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## MACROAUTOPHAGIC PROCESS WAS DIFFERENTIALLY MODULATED BY LONG-TERM MODERATE EXERCISE IN RAT BRAIN AND PERIPHERAL TISSUES

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The autophagic process is a lysosomal degradation pathway, which is activated during stress conditions, such as starvation or exercise. Regular exercise has beneficial effects on human health, including neuroprotection. However, the cellular mechanisms underlying these effects are incompletely understood. Endurance and a single bout of exercise induce autophagy not only in brain but also in peripheral tissues. However, little is known whether autophagy could be modulated in brain and peripheral tissues by long-term moderate exercise. Here, we examined the effects on macroautophagy process of long-term moderate treadmill training (36 weeks) in adult rats both in brain (hippocampus and cerebral cortex) and peripheral tissues (skeletal muscle, liver and heart). We assessed mTOR activation and the autophagic proteins Beclin 1, p62, LC3B (LC3B-II/LC3B-I ratio) and the lysosomal protein LAMP1, as well as the ubiquitinated proteins. Our results showed in the cortex of exercised rats an inactivation of mTOR, greater autophagy flux (increased LC3-II/LC3-I ratio and reduced p62) besides increased LAMP1. Related with these effects a reduction in the ubiquitinated proteins was observed. No significant changes in the autophagic pathway were found either in hippocampus or in skeletal and cardiac muscle by exercise. Only in the liver of exercised rats mTOR phosphorylation and p62 levels increased, which could be related with beneficial metabolic effects in this organ induced by exercise. Thus, our findings suggest that long-term moderate exercise induces autophagy specifically in the cortex.

**Key words:** *long-term treadmill exercise, autophagy, cortex, hippocampus, lysosome, skeletal muscle, liver, heart*

### INTRODUCTION

Lifestyle factors such as exercise and diet are increasingly recognized as determinants of successful aging. Clinical knowledge of the past two decades has established the beneficial effects of exercise in lowering hyperglycemia, hypercholesterolemia and hypertension (1, 2). Participation in recreational physical activity is correlated with a reduced risk of dementia in elderly humans (3). Running also enhances learning and memory in healthy adults (4). Consequently, exercise has been usually prescribed as part of treatment for diabetes, hypertension and cardiovascular disease, and also to lower the risk of metabolic syndrome and neurodegenerative diseases (5, 6). Moreover, exercise and its molecular/cellular consequences are consistent with longevity assurance pathways and life extension interventions (2, 7). Increasing evidence helps us to understand how it works at molecular and cellular level, although much remains unknown.

The autophagy-lysosome pathway and ubiquitin-proteasome system (UPS) are the main systems responsible for the digestion of most cytosolic and aggregated or misfolded proteins in the cells (8-10). The UPS selectively degrades the short-lived misfolded

proteins, while autophagy is less selective and contributes to the degradation of both long-lived proteins and damaged organelles. However, evidence gathered in recent years has shown interplay between these two mechanisms, as ubiquitylation can target substrates for degradation *via* both pathways (11).

There are various types of autophagy that differ in how the cytoplasmic material is delivered into the lysosome, being the macroautophagy the most investigated type of autophagy (hereafter referred to as autophagy). In this process, an isolation of a double-membrane (also known as phagophore) engulfs a portion of the cytoplasm to form the autophagosome, which finally fuses with lysosome and degrades its contents. This process constitutively takes place at lower levels in most cells in order to maintain the quality control of cellular components. On the other hand, it can be stimulated under stress conditions, such as starvation, to degrade cytoplasmic material into metabolites which can be used in biosynthetic processes or energy production, essential for cell survival (10, 12). However, the functional efficiency of the autophagic-lysosomal system clearly declines during aging, which compromises the maintenance of a healthy cell and can lead to certain neurodegenerative diseases

and other age-related disorders (9, 13, 14). In this respect, enhancing autophagic degradation with rapamycin or trehalose provides neuroprotection in different experimental models of neurodegenerative diseases, such as Huntington's, Parkinson's and Alzheimer's diseases (15-18).

The signalling pathways that regulate autophagy and the aging process are linked at molecular level. In particular, the repressive effects of the mammalian target of rapamycin (mTOR) and its modulation by AMP-activated protein kinase  $\alpha$  (AMPK $\alpha$ ) and longevity factors such as sirtuin 1 (SIRT1) and p53 (8, 13). There is a growing interest in the relationship between the activation of SIRT1 and the onset of autophagic processes, as SIRT1 can interact with and deacetylate several autophagic components (19) as well as its involvement in the AMPK $\alpha$ -mTOR axis (20, 21). In previous studies, we demonstrated that long-term moderate exercise leads to SIRT1 and AMPK $\alpha$  activation at cerebral level (22) as well as SIRT1 activation in peripheral tissues (23).

Recent findings have shown that a single bout of exercise activates autophagy in mice, not only in skeletal and cardiac muscles but also in brain (24, 25). If we consider exercise training, some authors have also described an autophagy activation in mice skeletal muscle (26, 27), but none of them have focussed on brain.

For this reason, the goal of this study was to examine the effects of long-term treadmill exercise on autophagy activation in the brain as well as in peripheral tissues in rat, in order to establish a possible relationship among autophagic process as an additional mediator of exercise effects, as we reported previously (22, 23, 28).

## MATERIALS AND METHODS

### *Animals and general procedure*

The experimental protocol was approved by the Ethics Committee of the Universitat Autònoma de Barcelona and complied with 'Principles of Laboratory Animal Care' and the European Communities Council Directive (86/609/EEC).

The exercise protocol was described in earlier reports (22, 23, 28). Twenty-nine male Sprague-Dawley rats aged 5 weeks at the beginning of the experiment were semi-randomly distributed into three experimental groups: exercised (EXE; n=11), handled no-exercised (NoEXE; n=8) and sedentary (SED; n=8). At the end of the experiment rats were 10 months old. Eight-weeks-old male rats (YOUNG; n=6) were also killed on the same day in order to include young controls in the analysis. After isoflurane anesthesia, the animals were killed by decapitation between 9:00 and 12:00 h. The brain was immediately removed, and dissected into cortex (parietal and frontal) and hippocampus; the liver, heart and gastrocnemius muscle were extracted and frozen in powdered dry ice. All tissues were maintained at  $-80^{\circ}\text{C}$  until use. In addition, four brains were dissected and separated sagittally in two hemispheres, one for immunohistochemistry and the other for protein extraction. Briefly, the tissue was fixed in methacarn and processed for paraffin-embedded immunohistochemistry.

### *Exercise regime*

A treadmill consisting of three parallel runways (45 cm long, 11 cm wide and 12 cm deep) was used. The apparatus was placed at an inclination of  $0^{\circ}$  in the cage. On the first day, the rats were placed on the treadmill for 30 min without running in order to become habituated to the apparatus and to diminish subsequent exercise-induced stress. Treadmill sessions were conducted

between 13:30 h and 16:30 h, 4-5 days per week for 36 weeks. Neither electrical shock nor physical prodding was used to force training. For the EXE group, the treadmill started at a speed of 4.2 m/min, and increased progressively by 1 m/min every 30 s to a speed of 12 m/min (which was reached between minute 3 and 12). During the first sessions, some animals slowed their gait over the session and displaced towards the back wall of the lane; on such occasions they were gently pushed by hand for a few seconds to stay at the front part of the lane. Despite these cautions, 25% of the rats had to be rejected from the experiment because they refused to run. NoEXE rats were placed individually on another treadmill (0 m/s) for the same number of sessions and the same duration as the EXE group. Thus control and exercised rats were handled in the same manner and spent the same amount of time out of the home cage. The SED group was handled once a week for cage cleaning and tail marking.

### *Protein extraction and Western blot*

Tissues samples were homogenized in lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100) containing phosphatase and protease inhibitors (Cocktail II, Sigma). After remaining on ice for 30 min, samples were centrifuged at  $10,000 \times g$  for 10 min and the supernatant with total protein content was collected. All the protein extraction steps were carried out at  $4^{\circ}\text{C}$ . The protein concentration was determined by the Bradford method.

For Western blot analysis, 20  $\mu\text{g}$  of protein per sample was denatured at  $95^{\circ}\text{C}$  for 5 min in sample buffer (1 M Tris-HCl pH 6.8, 10% glycerol, 2% sodium dodecyl sulfate (SDS), 5%  $\beta$ -mercaptoethanol, 0.05% bromophenol blue), separated by SDS-PAGE on 8-18% polyacrylamide gels and transferred to PVDF membranes (Millipore). The membranes were blocked in 5% non-fat milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) for 1 h at room temperature, followed by an overnight incubation at  $4^{\circ}\text{C}$  with antibodies against p-mTOR (Ser2481) and mTOR (1:500 and 1:1000 respectively; Millipore), Beclin 1 (1:1000 for Western blot; 1:100 for co-immunoprecipitation; Abcam), LC3B (1:2000; Cell Signaling), LAMP1 (1:1000; GeneTex), p62 (1:1000; Abcam), Ubiquitin (1:500; Leica), Bcl-2 (1:1000; Cell Signaling). Protein loading was routinely monitored by immunodetection of  $\beta$ -actin (1:20,000; Sigma) or GAPDH (1:2000; Millipore). Membranes were then washed and incubated with secondary antibodies for 1 hour at room temperature. Immunoreactive proteins were visualized using a chemiluminescence-based detection kit (ECL kit; Millipore) and digital images were acquired using a ChemiDoc XRS+System (BioRad). Band intensities were quantified by densitometric analysis using Image Lab software (BioRad) and values were normalized to  $\beta$ -actin or GAPDH.

### *Immunohistochemistry*

Tissue brain samples were deparaffinized in xylene and rehydrated through a descending ethanol gradient (100%, 95% and 80%), 3 min in each solution. Afterwards, the brain sections were rinsed in phosphate-buffered saline (PBS, pH 7.2) before quenching of endogenous peroxidase activity with PBS containing 0.3%  $\text{H}_2\text{O}_2$  and 10% methanol, for 15 min at room temperature in the dark. After washing in PBS for  $3 \times 5$  min and in PBS containing 0.5% Triton X-100 for  $3 \times 5$  min, the tissues were blocked with 0.2% (w/v) gelatin in PBS containing 3% fetal bovine serum (FBS), 0.5% Triton, 0.2% bovine serum albumin (BSA) and 0.2% sodium azide for 1 hour at room temperature in a humidified chamber. They were then washed for  $3 \times 5$  min in PBS-Triton and incubated with the primary antibody (see below) diluted in the same buffer used in the blocking step, overnight at

4°C. After washing in PBS-Triton for 3×5 min, the brain sections were incubated with biotinylated secondary antibody (see below) diluted in 0.2% (w/v) gelatin in PBS containing 1% FBS, 0.1% Triton and 0.2% BSA for 2 hours at room temperature in a humidified chamber. Tissues were washed in PBS-Triton for 3×5 min and incubated with ABC reagent (see below) for 90 min at room temperature in a humidified chamber. After washing in PBS-Triton for 2×5 min and phosphate buffer (PB, pH 7.2) for 2×5 min, the brain sections were incubated in peroxidase substrate solution (PB containing 0.05% 3,3'-diaminobenzidine (DAB) and 0.005% H<sub>2</sub>O<sub>2</sub>) until optimal color developed at room temperature. Finally, slides were washed for 3×5 min in PB, dehydrated through an ascending ethanol gradient (70%, 90% and 100%) and xylene, 3 min in each solution, and mounted in DPX, then allowed to dry overnight at room temperature. Representative images of parietal cortex were taken with a microscope (BX41, Olympus, Germany) at 40× magnification.

We used rabbit polyclonal anti-LC3B (1:200; Cell Signaling) as the primary antibody, goat anti-rabbit IgG-biotin (1:200; Sigma) as the secondary antibody and VECTASTAIN® Elite ABC reagent (1:100; Vector Laboratories).

#### Data analysis

Data were analyzed statistically using GraphPad Prism software. The results are expressed as the mean ± S.E.M. from at least 6–8 samples. In all cases, data were analyzed by one-way ANOVA followed by post-hoc Tukey-Kramer multiple-comparison test. *p* values lower than 0.05 were considered significant.

## RESULTS

#### Effects of long-term exercise on autophagy activation at cerebral level

First, we assessed the activation of mTOR, a well-known inhibitor of the autophagic process (8). The results showed that the active form of this factor (phosphorylated mTOR in Ser2481) was significantly higher in the cortex of SED animals than in YOUNG, EXE or NoEXE animals (Fig. 1A). Interestingly, in hippocampus there was no significant variations in the phosphorylated mTOR levels between the different groups studied (Fig. 1B).

Beclin 1 plays an important role in autophagosome formation (29). Evaluation of Beclin 1 showed a decrease in its expression with age in cortex, which was reverted not only by exercise but also with handling (Fig. 2A). It was noted that the increases reached levels similar to YOUNG animals. Conversely, no changes were observed in hippocampus for Beclin 1 (Fig. 3A).

The microtubule-associated protein 1 light chain 3 (LC3) contributes to the elongation and the maturation of autophagosomes. When autophagy is induced, the cytosolic form LC3-I, converts to the phosphatidylethanolamine-conjugated form LC3-II, which is localized on autophagosome membranes (29). Therefore, higher LC3-II/LC3-I ratio determined by Western blot, concomitant with an increase in punctate LC3B observed by immunohistochemical analysis, are both related with autophagy activation (30). We determined higher LC3B-II/LC3B-I values in the cortex of EXE and NoEXE groups than in SED rats (Fig. 2B). The fact that we could not see a well defined band corresponding to LC3B-II could be related with the high amount of LC3B-I relative to LC3B-II present in brain tissue, which makes LC3B-II detection difficult (30). For LC3B-II quantification we considered the scrolled area that

appears under LC3B-I. Despite this fact, immunohistochemistry revealed an increase in LC3B puncta in EXE and NoEXE animals compared with SED rats (Fig. 2D), suggesting an autophagy induction in these groups. No differences in LC3B-II/LC3B-I were observed among the hippocampus of EXE, NoEXE and SED rats (Fig. 3B), but an increase associated with aging was found between these groups and YOUNG animals.

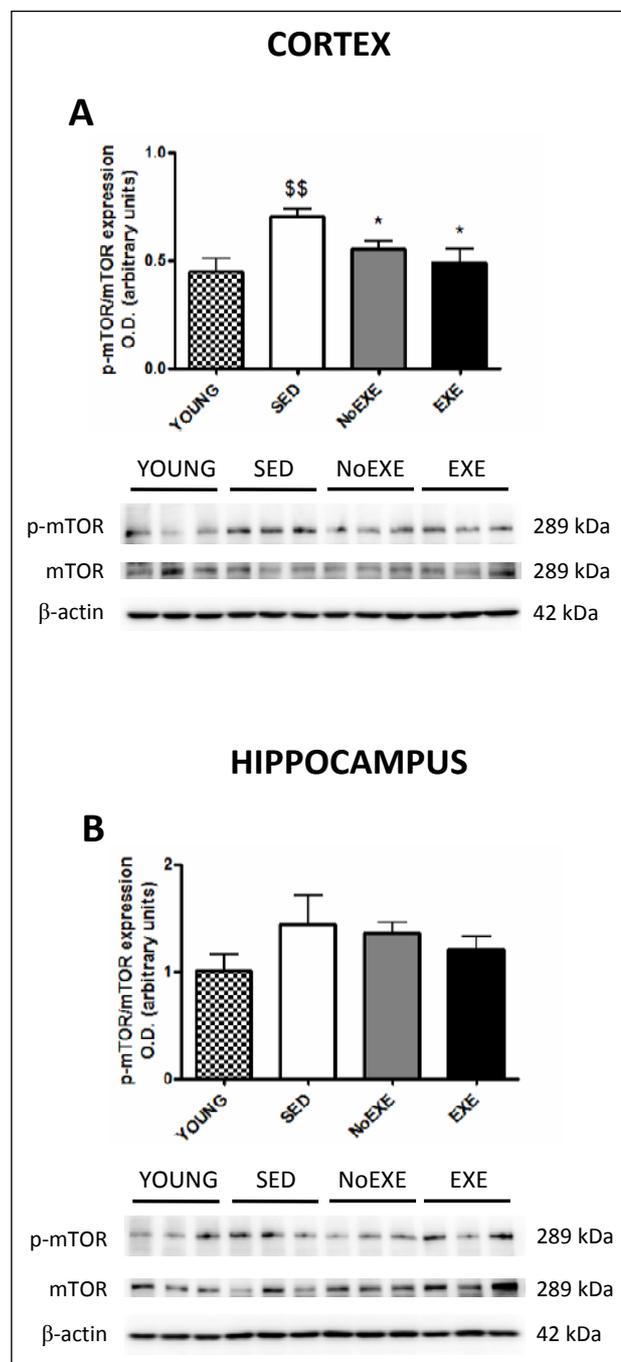
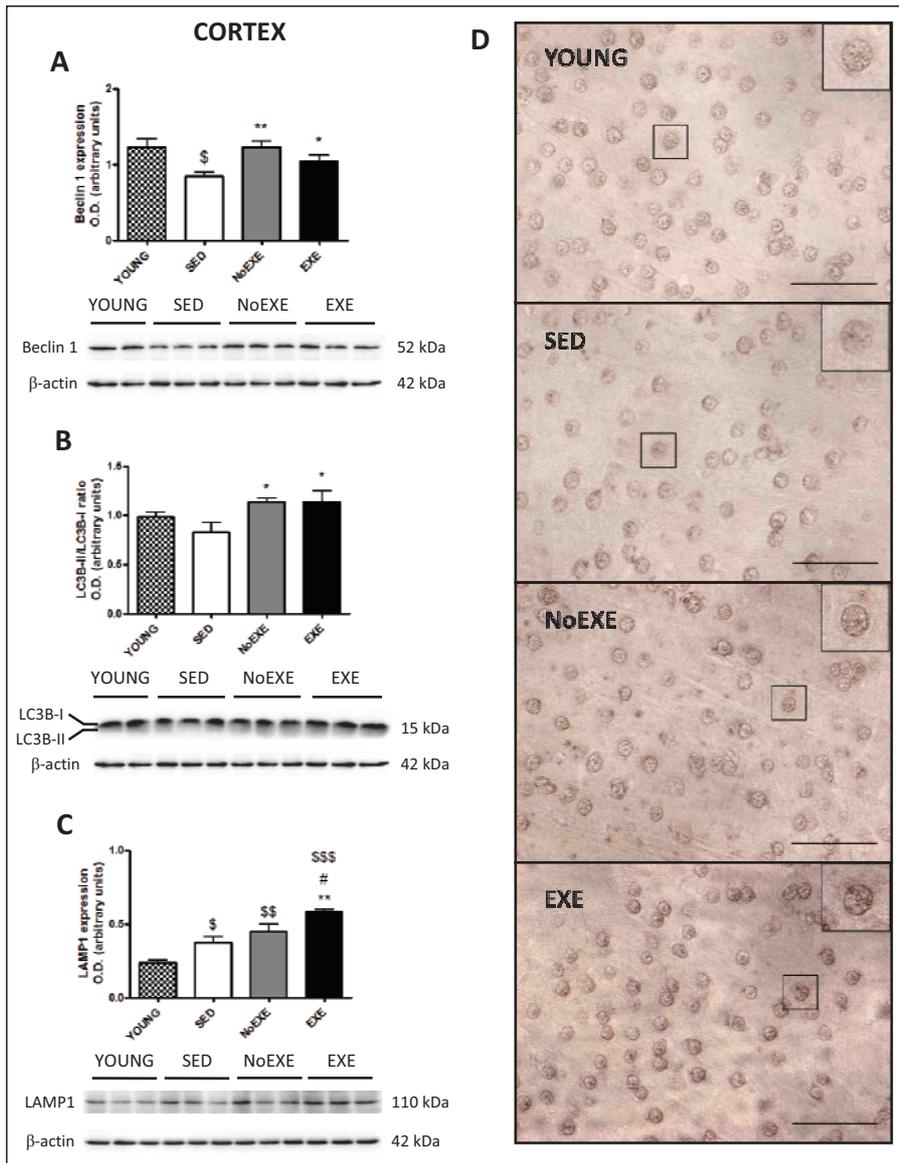


Fig. 1. p-mTORSer<sup>2481</sup>/mTOR protein levels in parietal and frontal cortex (A) and hippocampus (B) for young (YOUNG), sedentary (SED), non-exercised (NoEXE) and exercised (EXE) rats. Images show representative Western blots and bar graphs obtained from semi-quantitative image analysis, as described in the experimental procedures. Results are mean ± S.E.M. of 6–8 samples. \**p*<0.05 versus SED; \$\$*p*<0.01 versus YOUNG.



*Fig. 2.* Beclin1 (A), LC3B-II/LC3B-I (B) and LAMP1 (C) protein levels in parietal and frontal cortex for young (YOUNG), sedentary (SED), non-exercised (NoEXE) and exercised (EXE) rats. Images show representative Western blots and bar graphs obtained from semi-quantitative image analysis, as described in the experimental procedures. Results are mean  $\pm$  S.E.M. of 6–8 samples. \* $p$ <0.05, \*\* $p$ <0.01 versus SED; # $p$ <0.05 versus NoEXE; \$ $p$ <0.05, \$\$ $p$ <0.01, \$\$\$ $p$ <0.001 versus YOUNG.

(D) Representative images of parietal cortex stained with LC3B (40 $\times$  magnification) for young (YOUNG), sedentary (SED), non-exercised (NoEXE) and exercised (EXE) animals. Scale bar: 50  $\mu$ m.

The lysosome-associated membrane protein 1 (LAMP1) and its homologue LAMP2 are lysosomal transmembrane proteins, which are essential for lysosome motility and autophagosome-lysosome fusion (31). We found higher expression of LAMP1 associated with aging in cortex (*Fig. 2C*). Remarkably, LAMP1 increased particularly in EXE rats with respect to the other adult groups, which is corresponded to an increase of lysosomal biogenesis (17). On the other hand, significantly lower levels were found in the hippocampus of all adult groups (SED, NoEXE and EXE) (*Fig. 3C*).

Mammalian sequestosome 1 (SQSTM1), named p62, is one of the best characterized substrates of selective autophagy and is also able to act as cargo receptor for degradation of ubiquitinated substrates (32), thus reducing protein aggregates into the cell (33–35). p62 interacts with LC3, and subsequently is incorporated into the autophagosome being degraded. Therefore, accumulation of p62 is associated with autophagy inhibition (33, 36). Our results showed higher p62 levels in the cortex of SED and NoEXE rats (*Fig. 4A*), which is in agreement with a decline of autophagy with aging (8). Interestingly, a reduction of p62 was observed in the cortex of EXE animals. However, higher p62 levels remained unchanged in the hippocampus of the SED,

NoEXE and EXE groups (*Fig. 4C*). p62 contributes to eliminate ubiquitinated proteins not only by autophagy but also by proteasomal degradation (11). In this respect, we found that ubiquitinated proteins levels were significantly lower in the cortex of EXE animals than in NoEXE or SED rats, reaching levels similar to YOUNG animals (*Fig. 4B*). However, the higher levels of ubiquitinated proteins observed in the hippocampus of all adult groups were not reverted by exercise (*Fig. 4D*).

Thus, our results suggest higher rates of initiation and resolution of autophagic events (known as autophagy flux) in rat cortex by long-term exercise, as a combination of increased LC3-II/LC3-I ratio and reduced p62 were observed in the EXE group (30).

#### *Effects of long-term exercise on autophagy activation in peripheral tissues*

To determine whether the effects of exercise or animal handling were specific to brain areas, we studied other non-neuronal tissues from the same rats. mTOR was not modified either in skeletal muscle or in heart among experimental groups (*Fig. 6A* and *8A*, respectively). Conversely, higher

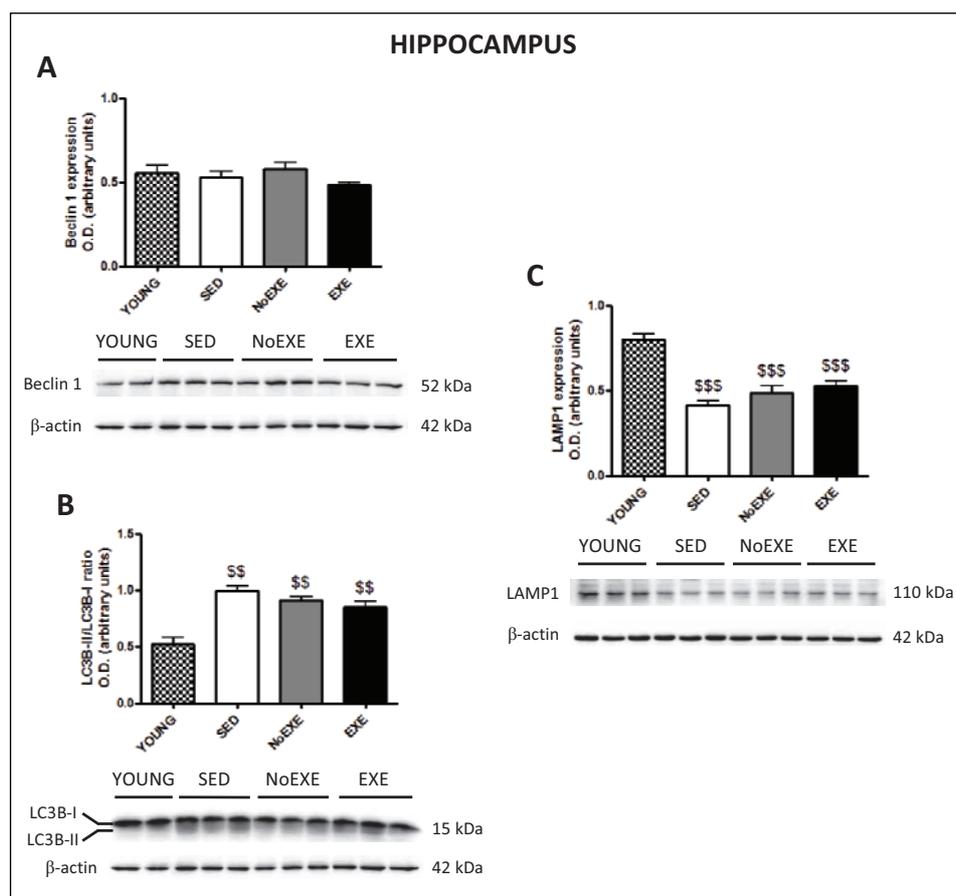


Fig. 3. Beclin1 (A), LC3B-II/LC3B-I (B) and LAMP1 (C) protein levels in hippocampus for young (YOUNG), sedentary (SED), non-exercised (NoEXE) and exercised (EXE) rats. Images show representative Western blots and bar graphs obtained from semi-quantitative image analysis, as described in the experimental procedures. Results are mean  $\pm$  S.E.M of 6–8 samples.  $^{ss}p < 0.01$ ,  $^{sss}p < 0.001$  versus YOUNG.

phosphorylated mTOR levels were observed in the liver of NoEXE and EXE animals than in SED rats (Fig. 5A). Regarding to Beclin 1 and LC3B-II/LC3B-I in liver, no significant changes were found (Fig. 5B and 5C), whereas a reduction in LAMP1 was observed in all adult groups (Fig. 5D). Also in muscle the expression of Beclin 1 and LC3B ratio (Fig. 6B and 6C) remained unchanged in the different experimental groups, but in this case we observed an increase in LAMP1 only in the EXE group (Fig. 6D). Different results were found for p62. In muscle, p62 levels were significantly higher in adult rats than in YOUNG animals (Fig. 7C), while in liver, an increase of p62 was observed only in EXE rats compared with the other groups (Fig. 7A). In heart, no differences were observed in Beclin 1, LC3B ratio, LAMP1 and p62 between the different experimental groups (Fig. 8B, 8C, 8D and 9A). No one peripheral tissue showed changes in ubiquitinated proteins, apart from higher levels in adult rats than in YOUNG ones in liver and muscle (Fig. 7B and 7D, respectively), with no differences in heart (Fig. 9B).

## DISCUSSION

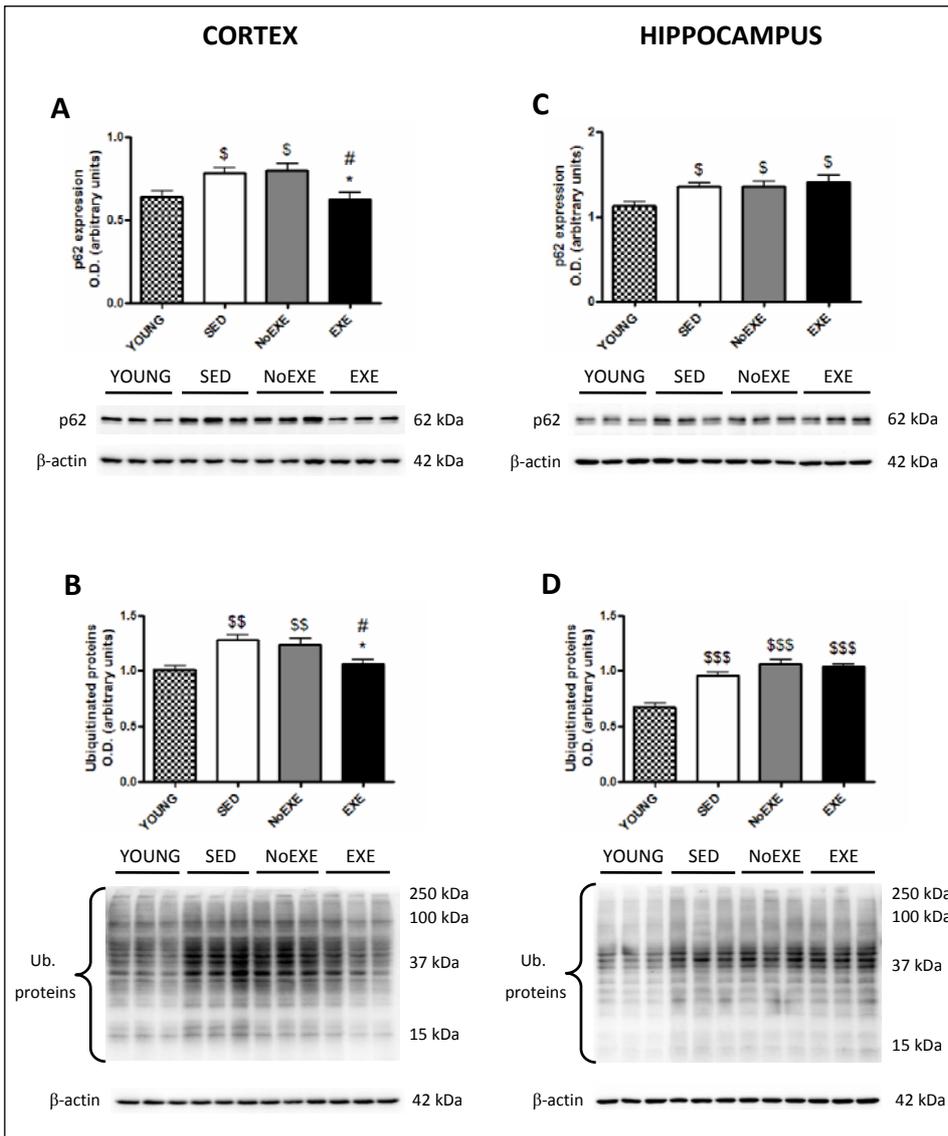
The present study is, to our knowledge, the first to assess the effects of long-term moderate treadmill exercise on macroautophagy process in brain. The results reported here suggest an activation of autophagy by exercise specifically in the cortex of adult rats.

In order to detect possible changes due to animal handling and the regular removal from cage environment, we included an additional control group to the sedentary one (SED), the handled no-exercised group (NoEXE), which was placed on the treadmill

with 0 m/s for the same number of sessions and the duration as the exercised (EXE) group. We incorporated this group because O'Callaghan *et al.* (37) found similar effects in middle-aged rats with both treadmill running and handling, reporting an improvement in age-related impairment of spatial learning and associated mechanisms. These authors proposed that the sensory stimulation of these animals and their regular exposure to an environment outside the home cage constituted a form of environmental enrichment when compared with the non-handled rats (sedentary group, SED), which remained always in their cages. In our case, although some changes in the autophagic machinery were found in the cortex of NoEXE group, they were not enough to result in the effects related with autophagy activation that we observed in the EXE group.

It has been stated that AMPK $\alpha$  activation by exercise decreases mTOR signalling, which facilitates autophagy and promotes lysosomal degradation (21, 38). On the other hand, resveratrol promotes longevity through a mechanism where autophagy induction by SIRT1 is implicated (39, 40). Moreover, it is known a close relationship between AMPK and SIRT1 controlling an integrated signaling pathways (21), where autophagy process is present (20). On the basis of our previous studies (22, 23), and particularly the activation of SIRT1 by long-term exercise in brain and peripheral tissues, we aimed to study related signals such as mTOR and the autophagy process under the same exercise regime.

Here, the reduced mTOR phosphorylated levels (therefore, its activation) we found in the cortex of exercised rats could be related with the SIRT1 and AMPK $\alpha$  activation induced by exercise reported previously (22). Surprisingly, lower mTOR phosphorylated levels were also observed in NoEXE animals,



*Fig. 4.* p62 and ubiquitinated protein levels in parietal and frontal cortex (*A* and *B*, respectively) and hippocampus (*C* and *D*, respectively) for young (YOUNG), sedentary (SED), non-exercised (NoEXE) and exercised (EXE) rats. Images show representative Western blots and bar graphs obtained from semi-quantitative image analysis, as described in the experimental procedures. Results are mean  $\pm$  S.E.M. of 6–8 samples. \* $p < 0.05$  versus SED; # $p < 0.05$  versus NoEXE; \$ $p < 0.05$ , \$\$ $p < 0.01$  \$\$\$  $p < 0.001$  versus YOUNG.

suggesting that handling/environmental stimulation could modulate other pathways involved in mTOR phosphorylation different from SIRT1-AMPK $\alpha$ .

Inhibition of mTOR by rapamycin extends lifespan in mice (41) and several authors have postulated this drug as potential therapeutic agent for the prevention or treatment of several age-related neurodegenerative diseases such as Alzheimer's and Parkinson's diseases (17, 18, 42). However, the search for long-life strategies that avoid the adverse effects of rapamycin and other drugs is one of the reasons for including physical exercise in the modern lifestyle.

In addition, we observed in both EXE and NoEXE animals higher Beclin 1 levels as well as LC3B-II/LC3B-I than in SED rats. The last fact, together with an increase in LC3B puncta in the cortex of EXE and NoEXE groups, were indicative of autophagy induction in these animals (30). However, LAMP1 levels were higher only in the cortex of exercised animals, which may be related to enhanced lysosomal content, aimed at increasing autophagic-mediated cellular clearance (17, 43). As Dehay *et al.* (17) reported, enhancement of lysosomal biogenesis may represent a new therapeutic strategy to attenuate neuron cell death in neurodegenerative diseases, specifically in Parkinson's disease.

Bcl-2 protein has been reported to be involved in autophagy, through interaction with the BH3 domain of the autophagic protein Beclin 1, and it is known that one of the mTOR-independent autophagy pathways acts through loosening the Beclin1-Bcl-2 interaction (24, 44). No changes were found in cortex either in the Bcl-2 protein levels or in the interaction between Bcl-2 and Beclin 1 determined by co-immunoprecipitation (data not shown). Therefore, we discarded this mTOR-independent autophagy pathway as a possible mechanism induced by long-term exercise in cortex.

Impairment of autophagy is accompanied by accumulation of p62 (33, 36) and different studies have shown that p62 is associated to polyubiquitinated protein aggregates present in affected brain regions of different neurodegenerative brain disorders (45, 46). Here, an increase of p62 levels were observed in cortex associated with aging, where exercise was able to revert it. Thus, increased LC3B-II/LC3B-I and the concurrent lower p62 levels suggest an induction of the autophagy process in cortex by exercise. In agreement with these results, ubiquitinated proteins levels decreased exclusively in EXE animals, reaching levels similar to YOUNG rats. It is worth noting that these effects of exercise seem to be specific in cortex, because the higher levels of both p62 and ubiquitinated proteins

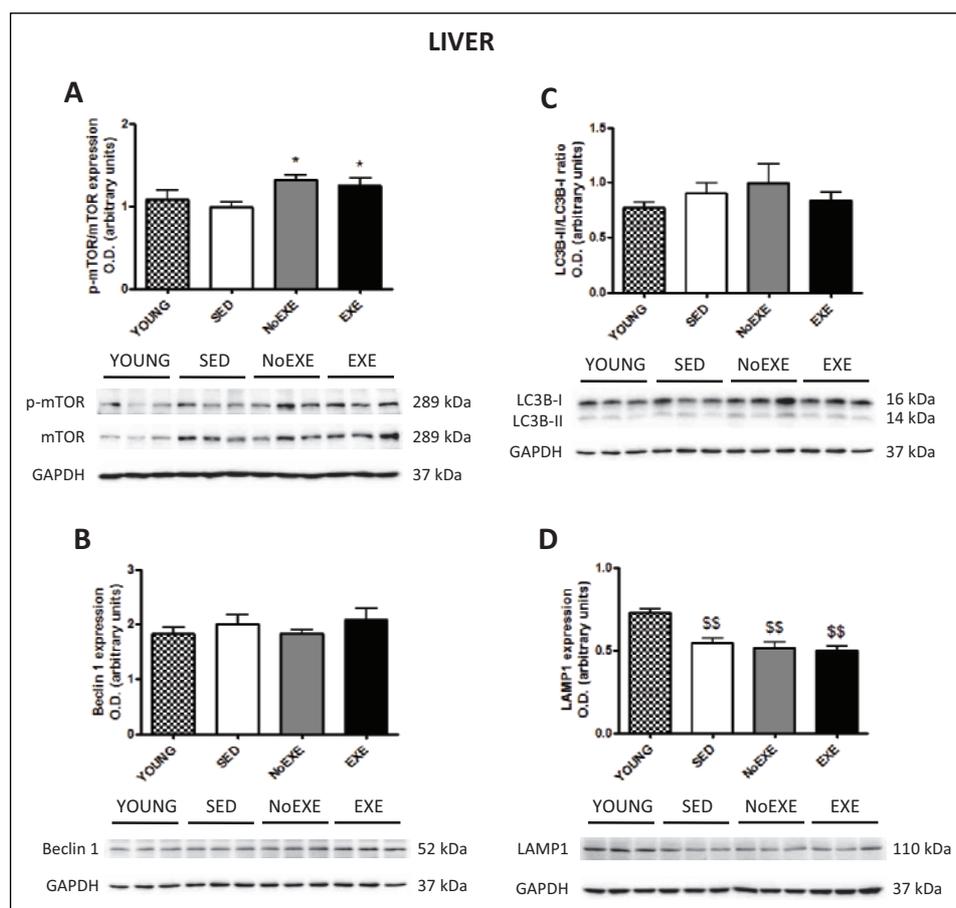


Fig. 5. p-mTORSer<sup>2481</sup>/mTOR (A), Beclin1 (B), LC3B-II/LC3B-I (C) and LAMP1 (D) protein levels in liver for young (YOUNG), sedentary (SED), non-exercised (NoEXE) and exercised (EXE) rats. Images show representative Western blots and bar graphs obtained from semi-quantitative image analysis, as described in the experimental procedures. Results are mean ± S.E.M. of 6–8 samples. \* $p < 0.05$  versus SED; <sup>SS</sup> $p < 0.01$  versus YOUNG.

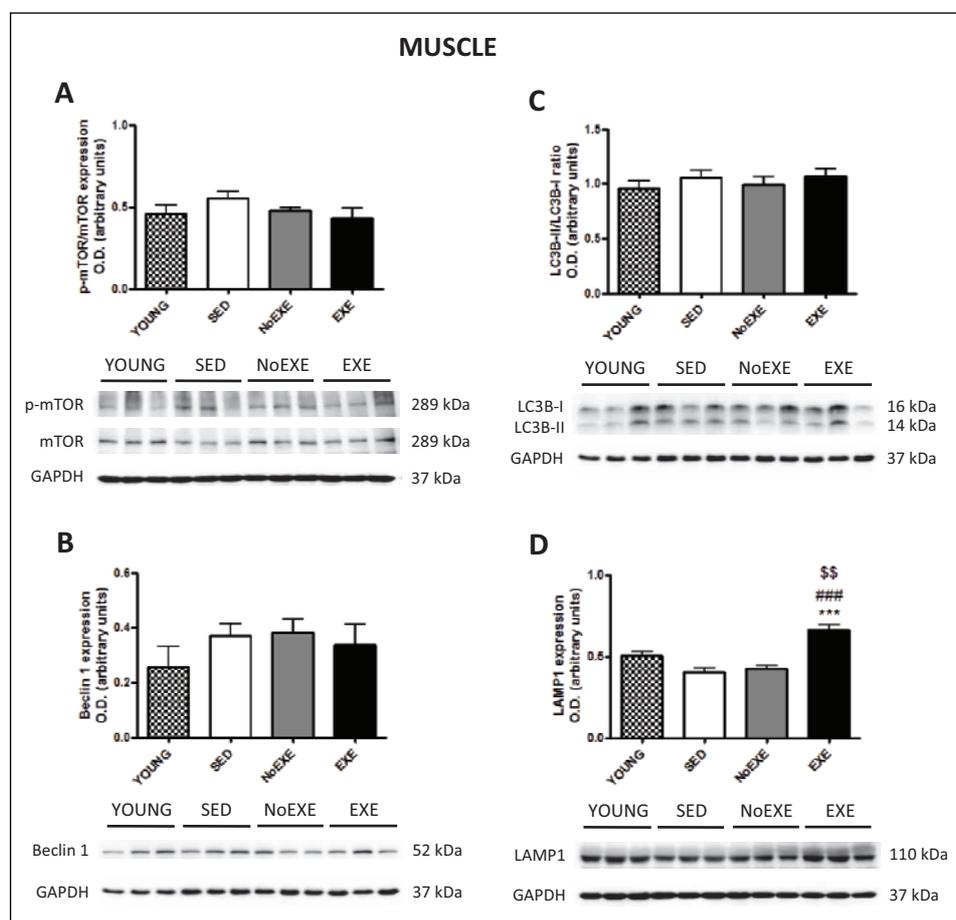


Fig. 6. p-mTORSer<sup>2481</sup>/mTOR (A), Beclin1 (B), LC3B-II/LC3B-I (C) and LAMP1 (D) protein levels in muscle for young (YOUNG), sedentary (SED), non-exercised (NoEXE) and exercised (EXE) rats. Images show representative Western blots and bar graphs obtained from semi-quantitative image analysis, as described in the experimental procedures. Results are mean ± S.E.M. of 6–8 samples. <sup>\*\*\*</sup> $p < 0.001$  versus SED; <sup>###</sup> $p < 0.001$  versus NoEXE; <sup>SS</sup> $p < 0.01$  versus YOUNG.

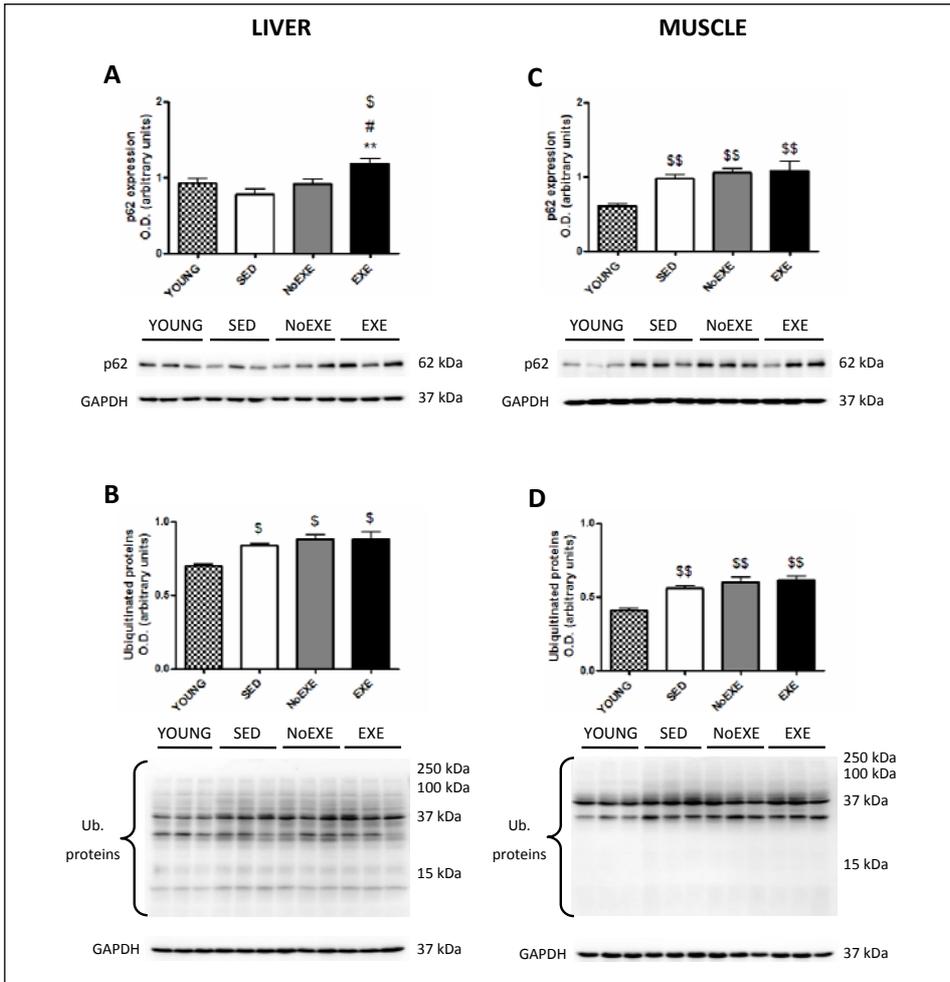


Fig. 7. p62 and ubiquitinated protein levels in liver (A and B, respectively) and muscle (C and D, respectively) for young (YOUNG), sedentary (SED), non-exercised (NoEXE) and exercised (EXE) rats. Images show representative Western blots and bar graphs obtained from semi-quantitative image analysis, as described in the experimental procedures. Results are mean  $\pm$  S.E.M. of 6–8 samples. \*\* $p < 0.01$  versus SED; # $p < 0.05$  versus NoEXE; \$ $p < 0.05$ , \$\$ $p < 0.01$  versus YOUNG.

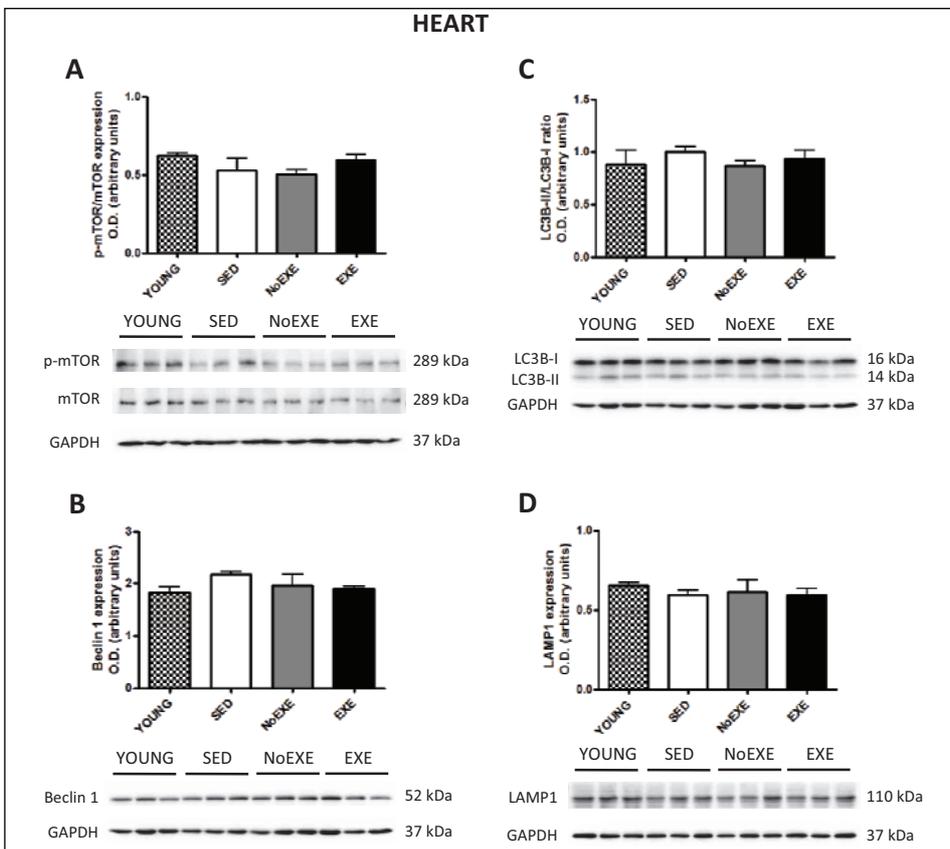


Fig. 8. p-mTORSer<sup>2481</sup>/mTOR (A), Beclin1 (B), LC3B-II/LC3B-I (C) and LAMP1 (D) protein levels in heart for young (YOUNG), sedentary (SED), non-exercised (NoEXE) and exercised (EXE) rats. Images show representative Western blots and bar graphs obtained from semi-quantitative image analysis, as described in the experimental procedures. Results are mean  $\pm$  S.E.M. of 6–8 samples.

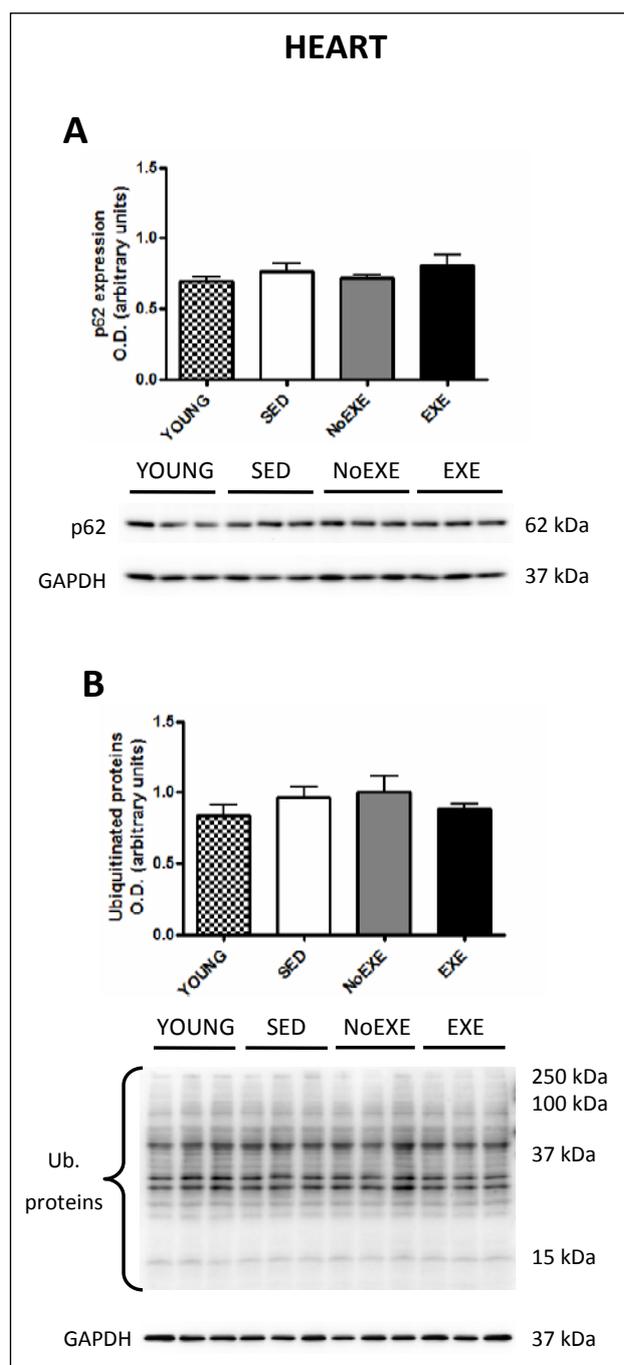


Fig. 9. p62 (A) and ubiquitinated protein levels (B) in heart for young (YOUNG), sedentary (SED), non-exercised (NoEXE) and exercised (EXE) rats. Images show representative Western blots and bar graphs obtained from semi-quantitative image analysis, as described in the experimental procedures. Results are mean  $\pm$  S.E.M. of 6–8 samples.

in the hippocampus of all adult groups were not reverted by exercise. As p62 is implicated in degradation of ubiquitinated proteins not only *via* autophagy but also by proteasome (11), we cannot rule out that long-term exercise could also induce proteasomal activation in cortex.

In brief, our results at cerebral level indicate that long-term exercise is able to revert the decline of autophagic process associated with aging specifically in cortex, increasing not only the LC3B-I conversion to LC3B-II, but also LAMP1 levels,

which in turn could be related to the decrease of both ubiquitinated protein levels and p62 in the cortex of exercised rats. As autophagy eliminates both protein aggregates and damaged organelles in neurons, activation of autophagy by exercise may represent an excellent non-pharmacological strategy to counteract or prevent neuronal degeneration associated with aging and neurodegenerative diseases (25, 45, 47). We are aware that further analyses will be required to determine more accurately if SIRT1 and AMPK function as positive regulators of autophagy. Moreover, it will be interesting to know in which cell types of the cortex the autophagic markers are modulated in response to exercise.

In reference to peripheral tissues, long-term moderate exercise did not induce any changes in mTOR activation or in autophagic proteins such as Beclin-1, LC3B and p62 in muscle or heart. It has been described an activation of autophagy and ubiquitin-proteasome pathway in skeletal human muscle during ultra-endurance exercise ( $\geq 24$  hours running) (38, 48). Moreover, a single bout of exercise activates autophagy in skeletal and cardiac muscle in rodents (24, 49). Long-term voluntary running also promotes autophagy in skeletal muscle (27), which may contribute to the beneficial metabolic effects of exercise improving glucose tolerance and metabolism (24). As Grumati *et al.* (50) have reported, some of the changes related to autophagy activation were not evident after 3 months of training, when skeletal muscle was likely to produce adaptive responses to exercise. Accordingly, we do not rule out that we could have detected some of the changes related with autophagy activation in skeletal and cardiac muscle as well as in hippocampus at the beginning of our protocol of long-term exercise. Finally in liver, an essentially metabolic tissue, results showed an increase in mTOR activation in both EXE and NoEXE groups, but this was not translated into changes in the autophagic process in this tissue. Moreover, exercised animals presented higher p62 levels. Thus, the mTOR activation and higher p62 protein levels observed in the liver of exercised rats could be related with metabolic effects rather than autophagy inhibition, regulating aspects such as the adipogenesis and energy control, which in turn, may prevent or counteract obesity and insulin resistance (51–54). Further studies should be carried out to clarify the beneficial physiological consequences of autophagy modulation by long-term exercise.

Apart from autophagy modulation, another molecular mechanisms related to the benefits of exercise training on brain function and metabolic effects should be taken in account, such as the increase of brain-derived neurotrophic factor (BDNF) in brain, skeletal muscle as well as in plasma and/or serum (55), the upregulation of chaperone heat shock protein 72 (Hsp72) in myocardium and skeletal muscles (56) and the increase of adiponectin levels in cardiac muscle and serum (57).

In conclusion, our study suggests that moderate exercise, such as fast walking or slow running from young ages, counteracts the decline of autophagy activation associated with aging in the cortex of adult rats. The results would reinforce the neuroprotective role of exercise previously reported (22), where we found a decrease of neurodegenerative hallmarks as well as a stimulation of mitochondrial biogenesis. Our study are in agreement with recent observations in mouse cortex after a single bout of exercise (25), but we present some evidence suggesting that exercise training also activates autophagy in the cerebral cortex.

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