0.05$ between 8E vs 18C. The medullary area showed significant interaction between AE and age [$F(1,44) = 23.7$, $p < 0.0001$]. The effect of age through the levels of the group factor showed significant differences between 8C vs 8E ($p < 0.0001$) and 18C vs 18E ($p < 0.018$). The effect of group through the levels of the age factor showed very significant differences between middle-aged and old rats ($p < 0.0001$), C vs E. This response indicated that the greatest differences among middle-aged animals are related to AE.

Morphometric study of the adrenal cortex three cellular layers (glomerular, fascicular y reticular) with three factors ANOVA [exercise *(group)* × age × layer] showed a significant triple interaction $F(2,541) = 3.010$, $p = 0.048$]. Differences between a two-way interaction [age × layer ($F(2,541) = 10$, $p < 0.0001$)] through the two levels of a third factor (exercise/group) and [group × layer $F(2,541) = 5.5$, $p < 0.02$)] through the two levels of a third factor (age) were evaluated by Bonferroni's tests. Multiple comparisons showed qualitative, non-constant, differences between the fascicular and reticular layer, but not in the glomerular layer in the following levels: (1) between middle-aged animals the double interaction (age × layer) presented significant differences when the fascicular layer was analyzed between the two levels of the group factor ($p = 0.007$, C vs E). E rats showed thicker fascicular layers than C animals. (2) Among old animals; the double interaction (age × layer) demonstrated significant differences when the fascicular layer was considered between the two levels of the group factor ($p = 0.014$, C vs E). The age effect on the fascicular layer affected further the C than the E animals as showed by the thinner fascicular layers of the C rats when compared with the E animals. (3) In the C group the double interaction (group × layer) presented significant differences in the fascicular layer between the two levels of the age factor ($p = 0.016$, middle-aged vs old rats). The effect of lack of exercise affected more the development of the fascicular layer in old than in middle-aged animals. The same effect was detected in the development of the reticular layer ($p = 0.030$, middle-aged vs old rats). Old C rats showed

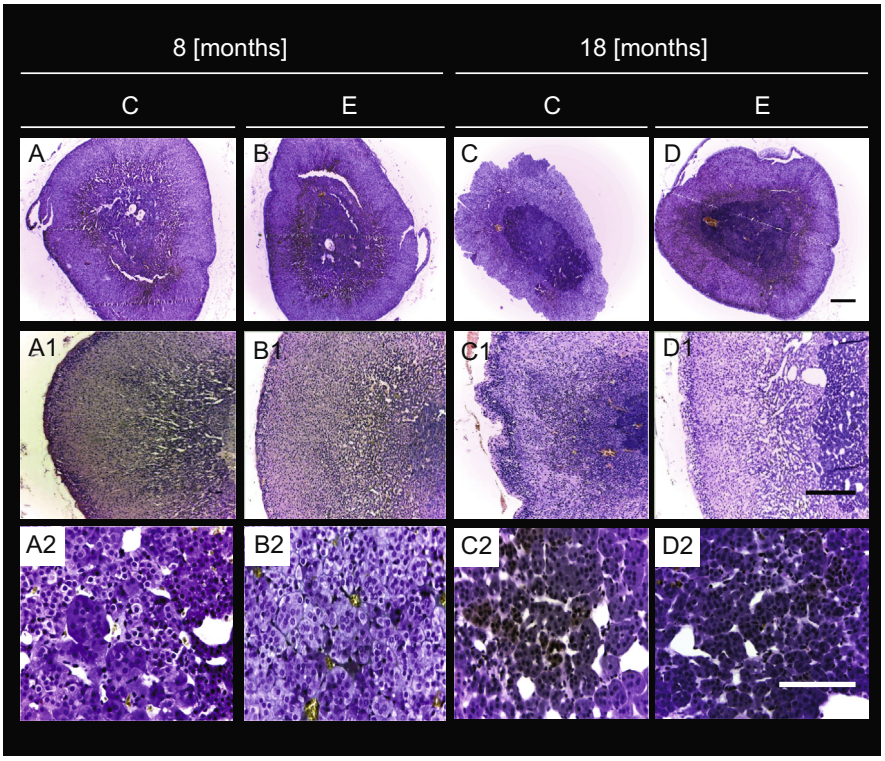

Fig. 7. Morphology of AGs. H-E stained sections showing the typical glandular histoarchitecture. Scale bar: 100 μm. C, 8 months (A, A1, A2) and E, 8 months (B, B1, B2); C, 18 months (C, C1, C2) and E, 18 months (D, D1, D2). (A-D, top panel): Panoramic view of the entire AGs at low magnification (2.5 X). (A1-D1, middle panel): Adrenal cortex (C). Photographs show a section of the three cortical layers: Glomerulosa (G); Fasciculata (F) and Reticularis (R). Note the larger cortical development in middle-aged animals (left) compared with old rats (right). Magnification: 10×. (A2-D2, bottom panel): Adrenal medulla (M). Magnification: 20×.

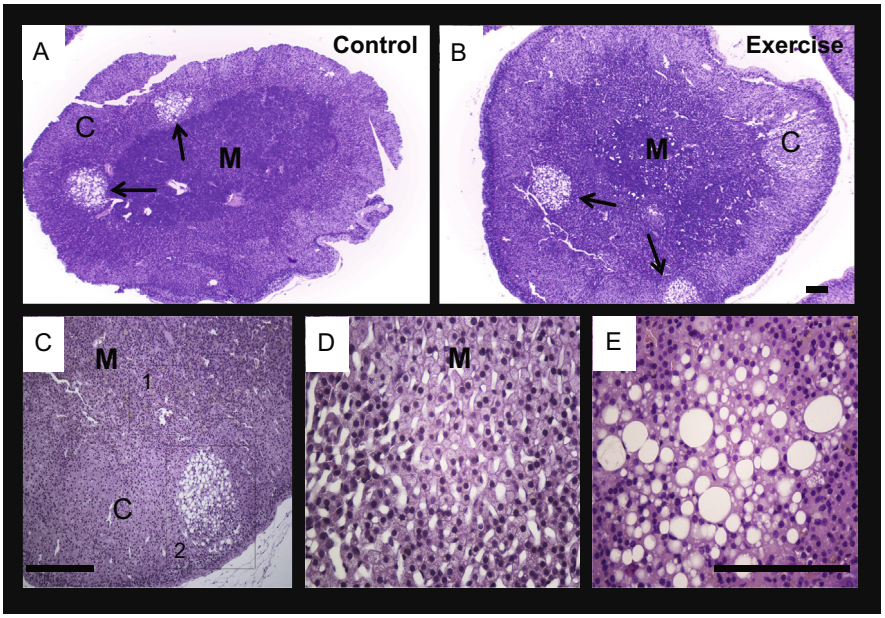

Fig. 8. Histopathology of AGs. Masson trichrome-stained sections of the aged rats, C-E. (A, B) Sections showing intracellular and/or interstitial vacuolation in both the cortical region and medullary area (indicated by arrows). Magnification 2.5×. Symbols: C, adrenal cortex; M, adrenal medulla. (C): 1 corresponds to the transition region between adrenal medulla and the R layer; and 2 indicates the adrenal cortex (20×). (D, E) Amplification of these regions with higher magnification (40×). Scale bar: 100 μm.

reticular layers thinner than younger C ones in the E group the double interaction (group × layer) presented significant differences when the reticular layer was considered between the two levels of the age factor ($p = 0.017$, middle age vs old). The effect of exercise affected more the development of the reticular layer in the old than in the middle-aged rats. Old rats of the E group presented reticular layers thinner than younger E animals. Results were reported in Table 1.

DISCUSSION

The aim of this work was to study the long-term effects of lifelong regular exercise on the adaptive response of the stress system, in middle-aged and old rats. These effects of AE on the behavioral response were studied by means of tests based on patterns of spontaneous behavior which evoke a state of anxiety caused by the conflict produced by stressors such as novelty, light–dark contrast, open-closed or elevated spaces. The analysis of results showed interesting general coincidences between the EPM and LDB tests. AE significantly decreased the main anxiety indicators and was a protective factor for the deregulation of affective and emotional behavior in old E rats. Consistently with previous findings (Fulk et al., 2004; Oliveira et al., 2010; Pietrelli et al., 2012), aging increased anxiety and emotionality, especially in old C rats, in parallel with a deterioration in locomotion, exploratory capacity, attention and motivation. This deficit can be explained, at least in part, by the dysfunction of the neuroendocrine axis (Campeau et al., 2010, 2011) and the osteo-articular system, loss of muscle mass and motor efficiency (Kenney et al., 2012). Data dispersion indicated a large heterogeneity, particularly in the EPM, confirming the importance of individual features in response to a new stressor when dealing with senescent animals (Segar et al., 2009; Oliveira et al., 2010).

The adrenal response was significantly affected by AE and showed a strong dependence on

Table 1. Morphometry of adrenal glands, thymus and physical parameters

	8 months		18 months	
	C $n = 12$	E $n = 12$	C $n = 12$	E $n = 12$
Physical parameters (weight)				
Body weight (BW, g) Absolute adrenal weight (mg)	546.35 ± 9.41 31.66 ± 1.34	516.17 ± 6.65 36 ± 2.44***	521.75 ± 37.45 22.33 ± 1.74$^{\#}$	435.35 ± 51.40 33.33 ± 3.09*
Relative adrenal weight (mg/g BW) Absolute thymus weight (mg)	0.057 ± 0.001 227.3 ± 16.69	0.069 ± 0.001 198.2 ± 17.65	0.043 ± 0.003 –	0.077 ± 0.009* –
Relative thymus weight (mg/g BW)	0.394 ± 0.014	0.405 ± 0.017	–	–
Morphometry of the adrenal glands (thickness and area)				
Cortex (width, μm)	566.44 ± 15.25	606.14 ± 14.20*	547.83 ± 13.10	570.76 ± 12.30
Glomerular layer (width, μm)	54.49 ± 8.57	58.78 ± 9.88	47.03 ± 7.23	55.89 ± 6.98
Fascicular layer (width, μm)	358.55 ± 8.50$^{\#}$	385.18 ± 6.88**	338.75 ± 8.80	360.46 ± 6.90*
Reticular layer (width, μm)	196.60 ± 8.55$^{\#}$	190.01 ± 9.70$^{\#}$	172.10 ± 7.29	160.65 ± 7.11
Medulla (area, mm^2)	0.05 ± 0.004$^{\#\#\#}$	0.070 ± 0.003***	0.06 ± 0.002	0.050 ± 0.003*

Results are expressed as mean ± SEM. Symbols: exercise (*) and age effects ($^{\#}$). Physical parameters, adrenal cortex and medulla were analyzed by a two-way ANOVA (exercise × age) with differences evaluated by *post hoc* Tukey's test. Significant differences were detected in: absolute adrenal weight (main effects), $^{\#}p < 0.05$ 8C vs 18C, $^{***}p < 0.001$ 8E vs 18C, $^{*}p < 0.05$ 18C vs 18E; relative adrenal weight (main effects), $^{*}p < 0.05$ 18C vs 18E; adrenal cortex (main effects), $^{*}p < 0.05$ 8E vs 18C. Medulla (simple effects): middle-aged rats, $^{***}p < 0.0001$C vs E, and old rats, $^{*}p < 0.05$C vs E. Control group, $^{\#\#\#}p < 0.0001$, middle-aged vs old rats and exercise group, $^{\#\#\#}p < 0.0001$, middle-aged vs old rats. The adrenal layers were analyzed by a three-way ANOVA (exercise × age × layer). Differences between two-way interactions through the levels of a third factor were evaluated by Bonferroni's test. Significant differences were detected in: fascicular layer $^{**}p < 0.01$ 8E vs 8C, $^{*}p < 0.05$ 18E vs 18C, $^{\#}p < 0.05$ 8C vs 18C, and reticular layer $^{\#}p < 0.05$ 8C vs 18C and $^{\#}p < 0.05$ 8E vs 18E.

age. The trophic and secretagog effect of exogenous ACTH was markedly potent in middle-aged E rats, considering the increase in CORT levels. This response indicated an increased AS to ACTH, although both the E and C groups had similar baseline levels of CORT. Using rats subjected to long-term running, Campbell et al. (2009) demonstrated that the adaptive response to exercise might be mediated by changes in AS via transient increases in ACTH receptors. These changes may result in an initial hyperactivation of the HPA axis and, after 8 weeks of exercise, a restoration to its baseline values. Similar baseline levels of CORT and most of the behavioral indicators described above support this notion. In contrast, older animals showed a significant loss of AS to ACTH action, in both the basal state and under stimulation, consistent with low levels of CORT synthesis and/or secretion and in agreement with previous reports on functionality loss (Stranahan et al., 2008; Kaliman et al., 2011; Heijnen et al., 2016). Alternative hypotheses have been formulated to explain the possible mechanisms regulating the secretion of GCs. Fulford and Harbuz (2005) reported no increases in ACTH basal levels in adrenalectomized rats subjected to voluntary exercise, which suggests the presence of additional intra-adrenal mechanisms to regulate the secretion of GCs and steroidogenesis. In line with these findings, Kosti et al. (2006) proposed that the adaptive response to stress might be mainly given by the intra-adrenal differences in neuropeptide Y levels, considered a stress marker in rats. Unlike Campbell et al., Kosti et al. postulate that animals with higher AS may be more easily capable of adapting to stress. In this line, it could be assumed that the greater AS presented by the E rats allowed them to adapt to effort without altering CORT levels. In turn, Ulrich-Lai and Engeland (2005) have proposed that changes in AS may be due to paracrine regulation from the chromaffin cells on the adrenal cortex, which may be consistent with the higher medullary development observed in middle-aged E rats.

The histopathologic analysis revealed the absence of relevant morphological or structural alterations in epithelial and interstitial cells of the adrenal cortex and medulla in middle-aged rats. However, and in agreement with previous reports, age altered some glandular morphological and morphometric parameters (Janjua and Khan, 1992; Rebuffat et al., 1992). A moderate 15–20% of aged rats presented histological changes in the cortex and medulla, associated with intra/interstitial vacuolization, probably due to interstitial fatty deposits or pigments of lipofuscin, as well as autophagia. The adrenal dysfunction observed in aged rats could be due, at least in part, to these alterations and even to undetected ultrastructural modifications. The absolute weights of the AGs remained stable, except in old C rats, and BW and thymus weights were also unaltered, in line with results reported by Patki et al. (2014) with exercised male Wistar rats. The thickness of the adrenal cortex remained conserved in most animals, with the exception of old C rats. Consistent with microscopy and morphometry results, fascicular development was remarkable in middle-aged E rats but slight in older C rats, perhaps due to hypotrophism or dehydration of the adrenal tissue. In addition, older C rats showed less reticular development, demonstrating an important decline in the production of androgens (Belloni et al., 1992). AE increased the medullary area in middle-aged E rats, although no changes followed in adrenal sympathetic activity and/or sympathetic tone. AE did not affect A or NA levels, but age markedly increased response heterogeneity. The present results differ from those obtained by Myers et al. (2012), who reported that long-term running decreases tyrosine hydroxylase expression and activity and, consequently, catecholamines levels in young rats, but fails to reduce the already elevated TH in senescent rats.

Discussion of the effects of chronic resistance exercise on circulating basal levels of CORT continues to generate controversy. Early reports on this topic (Dröste et al., 2003; Park et al., 2005; Yanagita et al., 2007) showed that voluntary exercise produced a basal hypersecretion of CORT associated with a chronic stress state. Further work by Dröste et al. (2007) and other groups using different models (Campbell et al., 2009; Campeau et al., 2010; Gradari et al., 2016; Heijnen et al., 2016) indicated that long-term exercise restored normal baseline of plasma CORT levels. The present results confirm that long-term regular AE restores and/or modulates the normal circulating levels of CORT but leave plasma CORT unaffected in old animals, as mentioned in previous paragraphs.

Within the conceptual framework of the neuroendocrine aging theory (Goosens and Sapolsky, 2007; Garrido, 2011), brain aging has been suggested to be conditioned by excessive GC secretion, which leads to damage in the cortex and hippocampus, brain areas involved not only in cognitive and emotional processes but also in the control of HPA axis activity. Although AE significantly increased brain CORT in middle-aged E rats, alterations compatible with a chronic stress condition or premature cerebral aging were not detected. The distribution, expression and affinity of the mineral and glucocorticoid receptors (MR and GR, respectively) vary and even overlap in different brain regions (Fulford and Harbuz, 2005). It is widely accepted that the cerebral cortex and hippocampus have the highest GC receptor densities, which plays a key role in the coordination and termination of stress response (McEwen, 2007; McEwen et al., 2015; Levone et al., 2015). In addition, GRs have a monophasic or biphasic response, depending on the brain region or the type of stressor (McEwen, 2007). On the basis of the present results, the increase in brain CORT could be assumed to be compensated by GC receptor down-regulation (Park et al., 2005; Zhou et al., 2008; Campbell et al., 2009), regional distribution changes or activation patterns in terms of dose and time. Previous work by our group (Pietrelli et al., 2011, 2012) has shown that Wistar rats subjected to chronic treadmill-running AE had a significant amplification of the cortical and hippocampal function, which could facilitate some of these processes, result in a faster adaptation to different stressors and prevent sustained hyperactivation. These results lead to the conclusions drawn by Stranahan and Mattson (2008), who argue that the runner adapts to running over time and that, at different points during adaptation to the same stressor, the response to a different stressor might be amplified or attenuated.

Brain, pituitary and hypothalamic CRF levels were significantly affected by age and AE. According to Soya et al. (2007), the hypothalamus shows a selective neuronal activation depending on a minimum intensity of treadmill-running exercise and responds with a threshold-like pattern. These concepts may explain, to some extent, why middle-aged E rats, despite being exposed to more brain CORT levels, had brain, pituitary and hypothalamic CRF levels apparently similar to controls. Moreover, CRF total levels do not predict the amount of bioactive free CRF (complex with its binding protein, CRF-BP) available to activate its receptors (Heinrichs, 2005). This adaptive response can be also interpreted in the light of the conclusions of Lowry and Moore (2006), who argued that different behavioral responses may involve interactions among CRF-related neuropeptides, CRF receptor subtypes and different neural circuits. Similarly, Heinrichs (2005) reported a specific regional modulation of anxiety through differential activation by CRF1 and CRF2 receptors in a dose-dependent manner. Alternatively, emotional regulation through paracrine actions may affect the sensitivity of these receptors. Additional CRF-dependent mechanisms may regulate ACTH levels, HPA axis activity and emotional and behavioral adaptation to stress, including well-known neuromodulatory systems such as the adrenergic and noradrenergic (Mammarella et al., 2016), dopaminergic, serotonergic (Lowry and Fox, 2013) and endocannabinoid systems (Gradari et al., 2016). In recent years, several authors (Martinowich and Lu, 2008; Zhou et al., 2008; Homberg et al., 2014) have highlighted the importance of two functionally related systems, i.e. brain-derived neurotrophic factor (BDNF) and serotonin (5-HT), which mediate brain remodeling in acute and chronic stress, hippocampal neurogenesis, neuronal survival, cognitive and emotional processes critically involved in resilience and susceptibility to stress (Taliaz et al., 2011; Levone et al., 2015; McEwen et al., 2015). Some of these mechanisms may underlie our current results in the E group, although not necessarily the same in middle-aged and old animals, to reduce and/or modulate the stress response and to amplify the cerebral energy metabolism, considering that the brain is an exclusively aerobic organ.

CONCLUSION

1) Treadmill-running AE, when performed in accordance with its basic principles concerning specificity, progressive overload, and variable intensity, and applied into the overcompensation period until 72 h, produced no adverse effects associated with chronic stress.
2) Aging increased behavior related to anxiety and emotionality. This increase may be due, among other factors, to HPA axis dysfunction by loss of AS and, perhaps, to alterations in ACTH receptors and intra-adrenal mechanisms and structural and/or ultrastructural histological changes leading to decreased plasma and brain CORT. Furthermore, the deregulation of the HPA axis feedback had a negative impact on pulsating control and inhibitory tone, which probably resulted in a delayed shutoff of the stress response;
3) AE had an anxiolytic effect. The main effect of AE indicates that E rats showed less anxiety and emotionality, regardless of age, in parallel with an increase in AS to ACTH without changes in sympathetic tone. In Mattson's terms (2008), AE, a hormetic eustressor, is capable of activating other hormetic mechanisms in a trans-cellular manner. These possible mechanisms, perhaps with various

regional and temporal patterns, could be activated by AE cumulative effects, resulting in the amplification of stress resilience.

4) This work could contribute to the knowledge of the adaptive effects of moderate lifelong physical activity as an adjuvant therapy in distress, depression and neurodegenerative diseases, and to shedding light on the importance regular AE and its positive impact on the stress resilience. Therefore, all recreational and non-competitive activities which are low intensity and long lasting such as cycling, dancing, swimming, rowing, walking, jogging, are recommended, especially at older ages.

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