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Exercise Increases Mitochondrial Complex I Activity and DRP1 Expression in the Brains of Aged Mice

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Abstract

Exercise is known to have numerous beneficial effects. Recent studies indicate that exercise improves mitochondrial energetics not only in skeletal muscle but also in other tissues. While exercise elicits positive effects on memory, neurogenesis, and synaptic plasticity, the effects of exercise on brain mitochondrial energetics remain relatively unknown. Herein, we studied the effects of exercise training in old and young mice on brain mitochondrial energetics, in comparison to known effects on peripheral tissues that utilize fatty acid oxidation. Exercise improved the capacity for muscle and liver to oxidize palmitate in old mice, but not young mice. In the brain, exercise increased rates of respiration and reactive oxygen species (ROS) production in the old group only while utilizing complex I substrates, effects that were not seen in the young group. Coupled complex I to III enzymatic activity was significantly increased in old trained versus untrained mice with no effect on coupled II to III enzymatic activity. Mitochondrial protein content and markers of mitochondrial biogenesis (PGC-1 α and TFAM) were not affected by exercise training in the brain, in contrast to the skeletal muscle of old mice. Brain levels of the autophagy marker LC3-II and protein levels of other signaling proteins that regulate metabolism or transport (BDNF, HSP60, phosphorylated mTOR, FNDC5, SIRT3) were not significantly altered. Old exercised mice showed a significant increase in DRP1 protein levels in the brain without

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Conflict of interest

The authors declare no conflicts of interest.

Contributions

A.M.G. designed and carried out the experiments, performed data analysis, and wrote the manuscript. J.C. performed experiments and approved the manuscript. G.D. performed experiments and edited the manuscript. R.M.O. designed the CLAMS experiments and approved the manuscript. B.H.G. contributed to the experimental design and edited the manuscript. P.M.C. designed the experiments, performed data analysis, and edited the manuscript. C.T.C. supervised the project, designed experiments, performed data analysis, and helped write the manuscript. The authors declare that they have no competing financial interests.

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changes in phosphorylation, while MFN2 and OPA1 protein levels were unchanged. Our results suggest that exercise training in old mice can improve brain mitochondrial function through effects on electron transport chain function and mitochondrial dynamics without increasing mitochondrial biogenesis.

Keywords

mitochondria; exercise; brain; cortex; complex I; DRP1

1. Introduction

Mitochondria have taken a central role in several hypotheses of aging for decades (Harman, 1956). Mitochondrial function, in particular respiration and the activities of electron transport chain (ETC) complexes I and IV, decline with aging in the brain, liver, heart and kidney, which also show changes in mitochondrial morphology (Manczak et al., 2005; Shigenaga et al., 1994). Mitochondrial DNA (mtDNA) mutations accumulate with age in the brain (Corral-Debrinski et al., 1992; Joseph et al., 2016), and altered brain energetics results in fission arrest and formation of abnormally fused presynaptic mitochondria in the prefrontal cortex (Hara et al., 2014; Zhang et al., 2016). Impaired mitochondrial function is critical in the pathogenesis of age related neurodegenerative disorders (Esteves et al., 2010; Lin and Beal, 2006), making it pivotal to develop strategies aimed at improving mitochondrial function.

Recent work indicates that exercise training increases mitochondrial biogenesis in skeletal muscle and improves lipid oxidation through activation of upstream signaling pathways such as peroxisome proliferation-activated receptor gamma co-activator 1 α (PGC-1 α) and mitochondrial transcription factor A (TFAM) (Joseph et al., 2016; Yan et al., 2012). However, β -oxidation is not favored in the brain as neurons exhibit a greater dependence on aerobic glucose metabolism than peripheral cells (Schönfeld and Reiser, 2013). Thus, the effects of exercise on brain mitochondrial function may differ from its effects in muscle and other peripheral tissues.

In addition to its cardiovascular benefits, exercise improves brain health and neuronal plasticity (Voss et al., 2013). In mouse models, exercise increases neurogenesis and synaptic plasticity (Cotman and Berchtold, 2002; van Praag et al., 2005). Some of these effects may be due to upregulation of brain-derived neurotrophic factor (BDNF) (Marosi and Mattson, 2014), which occurs in the hippocampus, cortex, and cerebellum in response to exercise (Neeper et al., 1996). The effects of exercise are particularly pronounced in the hippocampus with exercise improving hippocampus-dependent spatial memory in rodent models (Erickson et al., 2011). Exercise may also have protective effects against the development of neurodegenerative conditions (Paillard et al., 2015). Upregulation of BDNF, increased mitochondrial biogenesis, and modulation of autophagy have been suggested as beneficial effects in this context (reviewed in (Mattson, 2014)), particularly as BDNF stimulates PGC-1 α expression in hippocampal neurons (E et al., 2014; Khabour et al., 2013). However,

relatively little is known about the effects of exercise on brain mitochondrial function with aging.

Here, we analyzed mitochondrial function and pathways implicated in regulating mitochondrial biogenesis in young and old mice after three weeks of exercise. We demonstrate that in concert with improvements in oxidative capacity of liver and muscle, complex I linked mitochondrial function is improved in old mice after exercise training without affecting complex II linked mitochondrial function. We found no evidence of increased brain mitochondrial protein expression or mitochondrial biogenesis, however protein levels of DRP1 were significantly elevated by exercise in brains of old mice, implicating enhanced mitochondrial transport or turnover.

2. Methods

2.1 Animal care and maintenance

C57BL/6J mice, both 4-week and 24-month old males, were obtained from the National Institute on Aging aged rodent colony (Charles River Laboratories, Madison, WI). Prior to the initiation of each experiment, mice were maintained on a constant 12-h light and 12-h dark cycle with free access to water, were fed ad libitum with a standard chow diet, and were allowed to acclimate for a week prior to the experimental protocol. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Pittsburgh, and were in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals.

2.2 Treadmill running protocol

Exercise training was performed similarly to previously published treadmill running protocols (Perrino et al., 2011). The 24-month old mice were randomly divided between control (old control, OC) and treadmill trained groups (old trained, OT). The 4-week old mice were divided into a young control group (YC) and treadmill trained group (YT). The OT and YT mice ran on a motor-driven treadmill (AccuScan Instruments, Columbus, OH) 5 days a week for 3 weeks and two days, for a total of 17 days of training. Training always started with 5 min of warm-up running at a velocity of 10 m/min. On the first day, and following 5 min warm-up, mice ran for 20 min at a 0° incline, at a velocity of 15 m/min. Over 4 days, the time of training progressively increased to 60 min at a 10° incline and a velocity of 15 m/min. During the second week, the mice ran for 60 min at 10° incline and a velocity of 15 m/min. During the third week the velocity increased to 17 m/min, and for the final two days of training the velocity was increased to 19 m/min. To encourage animals to move onto the treadmill, a mild electric shock was applied from a grid behind the treadmill. All mice in both groups were able to complete the training regimen without developing behavioral signs of fatigue. For control purposes, the YC and OC mice were handled in exactly the same way, but the treadmill was not turned on.

2.3 Whole body physiology and tissue harvest

Forty-four hours following the final bout of exercise, oxygen consumption (VO_2), expired CO_2 , and respiratory quotient (RQ) were determined over a 24 hour period using a

Comprehensive Laboratory Animal Monitoring System (CLAMS) (Columbus Instruments, Columbus, OH). Following CLAMS, mice were fasted for 6-hours and then euthanized. Liver, gastrocnemius, and brain were harvested immediately and flash frozen in liquid nitrogen and stored at -80°C for Western blot, or processed for mitochondrial or fatty acid oxidation assays. Each group (OC, OT, YC, YT) consisted of 7–8 biological replicates

2.4 Experiments with isolated mitochondria

2.4.1 Mitochondrial Isolation—After being euthanized, the brains were promptly removed, a portion of the cortex and striatum were dissected from one side and snap frozen in liquid nitrogen for Western blot analysis, with the remainder of the brain used for mitochondrial isolation as previously described (Gusdon et al., 2009). Briefly, dilutions of Percoll were prepared in isolation buffer I (IB I, containing 225 mM mannitol, 75 mM sucrose, 10 mM HEPES Potassium, 1 mM EDTA, 0.1% fatty acid free BSA, pH 7.4). All steps were performed at 4°C . Brain tissues were homogenized in 12% Percoll, layered on top of a gradient of 24% and 42% Percoll, and centrifuged at $27,000 \times g$ for 10 minutes. The mitochondrial fraction was located between the 24% and 42% layers and was removed with a syringe, diluted in IB I, and centrifuged at $10,000 \times g$ for 10 minutes. The supernatant was discarded, and the pellet was re-suspended in isolation buffer II (IB II, containing 225 mM mannitol, 75 mM sucrose, 10 mM HEPES potassium, 0.1 mM EDTA, pH 7.4) and centrifuged at $10,000 \times g$ for 10 minutes. The supernatant was again discarded, and mitochondria were cryopreserved as described (Nukala et al., 2006) by re-suspending the pellet in 1.5 mL of 10% dimethyl sulfoxide (DMSO) in IB II. This solution was then placed in a Nalgene freezing container for cooling to -80°C at a uniform rate of about $1^{\circ}\text{C}/\text{min}$. Protein concentrations for cortical and striatal mitochondrial preparations were determined using the bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific, Rockford, IL).

2.4.2 Mitochondrial respiration—Mitochondrial respiration was determined by measuring oxygen consumption using an Oxygraph-2k (Oroboros Instruments, Innsbruck, Austria). Incubation media was supplemented with 5 mM L-malate and 5 mM L-glutamate to support complex I respiration or 5 mM L-succinate to support complex II respiration. For each measurement, a concentration of 0.2 mg/mL mitochondrial protein was added. State 4 respiration was recorded for two minutes after the addition of mitochondria. State 3 respiration was induced by the addition of 0.5 mM ADP. The rate of state 3 respiration was measured for at least three minutes after maximum phosphorylating respiration was reached.

2.4.3 Mitochondria ROS production—Mitochondrial ROS production was assessed as previously described (Votyakova and Reynolds, 2005). Briefly, 300 μg of mitochondrial protein was added to incubation media supplemented with either 5 mM L-glutamate and 5 mM L-malate or 5 mM succinate. ROS production was measured by recording the real-time oxidation of 2 μM Amplex Red dye (Molecular Probes, Eugene, OR) to the fluorescent molecule resorufin as catalyzed by 1 U/mL horseradish peroxidase. Fluorescence was measured at an excitation wavelength of 560 nm and an emission wavelength of 590 nm. Rates of ROS production were measured over 5 minutes for each treatment, and all rates were linear over the time intervals used. Slopes were converted into units of $\text{pmol H}_2\text{O}_2/\text{min}/\text{mg}$.

2.4.4 ETC enzymatic activity—The electron shuttling activity between complex I and complex III was determined as described (Gusdon et al., 2008) by monitoring the reduction of ferricytochrome *c* to ferrocycytochrome *c* at 550 nm using a Spectramax E5 spectrofluorophotometer (Molecular Devices). The assay medium contained 25 mM potassium phosphate (pH 7.2 at 20°C), 2.5 mM MgCl₂, 2.5 mg/mL bovine serum albumin, and 2 mM KCN. KCN was included in order to inhibit cytochrome *c* oxidase and prevent the reoxidation of ferrocycytochrome *c*. A baseline was recorded for 1 minute at 550 nm after the addition of alamethacin (30 µg/mL), ferricytochrome *c* (15 µM), NADH (0.13 mM), and malonate (20 mM). In parallel wells, rotenone (2 µM) was added. Reactions were initiated by the addition of coupled mitochondria (200 µg/mL), and the rate of reduction of ferricytochrome *c* to ferrocycytochrome *c* was recorded for three minutes. Complex I-III activity was determined by subtracting the rotenone insensitive activity from the total activity.

2.4.5 Complex II-III Activity—Enzymatic activity was determined as described (Kennaway et al., 1984) by monitoring the reduction of ferricytochrome *c* to ferrocycytochrome *c* at 550 nm. The same assay medium as for complex I-III activity was used. A baseline was recorded for 1 minute at 550 nm after the addition of alamethacin (30 µg/mL), ferricytochrome *c* (15 µM), and rotenone (2 µM). In parallel wells, malonate (20 mM) was added. Reactions were initiated with the addition of mitochondria (200 µg/mL) preincubated with 20 mM succinate at 30°C for 20 minutes in potassium phosphate buffer. The rate of reduction of ferricytochrome *c* was recorded for three minutes. Coupled complex II-III activity was determined by subtracting the malonate insensitive activity from the total activity.

2.5 Western blotting

Western blotting was performed as described (Zhu et al., 2007). Protein concentration was determined using a BCA protein assay (Thermo Fisher Scientific, Rockford, IL), and equal amounts of protein (25 µg) were added to each well. The following primary antibody dilutions were used: 1:5,000 mouse monoclonal MitoProfile Total OXPHOS antibody cocktail (MitoSciences, Eugene, OR), 1:1,000 rabbit anti-FNDC5 (Abcam, Cambridge, MA), 1:1,000 rabbit anti-PGC-1 α (Santa Cruz, Dallas, TX), 1:10,000 rabbit anti-TFAM (courtesy of Dr. Craig Cameron, Penn State University), 1:1,1000 rabbit anti-HSP60 (Abcam), 1:1,000 rabbit anti-BDNF (Abcam), 1:500 mouse monoclonal anti-LC3 (5F10 clone, Nanotools, Teningen, Germany), 1:1,000 rabbit anti-Phospho-mTOR (Ser2448, Cell Signaling, Danvers, MA), 1:1,000 rabbit anti-mTOR (Cell Signaling, Danvers, MA), 1:1000 anti-DRP1 (BD Biosciences, Franklin Lakes, NJ), 1:1000 anti-SIRT3 (courtesy of Dr. Eric Verdin, UCSF, CA), 1:2,000 anti-MFN2 (Abcam), 1:5,000 anti-TOM20 (Santa Cruz), and 1:1,000 anti-MnSOD (Abcam) followed by ECL detection (GE Healthcare, Little Chalfont, UK). Membranes were stripped and reprobred with 1:10,000 rabbit anti-GAPDH (Abcam). Densitometry was performed using ImageJ's Gel analyzer (NIH, Bethesda, MD).

2.6 Muscle and liver palmitate oxidation assay

The assay was carried out using gastrocnemius and liver homogenates as described previously (Huynh et al., 2014). Following tissue harvesting, 60 mg of the specimen was

placed into ice-cold sucrose-EDTA medium (SETH buffer; 250 mM sucrose, 1 mM EDTA, and 10 mM Tris-HCl, pH 7.4). The sample was then blotted dry, and placed in 200 μ l of SETH buffer, minced thoroughly with scissors (~200 snips) and diluted 20-fold with additional SETH buffer. The minced tissue was then homogenized (12 passes) on ice using a Teflon coated pestle and glass homogenizer (Kontes Dual, Kimble Chase, Vineland, NJ). Forty microliters of homogenate were then added to the incubation well of a modified 48-well plate with a channel cut between the adjacent trap wells (Nunc, Thermo Fisher Scientific, Rochester, NY). The trap wells contained 200 μ L of 1N sodium hydroxide for the collection of liberated 14 -CO₂. To the homogenate, 160 μ l of incubation buffer (0.2 mM palmitate ([1- 14 -C] palmitate at 0.5 Ci/ml), 100 mM sucrose, 10 mM Tris-HCL, 5 mM potassium phosphate, 80 mM potassium chloride, 1 mM magnesium chloride, 0.1 mM malate, 2 mM ATP, 1 mM dithiothreitol, 0.2 mM EDTA, 1 mM L-Carnitine, 0.05 mM coenzyme A, and 0.5% fatty acid free bovine serum albumin, pH 7.4) was added to initiate the reaction. The plate was quickly sealed and incubated in a shaking water bath at 37°C for 30 minutes. Following the incubation, 100 μ L of 70% perchloric acid was added to terminate the reaction. One hundred and fifty microliters of the sodium hydroxide from the trap wells was counted for label incorporation into trapped 14 -CO₂ as an index of complete palmitate oxidation. This was determined by scintillation counting using 4ml of EcoScint (National Diagnostics, Atlanta, GA).

2.7 Statistical analysis

Statistical significance among groups was determined using one-way ANOVA analysis and protected Student's *t*-test. Results were considered statistically significant when $P < 0.05$. All figures were created with and statistical analysis was performed using R (R Foundation for Statistical Computing).

3. Results

3.1 Whole body physiology

Analysis of whole body physiology by CLAMS in the four groups revealed diminished systemic metabolism in aged mice that was reversed by exercise training. Both VO₂ and VCO₂ were lower in the OC group and were restored with exercise training in the OT group (Figure 1A and B, respectively). Similarly, RQ was decreased in the OC and was restored after exercise training in the OT mice (Figure 1C). The rates of fatty acid oxidation (FAO) were determined in homogenates from the gastrocnemius and liver as described (Huynh et al., 2014) by measuring the oxidation of radiolabeled palmitate. Rates of FAO were decreased in the OC group in both gastrocnemius and liver and were restored with exercise training in OT mice (Figure 1D).

3.2 Effects of exercise on mitochondrial function

3.2.1 Respiration—To investigate the effects of exercise training on brain mitochondrial function, a tissue that is dependent on glucose metabolism rather than FAO, the rates of mitochondrial oxygen consumption were measured using glutamate and malate to support complex I mediated respiration and succinate to support complex II mediated respiration. State 4 respiration was assessed by quantifying mitochondrial oxygen consumption in the

presence of complex I or complex II substrates without adding ADP. State 4 respiration was not significantly different among the groups utilizing complex I substrates (Figure 2A). However, when utilizing complex II substrates, respiration in old control OC and OT was significantly elevated compared with YC and YT (Figure 2B), but there was no effect of exercise training. State 3 respiration was assessed after the addition of ADP. Utilizing either complex I or complex II substrates, both OC and OT exhibited increased rates of respiration compared with YC and YT. Additionally, exercise training significantly increased complex I, but not complex II state 3 respiration in the OT group compared with OC (Figure 2A,B).

3.2.2 ROS production—Mitochondrial ROS production was assessed by measuring the rate of oxidation of Amplex red by hydrogen peroxide in the presence of superoxide dismutase. Utilizing complex I substrates, brain mitochondria produce characteristically low levels of ROS, and there was no significant difference among the groups (Figure 2C). Inhibiting distally within complex I with rotenone markedly increased ROS production from complex I redox centers, and after rotenone treatment, the rate of ROS production was increased in OT compared with OC (Figure 2C). Utilizing complex II substrates, brain mitochondria produce higher basal rates of ROS when membrane potential is maximum due to reverse electron transport (Votyakova and Reynolds, 2001), and there was no significant difference among the groups (Figure 2D). Dissipating membrane potential with FCCP decreases ROS production, and again there was no significant difference among the groups (Figure 2D).

3.2.3 Enzymatic activities—Changes in enzymatic activity could account for the increased rates of respiration observed in mitochondria from the OT group in the presence of glutamate, malate and ADP (Figure 2A). ETC activity was assessed by determining the rates of coupled enzymatic activity. Complex I-III activity was determined by following the reduction of cytochrome *c* in the presence of NADH, as a source of electrons for complex I, and malonate to inhibit complex II. Complex II-III activity was determined by following the reduction of cytochrome *c* by mitochondria, which had been preincubated with succinate, in the presence of rotenone to inhibit complex I. Complex I-III activity was significantly decreased in OC compared to YC, and this decrease was reversed by exercise training (Figure 2E). Complex II-III activity did not differ among the four groups (Figure 2F).

3.2.4 Mitochondrial protein content and biogenesis—To investigate whether the increase in respiration and coupled I-III activity observed in old trained mice could be due to increased mitochondrial content, cortical and striatal lysates were immunoblotted for components of each of the ETC complexes. Consistent with previous reports (Haase et al., 2011; Menshikova et al., 2006), mitochondrial protein content was significantly increased with exercise training in skeletal muscle (Supplementary Figure 1). In contrast, there were no significant differences in protein content of complex I, II, III, IV, or V subunits in the cortex or striatum in old or young mice after exercise training (Figure 3A–J; Supplementary Figure 2A). Additionally, we observed no significant difference in the protein content of TOM20 or MnSOD (Supplementary Figure 2B). PGC-1 α and TFAM each play important roles in controlling mitochondrial biogenesis and their expression in the hippocampus has been shown to be affected by exercise training (Yan et al., 2012). In the cortex and striatum,

exercise training did not alter expression of PGC-1 α or TFAM (Figure 4; Supplementary Figure 3). There were also no effects on levels of the mitochondrial chaperonin heat shock protein 60 (Hsp60) (Figure 4; Supplementary Figure 3) or the mitochondrial deacetylase SIRT3 (Figure 4; Supplementary Figure 3), a regulator of mitochondrial biogenesis, ATP generation, ROS production, and the uncoupled protein response (McDonnell et al., 2015).

3.2.5 Upstream regulation of mitochondrial biogenesis—BDNF has been shown to be increased in the brain in response to exercise (Neeper et al., 1996), where it drives PGC-1 α expression. However, we found no difference in protein levels of BDNF after exercise training in the cortex or striatum of old or young mice (Figure 5; Supplementary Figure 4). The membrane protein fibronectin type III domain containing 5 (FNDC5) is induced by PGC-1 α , and can be cleaved in exercised muscle to form the hormone irisin (Boström et al., 2012). We found no significant difference in protein levels of FNDC5 after exercise training in the cortex or striatum of old or young mice (Figure 5; Supplementary Figure 4).

3.2.6 Effect of exercise on autophagy—Autophagy is critical for the response of muscle tissue to exercise and has also been shown to be increased in the brain in response to exercise (He et al., 2012). However, we found no difference in the amount of LC3-II after exercise training in the cortex or striatum of old or young mice (Figure 5; Supplementary Figure 4). The mammalian target of rapamycin (mTOR) plays an important role in suppressing autophagy, and activity of mTOR has been shown to be increased with exercise (Watson and Baar, 2014). We found that the ratio of phosphorylated to unphosphorylated mTOR was not changed with exercise training in the cortex or striatum of old or young mice (Figure 5; Supplementary Figure 4). Additionally, absolute levels of phosphorylated and total mTOR did not differ with exercise training in the cortex or striatum between old or young mice (Supplementary Figure 4).

3.2.6 Mitochondrial dynamics—Mitochondria are dynamic organelles and constantly undergo fission and fusion. Alterations in mitochondrial homeostasis and dynamics have been implicated in aging and age-related disorders (Hara et al., 2014; Zhang et al., 2016) and may also be influenced by exercise training (Santos-alves et al., 2015). Protein levels of DRP1, a protein involved in mitochondrial fission, were significantly increased after exercise training in the cortex of old mice (Figure 6A), while levels of DRP1 were not affected after exercise training in the brains of young mice (Figure 6B). DRP1 protein levels in old trained mice were significantly higher than in young control and exercised mice ($P < 0.05$). Protein level phosphorylated DRP1 to total DRP1 was not changed with exercise training (Supplementary Figure 5A). Exercise training did not affect the levels of the mitochondrial fusion proteins MFN2 (Figure 6C,D) or OPA1 (Supplementary Figure 5B).

4. Discussion

Mitochondria play a key role in aging (Britic and Larsson, 2013; Joseph et al., 2016), and interventions that improve mitochondrial function may ameliorate age-associated pathophysiological changes. Exercise has long been known to have beneficial effects on whole body metabolism, skeletal muscle oxidative capacity, and mitochondrial function, in

part through stimulating mitochondrial biogenesis (Yan et al., 2012). Aging has been shown to result in mitochondrial dysfunction as well as reduced FAO in skeletal muscle (Hebert et al., 2010; Picard et al., 2011). We show that exercise training improved rates of FAO in old mice (Figure 1D) confirming the ability of exercise in our model to result in positive physiological adaptations. This is in line with studies in rodents and humans showing that exercise has a powerful effect on skeletal muscle FAO in older individuals (Chow et al., 2007; Lanza et al., 2008). This is in contrast to some reports suggesting that aged animals have lost the capacity to adapt to exercise stimuli (Ballak et al., 2015). These tissue specific changes were reflected in positive adaptations at the whole body level as demonstrated by improved VO_2 and VCO_2 , (Figure 1A–C). While exercise yields a number of benefits in the central nervous system, the ability of exercise to affect mitochondrial function in the brain with aging has not been systematically studied.

4.1 Improvements in complex I substrate linked mitochondrial function

Our results demonstrate for the first time, that brain mitochondria from aged animals respond to exercise training by selectively improving the activity of complex I of the ETC. While rates of state 3 respiration were increased in old mice, the overall quality of electron transport appears to decrease with aging, as shown by the lower complex I-III coupled activity in old control mice (Figure 2E) and increased rates of state 4 respiration (Figure 2A,B), leading to a lower RQ. After exercise training, mitochondria isolated from the old group exhibited further increases in respiration (Figure 2A) accompanied by slight increases in rotenone-driven ROS production (Figure 2C) while utilizing complex I substrates. The increased ROS production in the old group after exercising training likely occurred as a result of increased electron flux through the ETC, and is in accord with increased rates of respiration. Inhibiting distal to complex I redox centers with rotenone results in supra-physiological increases in ROS production, which can make slight differences in ROS production more apparent. *In situ* ROS production during exercise is likely much lower given the presence of ADP and reduced membrane potential during active ATP generation (Goncalves et al., 2014). Notably, the decrease in complex I-III coupled activity observed in old mice relative to young mice was fully reversed by exercise training (Fig. 2E). The RQ was also improved by exercise training due to the increase in state 3 respiratory rates (Fig. 2A).

4.2 Role of mitochondrial biogenesis

While increased mitochondrial mass could result in increased rates of respiration, we did not observe an increase in the brain expression of the two most important factors involved in mitochondrial biogenesis: PGC-1 α and TFAM (Figure 4A–D), the latter of which is tightly correlated with mtDNA content (Ekstrand et al., 2004; Ikeda et al., 2015; Lu et al., 2013; Mei et al., 2015). Nor were there changes in HSP60, which is responsible for transport of proteins into the matrix (Kaufman et al., 2003), or in SIRT3 (Figure 4E–H), which also regulates mitochondrial biogenesis (McDonnell et al., 2015). Likewise, there were no significant changes in levels of either nuclear DNA-encoded or mtDNA-encoded respiratory complex subunits (Figure 3, Supplementary Figure 2A) or of mitochondrial outer membrane or matrix proteins (Supplementary Figure 2B), although we did confirm that mitochondrial protein content was increased in liver and muscle after exercise training in our experimental

model (Supplementary Figure 1), consistent with previous reports (Haase et al., 2011; Menshikova et al., 2006).

The fact that we did not observe changes in mitochondrial protein content or markers of biogenesis is not unexpected when considering the pattern of exercise-induced changes in respiration and ROS production. As shown in Figure 2, increased mitochondrial respiration and ROS production was only found when mitochondria were utilizing complex I substrates. If mitochondrial content were increased as a result of increased mitochondrial biogenesis, respiration and ROS production would also be increased while utilizing complex II substrates. Consistently, the activity of both complexes I and II were elevated in the studies that demonstrated increased mitochondrial biogenesis and content in skeletal muscle (Menshikova et al., 2006). The different physiology of muscle and brain cells may contribute, although a recent study showed that complex I linked mitochondrial function was also affected by aging in muscle (Kruse et al., 2016). Local augmentation of mitochondrial mass would allow skeletal muscle to perform a larger proportion of work by aerobic rather than anaerobic metabolism, as less ADP would be required to stimulate the same degree of mitochondrial respiration (Constable et al., 1987). Because neurons function exclusively through aerobic metabolism, these adaptive mechanisms may be less relevant to brain mitochondrial function.

One recent study indirectly suggested that six weeks of exercise training could increase mitochondrial biogenesis in the hippocampus, as evidenced by increased mRNA expression of TFAM and an accessory subunit of complex I (Aguiar et al., 2014). This study also showed that exercise training increased the activity of complex I and made complex I more resistant to inhibition by rotenone, consistent with increased mitochondrial content, as both rotenone-sensitive and insensitive activity would be expected to increase in proportion to increased mitochondrial mass. The fact that increased brain mitochondrial biogenesis or mass was not observed in our study might be due to the shorter duration of exercise (3 weeks) compared with other studies (6–8 weeks) (Aguiar et al., 2014; E et al., 2014; Steiner et al., 2011). However, another study showed that 24 weeks of exercise training increased complex I activity without affecting complex II activity, similar to our results (Navarro et al., 2004). Alternatively, increased biogenesis may be more pronounced in the hippocampus, which is capable of undergoing exercise-induced neurogenesis, a process that requires a net gain in mitochondrial mass (van Praag et al., 2014). In any case, the data presented here indicate that a shorter duration of peripheral exercise in old animals is sufficient to enhance brain mitochondrial function through mechanisms other than upregulation of mitochondrial mass.

4.3 Effect of exercise on upstream signaling pathways

We also evaluated the contribution of other upstream signaling pathways. BDNF is implicated in the beneficial effects of exercise on memory, neurogenesis, and synaptic plasticity (Voss et al., 2013), acting upstream of PGC-1 α in stimulating mitochondrial biogenesis (Cheng et al., 2012). However, we did not detect a significant difference in the expression of BDNF after exercise (Figure 5A,B). Recently, the myokine irisin (generated by the cleavage of the membrane protein FNDC5) was shown to be induced by PGC-1 α ,

playing an important role for energy expenditure by stimulating UCP1 expression (Boström et al., 2012). We did not find a difference in the expression of FNDC5 (Figure 5C,D) in the cortex or striatum. Another study showed that seven days of running upregulated SIRT3 expression in the hippocampus, and led to a pattern of protein acetylation contributing to resistance against cellular stress (Cheng et al., 2015). We did not find an increase in SIRT3 expression after exercise (Figure 4). The effects of exercise on hippocampal BDNF and SIRT3 were observed after only 2–7 days of exercise (Neeper et al., 1996) and may not be sustained. Alternatively, there may be brain region selective effects of exercise.

Induction of autophagy during exercise is believed to be important to supply amino acids to skeletal muscles during exercise (Jamart et al., 2013) and may also protect against oxidative stress (Dutta et al., 2013). Furthermore, some studies have suggested that a brief duration of exercise induces autophagy in the brain (He et al., 2012). However, we did not observe a difference in levels of the autophagosome marker LC3-II after exercise (Figure 5E,F). Activation of mTOR down-regulates autophagy and up-regulates protein synthesis in skeletal muscle, and its activation may be caused by increased levels of BDNF in response to exercise (Watson and Baar, 2014). The fact that we did not observe changes in phosphorylated mTOR in the cortex or striatum after exercise (Figure 5G,H) is consistent with the lack of alteration in LC3-II and in BDNF. It should be noted, however, that a lack of change in steady state levels of LC3-II may be observed in the context of increased autophagic flux, if autophagy and lysosomal degradation are upregulated in parallel. Further studies, as new methods to monitor autophagy activity *in vivo* are developed, will be necessary to determine the effects of transient or sustained exercise on levels of brain autophagy.

4.4 A role for mitochondrial dynamics

Interestingly, our data demonstrated that improved brain complex I activity due to exercise training in old mice was correlated with a significant increase in the protein levels of the mitochondrial fission protein DRP1 in the cortex (Figure 6A,B). We found no change in the protein levels of the key fusion proteins MFN2 (Figure 6) or OPA1 (Supplementary Figure 5). Additionally, there was no change in DRP1 that is phosphorylated at the inactivating PKA phosphorylation site (Supplementary Figure 5). Taken together, these data suggest that a shift in the mitochondrial fission-fusion balance toward fission contributes to improved mitochondrial function in the cortex after exercise. As altered brain energetics induces mitochondrial fission arrest (Zhang et al., 2016) in Alzheimer's disease, upregulation of the DRP1 may act to reverse age-related alterations in the brain.

There are several mechanisms by which upregulation of DRP1 may be beneficial to neurons. Mitochondrial fission is necessary for effective autophagic clearance of damaged mitochondria in both PINK1-Parkin dependent and independent mitophagy pathways (Dagda et al., 2009; Kageyama et al., 2014). Moreover, mitochondrial fission is required for the development and maintenance of proper mitochondrial and synaptic function in neurons (Fukumitsu et al., 2016; Ishihara et al., 2009), and improves neuronal survival by decreasing oxidative stress (Kageyama et al., 2012). DRP1 has also been shown to be important for exercise-induced hippocampal neurogenesis and plasticity (Steib et al., 2014). This may be

mediated in part by a requirement for fission in order to achieve optimal delivery of mitochondria to both presynaptic and postsynaptic terminals (Fukumitsu et al., 2016). Indeed, the requirement for local ATP production and calcium buffering at synapses results in more mitochondrial traffic toward synapses and increased synaptic mitochondrial content, while impaired mitochondrial transport precedes neurodegeneration (Chang et al., 2006; MacAskill et al., 2010; Yamada et al., 2016; Yu et al., 2016). Alternatively, fission serves to limit propagation of mitochondrial calcium, with dysregulation of this process implicated in excitotoxicity and Parkinsonian neurodegeneration (Cherra et al., 2013). Modulation of mitochondrial dynamics and calcium buffering have also been implicated in synaptic plasticity in the aging brain (Cheng et al., 2010).

The increased activity-dependent synaptic plasticity and glutaminergic synaptic activity in the cortex compared with the striatum, as well as brain regional differences in metabolism (Pandya et al., 2016; Sauerbeck et al., 2011), may help to explain different effects of exercise training noted in different brain regions. The relative levels of fission-fusion proteins are known to vary according to body region (Santos-alves et al., 2015). Of note, fission-fusion proteins also varied with duration of exercise, with one study in skeletal muscle showing a decrease in MFN2 levels in skeletal muscle after 45 minutes of exercise with subsequent recovery to levels higher than the pre-exercise baseline (Ding et al., 2010). Additionally, abnormally fused mitochondria are observed specifically in presynaptic regions of the aging primate cortex (Hara et al., 2014), suggesting that increased levels of fission proteins induced by exercise may serve to normalize these aging effects.

Since we did not observe a difference with exercise training on a number of signaling pathways that could affect mitochondrial homeostasis (Figures 4 and 5), we can only speculate as to the mechanism linking exercise to mitochondrial homeostasis in the brains of old, but not young, animals. Recently, the role of microRNAs in aging has been demonstrated, including those that affect mitochondrial homeostasis. The miR-30 family members are upregulated during aging and cellular senescence, but are reduced by caloric restriction in mice with improved longevity (Smith-Vikos and Slack, 2012). As miR-30 family microRNAs act to suppress expression of DRP1 by inhibiting the p53 mediated activation of the DRP1 promoter (Li et al., 2010), as well as a number of autophagy mediators, it is possible that exercise training resulted in increased DRP1 expression and improved mitochondrial quality control and function by alleviating aging related miRNA changes in old mice.

Our results are consistent with prior reports demonstrating that preventing mitochondrial fission leads to decreased mitochondrial respiration and coupling with increased susceptibility to oxidative stress (Kageyama et al., 2012; Parone et al., 2008). However, this does not necessarily explain the complex I specificity of our findings. One explanation could be that exercise drives structural alterations in the ETC. Exercise was shown to be able to alter the membrane-dependent interactions among mitochondria in mice (Picard et al., 2013), which could affect mitochondrial morphology and superstructure. The redox status of the cell, which could be modified by exercise, has also been shown to modify mitochondrial morphology (Shutt et al., 2012). In addition, mitochondrial function is governed by supercomplexes formed between complexes I and III and also among complexes I, III, and

IV (Genova and Lenaz, 2014). Thus, exercise may improve the stability of the complex I/III/IV supercomplex, increasing electron flow through complex I while having little effect on the flow of electrons delivered through complex II. This mechanism would be consistent with our finding that complex I linked enzymatic activity was increased after exercise training (Figure 2E) without having an effect on complex II linked enzymatic activity (Figure 2F).

5. Conclusions

Our results indicate that exercise training improves muscle and liver FAO in aged mice, changes that are reflected in improved whole body metabolism. In brain mitochondria from aged mice, exercise selectively improved mitochondrial complex I function. Moreover, exercise increased levels of DRP1 without affecting indices of mitochondrial biogenesis or other stress response pathways. These data indicate that changes in mitochondrial dynamics and possibly structural alterations to complex I occur in response to exercise in the absence of, or prior to, changes in overall brain mitochondrial content. Importantly, the specific improvements elicited by exercise for brain complex I activity was observed in the old age group, suggesting that beneficial effects of exercise observed with aging and neurodegenerative diseases may be mediated in part by improved brain mitochondrial function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

PGC-1α	peroxisome proliferator-activated receptor gamma co-activator 1 α
TFAM	mitochondrial transcription factor A
BDNF	brain-derived neurotrophic factor
ETC	electron transport chain
OC	old control group
OT	old trained group
YC	young control group

YT	young trained group
DMSO	dimethyl sulfoxide
IB I	isolation buffer I
IB II	isolation buffer II
BCA	bicinchronic acid
ROS	reactive oxygen species
DRP1	dynamamin-related protein 1
MFN2	mitofusin-2
SIRT3	sirtuin 3
mTOR	mammalian target of rapamycin
FCCP	carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazine
FNDC5	fibronectin type III domain containing 5
HSP60	heat shock protein 60
COX7RP	cytochrome <i>c</i> oxidase subunit 7a-related protein
TOM20	translocase of the outer membrane 20
MnSOD	manganese superoxide dismutase

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Highlights

- Exercise training increased whole body oxygen consumption and fatty acid oxidation.
- Complex I-III coupled mitochondrial activity was decreased in the brains of old mice.
- Exercise increased complex I linked respiration and complex I-III coupled activity in old mice.
- Exercise training did not affect mitochondrial protein content or biogenesis in the brain.
- Expression of the fission protein DRP1 was increased in the brains of old exercised mice.

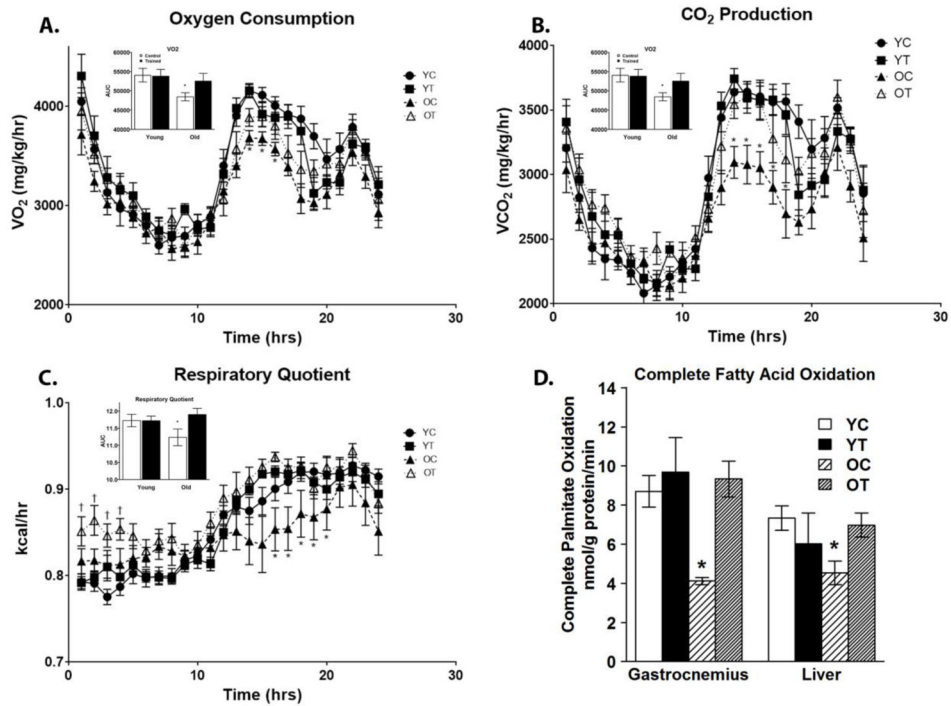


Figure 1.

Exercise training affects whole body physiology and fatty acid oxidation. Forty-four hours after the final round of exercise training, total body oxygen consumption (VO₂), expired CO₂ (VCO₂), and respiratory quotient (RQ) were determined over a 24-hour period using a Comprehensive Laboratory Animal Monitoring System (CLAMS). Rates of fatty acid oxidation were determined using tissues homogenates from the gastrocnemius and liver. Rates of oxygen consumption were decreased in OC mice which were restored after exercise training in OT mice (A). Rates of CO₂ production were decreased in OC mice which were restored after exercise training in OT mice (B). The respiratory quotient was decreased in OC mice which was restored with exercise training in OT mice (C). Rates of palmitate oxidation were decreased in OC mice in tissue homogenates from both gastrocnemius and liver, which was restored with exercise training in OT mice (D). Data are presented as means \pm SD. In Panels A–C, bar graphs at the top left represent areas under the curve (AUC) from 8 to 24 hr. In Panel A: * P < 0.05 in OC compared with YC, YT, and OT. In Panel B: * P < 0.05 in OC compared with YC, YT, and OT. In Panel C: * P < 0.05 in OC compared with YC, YT, and OT and † P < 0.05 in OT compared with YC, YT, and OC. In Panel D: * P < 0.05 in OC compared with YC, YT, and OT. YC, N=8; YT, N=8; OC, N=7; OT, N=7.

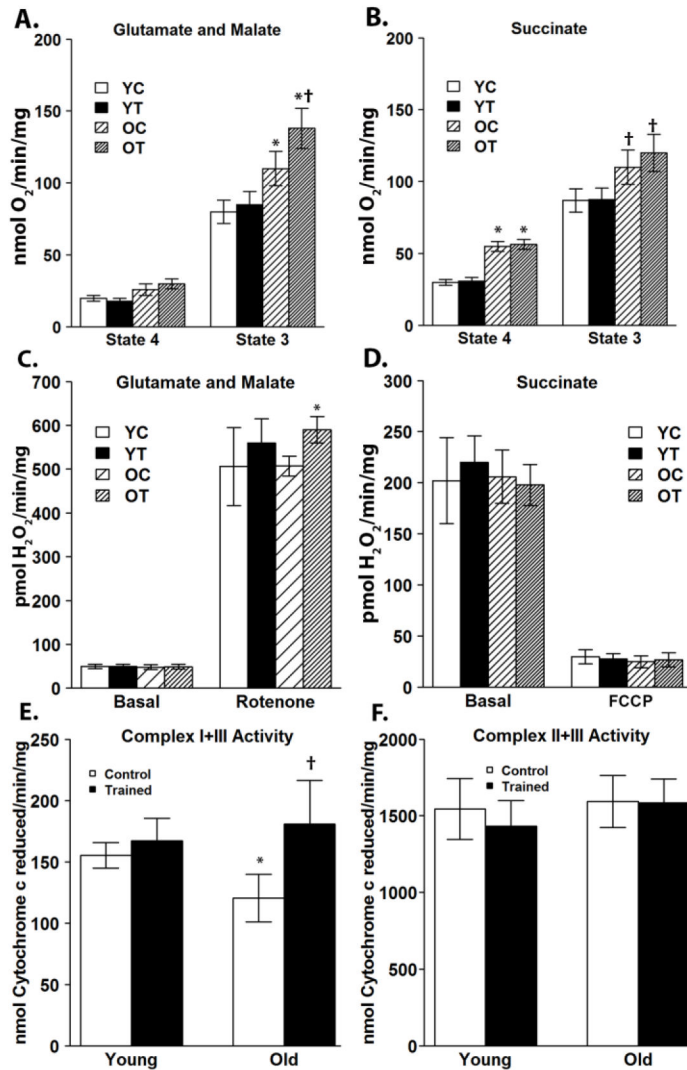


Figure 2.

Exercise training increases complex I substrate driven state 3 respiration, rotenone-induced ROS production, and complex I–III enzymatic activity in the brains of old mice. Utilizing complex I substrates, rates of state 3 respiration were increased in both old groups compared to the young groups, and in old mice, exercise training further increased the rate of state 3 respiration (A). Utilizing complex II substrates, rates of state 4 and 3 respiration were increased in old mice compared with young, however exercise training had no significant effect on succinate-supported state 3 respiration in the old mice (B). Utilizing complex I substrates, rates of basal ROS production were characteristically low (C). After inhibition with rotenone, rates of ROS production were significantly increased in the OT group (C). As expected, rates of ROS production utilizing succinate were basally elevated due to reverse electron transport, which was ablated after elimination of membrane potential with the uncoupling agent FCCP (D). There was no difference in ROS production utilizing complex II substrates among the four mouse groups (D). Complex I–III coupled activity was significantly increased in old mice with exercise training (E). Complex II–III coupled

activity showed no significant difference among the four groups (F). Data are presented as means \pm SD. In Panel A: * $P < 0.05$ vs YC and YT in state 3 respiration; † $P < 0.05$ vs OC in state 3 respiration. In Panel B: * $P < 0.05$ vs YC and YT in state 4 respiration; † $P < 0.05$ vs YC and YT in state 3 respiration. In Panel C: * $P < 0.05$ vs OC after rotenone treatment. In Panel E: * $P < 0.05$ vs YC. † $P < 0.05$ vs OC. YC, N=8; YT, N=8; OC, N=7; OT, N=7.

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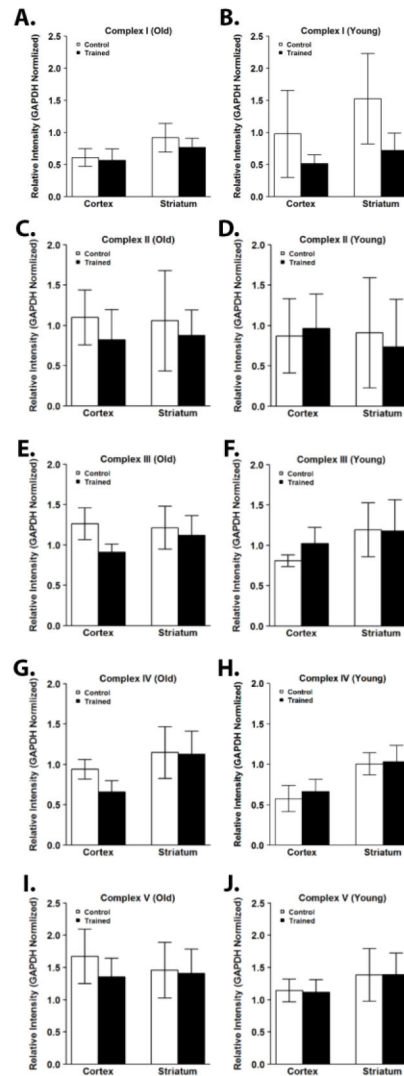


Figure 3.

Exercise training resulted in no significant difference in brain mitochondrial content. Mitochondrial content was determined by Western blotting for a component of each of the ETC complexes. There were no significant differences after exercise training in the protein content of mitochondrial complex I (A), complex II (B), complex III (C), complex IV (D), or complex V (E) subunits in the cortex and striatum of young and old mice. Data are presented as means \pm SD. YC, N=8; YT, N=8; OC, N=7; OT, N=7.

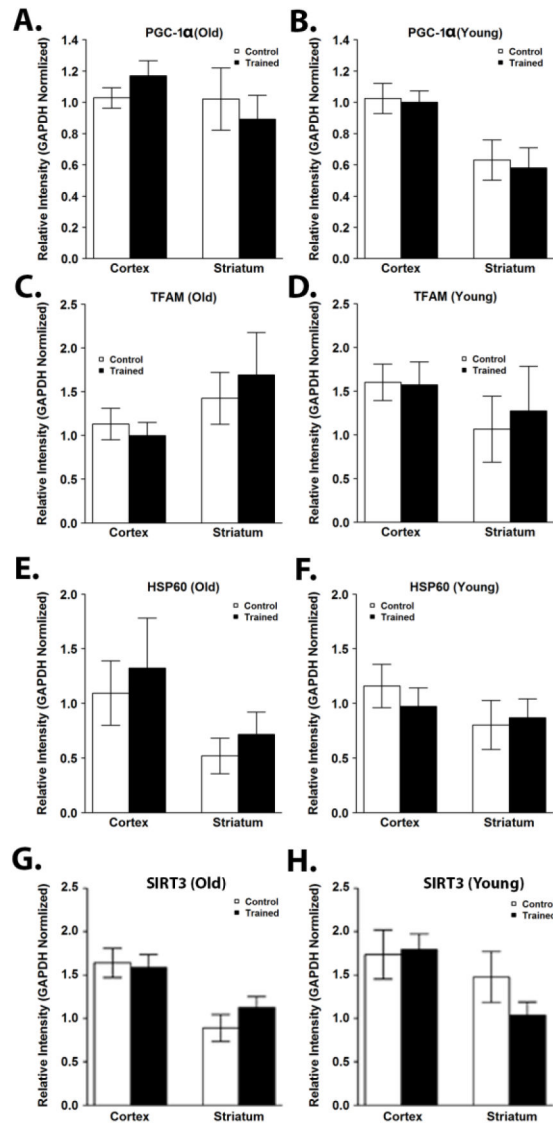


Figure 4.

Exercise training does not affect markers of mitochondrial biogenesis, HSP60, or SIRT3 in the brain. Mitochondrial biogenesis was assessed by Western blotting for PGC-1 α and TFAM. Western blotting was also performed for HSP60 and SIRT3. Protein levels of PGC-1 α were unchanged after exercise training in the cortex or striatum of old (A) or young (B) mice. Protein levels of TFAM were also not significantly different in the cortex or striatum of old (C) or (D) mice. Protein levels of HSP60 (E, old; F, young) and SIRT3 (G, old; H, young) were unchanged after exercise training in the cortex or striatum of old or young mice. Data are presented as means \pm SD. YC, N=8; YT, N=8; OC, N=7; OT, N=7.

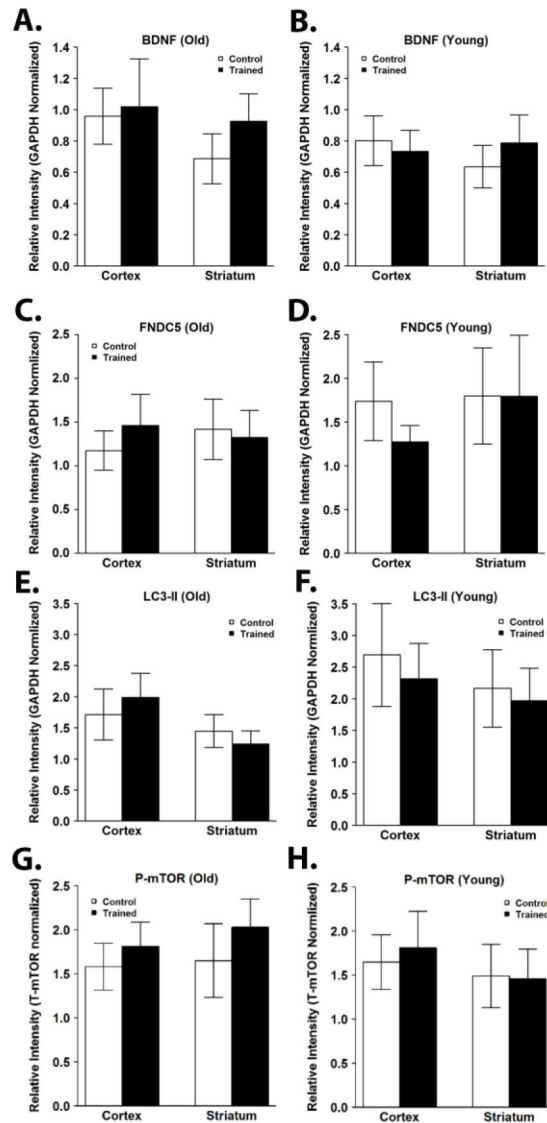


Figure 5.

Exercise training does not affect brain levels of BDNF and FNDC5, markers of autophagy, or phosphorylation of mTOR. Expression levels of BDNF, FNDC5, LC3-II, phosphorylated mTOR (P-mTOR) were assessed by Western blotting. P-mTOR levels were normalized to total mTOR levels. Protein levels of BDNF (A,B), FNDC5 (C,D), LC3-II (E,F), and P-mTOR (G,H) were not significantly different after exercise training in the cortex or striatum of old (A,C,E,G) or young (B,D,F,H) mice. Data are presented as means \pm SD. YC, N=8; YT, N=8; OC, N=7; OT, N=7.

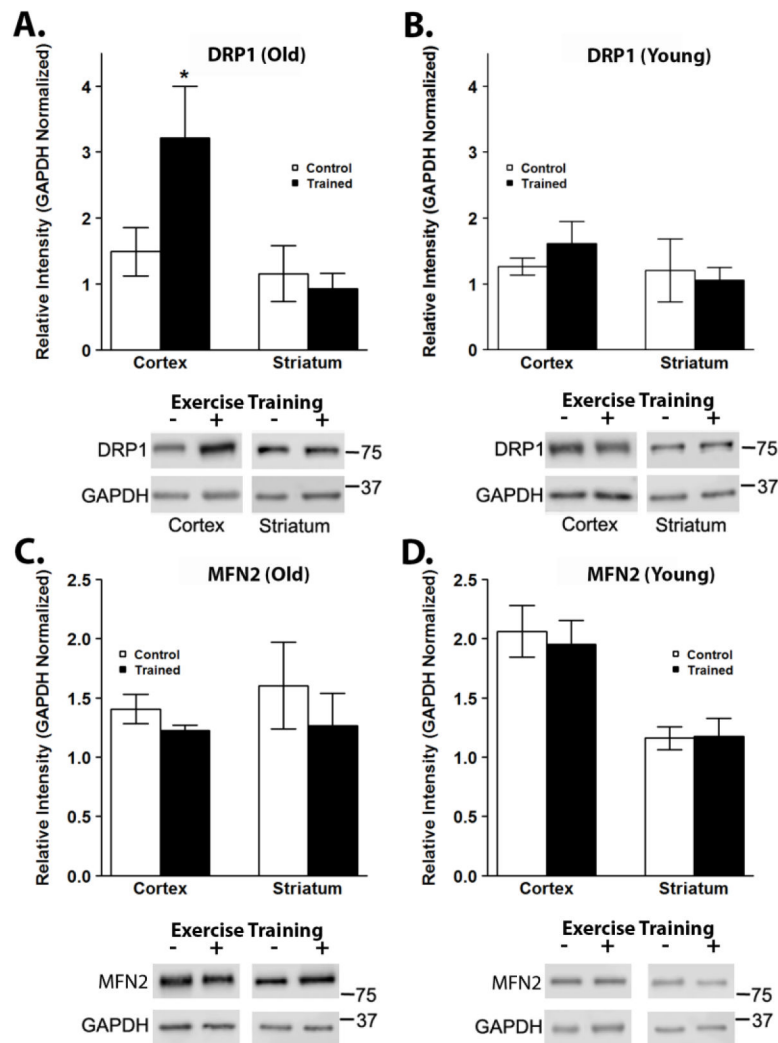


Figure 6. Exercise training affects markers of mitochondrial dynamics in the brain. Mitochondrial dynamics was assessed by Western blotting for markers of mitochondrial fission and fusion, DRP1 and MFN2, respectively. Protein levels of DRP1 were significantly elevated in the cortex of old, exercised mice (A), however levels were unchanged in young mice in response to exercise training (B). * $P < 0.05$ vs. OC, YC and YT. Protein levels of MFN2 were unchanged after exercise in either striatum or cortex of old (C) or young (D) mice. Data are presented as means \pm SD. YC, N=8; YT, N=8; OC, N=7; OT, N=7.