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EFFECTS OF PHYSICAL ACTIVITY ON BRAIN MITOCHONDRIAL FUNCTION

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Dedicatória

“Aconteça o que acontecer, aprendo. Ganho sempre.”

Marguerite Yourcenar

À Mana,

Por fazeres da insegurança

dos meus passos o caminho mais leve

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ABSTRACT

The main purpose of the present work was to analyse the effect of physical activity and exercise training on brain mitochondrial function. Eighteen Sprague-Dawley male rats were randomly divided into three groups (n=6/group): sedentary, endurance treadmill training (TM, 5days/week with progressive increase of time and speed during 12-weeks), free wheel voluntary physical activity (FW, 24h/day with an unlimited access to running wheel). Behavioral tests were performed to measure spontaneous alternation behaviour and exploratory activity in the open field. *In vitro* brain mitochondrial oxygen consumption rates, transmembrane potential fluctuations, mitochondrial calcium accumulation until mitochondrial permeability transition pore (mPTP) opening and mitochondrial oxidative damage were evaluated. Both exercise groups increased spontaneous alternation and exploratory activity; however, on open-field, the evaluated behaviours were only increased in TM group. TM and FW exercise induced a significant increases in state 3, respiratory control and ADP/O ratios. In addition, only TM lowered the lag phase and accumulated significantly more Ca^{2+} before mPTP induction than the sedentary group. No significant differences was observed on brain mitochondrial oxidative damage (-SH content and MDA levels). These data suggest that physical activity and more importantly forced exercise training results in improvements on the brain mitochondrial function.

KEYWORDS: PHYSICAL ACTIVITY; EXERCISE; BRAIN; BIOENERGETICS

RESUMO

O presente trabalho teve como principal objetivo analisar o efeito do treino de endurance e da atividade física voluntária na função mitocondrial do cérebro. Foram divididos, aleatoriamente, dezoito machos Sprague-Dawley em três grupos (n = 6/grupo): sedentário, exercício em tapete rolante (TM, 5 dias/semana com aumento de tempo e velocidade, progressivamente, durante 12 semanas) e exercício na roda livre (FW, 24h / dia com acesso ilimitado à roda). Foram realizados testes para avaliar o comportamento alternado e espontâneo e, também, a atividade exploratória em espaço aberto. Foram avaliadas, *in vitro,* as taxas de consumo de oxigénio mitocondrial, as flutuações de potencial elétrico transmembranar, a quantidade de cálcio acumulada até a abertura do poro de permeabilidade transitória mitocondrial (mPTP) e o dano oxidativo mitocondrial. Os dois tipos de exercício induziram um aumento das alterações de comportamento e da atividade exploratória. No entanto, apenas o grupo TM apresentou um aumento significativo dos comportamentos avaliados em espaço aberto. O treino em tapete rolante e atividade voluntária induziram um aumento significativo do estado 3, do controlo respiratório e da razão ADP/O. Adicionalmente, no grupo TM foi observada uma redução da *lag phas*e e uma maior capacidade de acumular Ca^{2+} antes da indução mPTP comparativamente ao grupo sedentário. Não foram observadas diferenças entre grupos nos marcadores de dano oxidativo mitocondrial (contéudo de grupos -SH e de MDA). Estes dados sugerem que a atividade física voluntária e, principalmente, o treino de endurance forçado promovem melhorias na função mitocondrial do cérebro.

PALAVRAS-CHAVE: ACTIVIDADE FÍSICA; EXERCÍCIO; BIOENERGÉTICA

ABBREVIATIONS AND SYMBOLS

A.M.	Before Midday
Ach	Acetylcholine
ADP	Adenosine Diphosphate
ADP/O	Ratio between Phosphorylated ADP and Consumed Oxygen
AIF	Apoptosis Inducing Factor
AMP	Adenosine Monophosphate
AMPK	Adenosine Monophophate Kinase
ANT	Adenine Nucleotide Translocase
Apaf-1	Apoptosis Protease Activating
ATP	Adenosine Triphosphate
BDNF	Brain-Derived Neurotrophic Factor
BSA	Bovine Serum Albumin
Ca^{2+}	Calcium Ion
CAT	Catalase
CS	Citrate Synthase
COX	Cytochrome c Oxidase
CO_2	Carbon Dioxide
CsA	Cyclosporin A
Cyp D	Cyclophilin D
d	Day
Da	Dalton
DA	Dopamine
DNA	Deoxyribonucleic Acid
Drp1	Dynamin Related Protein 1

EndoG	Endonuclease G
ETC	Electron Transport Chain
FAD	Oxidied Flavin Adenine Dinucleotide
$FADH_2$	Reduced Flavin Adenine Dinucleotide
Fis1	Fission 1
FW	Free Wheel
G	Glutamate
GABA	Gamma-Aminobutyric Acid
GPX	Glutatione Peroxidase
GSH	Reduced Glutathione
GSSG	Oxidied Glutathione
h	Hours
H_2O_2	Hydrogen Peroxide
HSP70	Heat Shock Protein of 70 kDa
KCl	Potassium Chloride
Kg	Kilogram
KH_2PO_4	Monopotassium Phosphate
m	Meters
M	Malate
MAPK	Mitogen-Activated Protein Kinase
Mfn 1	Mitofusin 1
Mfn 2	Mitofusin 2
mg	Milligrams
min	Minute
mL	Millliter
Ml	Microliter
µM	Micramolar

mM	Millimolar
mmol	Millimoles
MnSOD	Manganese Superoxide Dismutase
mPTP	Mitochondrial Permeability Transition Pore
mtDNA	Mitochondrial Deoxyribonucleic Acid
mtNOS	Mitochondrial Nitric Oxide Synthase
mRNA	Messenger Ribonucleic Acid
MΩ	Megaohm
NA	Noradrenaline
NaCl	Sodium Chloride
NAD	Oxidized Nicotinamide Adenine Dinucleotide
NADH	Reduced Nicotinamide Adenine Dinucleotide
NADPH	Reduced Nicotinamide Adenine Dinucleotide Phosphate
NGF	Nerve-Growth Factor
Nmol	Nanomol
nM	Nanomolar
NO	Nitric Oxide
NRF1	Nuclear Respiratory Factor 1
NRF2	Nuclear Respiratory Factor 2
NT-3	Neurotrophin-3
NT-4	Neurotrophin-4
O_2	Oxygen
O_2^-	Superoxide Radical
ºC	Degrees Celsius
$OH^·$	Hydroxyl Radical
Opa 1	Optic atrophy 1
PGC - 1α	Peroxisome Proliferator-Activated Receptor Gamma coactivato

RCR	Respiratory control ratio
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
s	Second
SED	Sedentary group
SEM	Standard Error of the Mean
SIRT 1	Silent information regulator T1
SOD	Superoxide dismutase
TM	Treadmill
TNF	Tumor necrosis factor
TPP^+	Tetraphenylphosphonium
tRNA	Transfer ribonucleic acid
UCP	Uncoupling proteins
VDAC	Voltage-dependent Anion Channel
xg	Centrifugal Force
$\Delta\mu H^+$	Transmembrane Chemical Potential
$\Delta\psi$	Transmembrane Electric Potential

1. INTRODUCTION

Nowadays, it is well recognized that the adoption of a healthy life style is related to increased levels of physical activity. Evidences suggest that physical activity is able to induce overall benefits in skeletal muscle, cardiovascular system, liver and kidney metabolism and also in brain function (Cotman and Berchtold 2002, Klaus and Amrein 2012). At least in part, the improved mitochondrial function seems to be an important adaptation to physical activity and therefore an important target to counteract several chronic diseases associated with compromised mitochondrial viability (Bishop et al. 2010, Moreira et al. 2010).

Epidemiological studies demonstrate that cognition is improved with physical activity in children, adult and aged persons (Hopkins et al. 2011, LeMoyne et al. 2012). Similarly, data from animal research suggests increases in behavioral tests performance with exercise training or voluntary physical activity (Fulk et al. 2004). However, besides morphological, vascular, neurogenic and neurochemical adaptations induced by physical activity (Cotman and Berchtold 2002, Gomez-Pinilla et al. 2002, van Praag et al. 2005), brain mitochondrial machinery improvement also seems to have a crucial role in exercise-induced increased brain function (Ploughman 2008, Radak et al. 2001a).

Physical activity is involved in a series of adaptations usually leading to the upregulation of brain tissue protective mechanisms (Ang and Gomez-Pinilla 2007, Dishman et al. 2006, Ma 2008). These adaptations include increased mitochondrial biogenesis and function, and improvement in mitochondrial antioxidant capacity, leading to a more effective control of free radical production and suggest that physical activity is an important mediator of increased brain function through mitochondrial-mediated mechanisms (Navarro and Boveris 2007, Steiner et al. 2011). However, research focused on brain mitochondrial response to exercise training in the brain is still scarce.

Mitochondrial relevance to brain function is undoubted. Besides the classical oxidative phosphorylation and inherent adenosine triphosphate (ATP) production, these organelles are also critical to intracellular calcium (Ca^{2+}) regulation as well as for redox and apoptotic signaling. Viable and fit

mitochondria are required to several ATP-depend process in neurons such as ion transport, receptors function, vesicle release and recycling of neurotransmitters (Chan 2006, Hoppins et al. 2007, Steiner et al. 2011). Moreover, mitochondrial dynamics regulated by fusion and fission-related mechanisms are associated with the redistribution of mitochondria to distinct subcellular localizations, thus responding to high-energy requirements, and leading to biogenesis and mitochondrial deoxyribonucleic acid (mtDNA) mixing that is critical for the repair of defective mtDNA (Steiner et al. 2011).

Therefore, we aimed to study the effects of 12 weeks of endurance exercise and voluntary physical exercise on brain cortex and cerebellum mitochondrial bioenergetics with special reference to oxidative stress and mitochondrial permeability transition.

2. STATE OF ART

2.1. Neuro-adaptations to exercise

2.1.1. Neuroplasticity

Functional and structural alterations occur in the brain throughout lifespan. Neuroplasticity has replaced the formerly-held position that the brain is a physiological static organ. Actually, is known today that brain has the ability to change its structure and function throughout life (Pascual-Leone et al. 2005). Brain plasticity allows to acquire new information and learn skills which, in turn, enable an adequate response to environmental stimulation, and to recover from brain injuries (Gomes da Silva et al. 2012). Importantly, some studies demonstrated that brain plasticity decreases with age and is deregulated in patients with neurological disorders (Eisch et al. 2008, Lovden et al. 2013, Lynch et al. 2006). This lifelong brain capacity for behavioral flexibility is mainly driven by a mismatch between functional supply and environmental demand (Lovden et al. 2010).

Physical activity is known to impact the entire body and its regular practice is associated with general health benefits. It is well accepted that physical activities are considered among the non-pharmacological strategies to reduce the risk of developing cardiovascular diseases and diabetes among other distinct pathologies (Klaus and Amrein 2012). However, several of these pathophysiological conditions, including hypertension and hyperlipidemia, glucose intolerance, diabetes mellitus and obesity also contribute to vascular dementia and to increased risk to develop neurodegenerative process (Ahlskog et al. 2011).

Regular physical exercise has proven to accomplish significant outcomes against neurodegenerative-related mechanisms. The benefits of an active life style are associated with enhancing and protecting brain function. Several studies demonstrated that physical exercise is able to confer health protective

benefits against distinct neurological diseases and ischemic stroke (for refs see Marques-Aleixo et al. 2012).

Taking all together, behavioral and neurobiological manipulations, such as those related with exercise programs can have long-term and vigorous effects on brain function (Hopkins et al. 2011). In fact, the neuroplasticity appears to have a crucial association with environmental manipulations including physical exercise throughout the life. The main mechanisms that are involved in physical exercise-induced brain plasticity include structural, functional and neurochemical alterations, which we will further address in detail in the following sections.

2.1.1.1. Cognitive function

An active lifestyle and increased levels of regular physical activity have been associated with improvements on cognitive functioning in children, adults and in elderly persons (LeMoyne et al. 2012). The level of physical activity seems to decrease in the course of lifetime. However, it has been reported that an active lifestyle can have a preventive role in cognitive health and in the aging-related decrease in cognitive function (Kaliman et al. 2011, Pluncevic 2012).

Hopkins et al. (2011) demonstrated that the effects of regular exercise in anxiety, mood and cognitive function are significant. Similarly, Hillman et al. (2008) reviewed the benefits of exercise for the treatment of depression and some neurodegenerative disorders. In fact, intellectual appointment, social interaction and physical activity are associated with the maintenance of cognitive function and also with the protection against the onset of chronic diseases, including neuropathologies such as Alzheimer´s and Parkinson´s (for refs see Marques-Aleixo et al. 2012).

Other authors described that children with low physical activity levels showed decreased neuroelectric activity and inferior cognitive performance when compared to physically fit children (Chaddock et al. 2010a). Moreover, it seems that they present better hippocampal plasticity and spatial memory in adulthood

and life (Gomes da Silva et al. 2012). Likewise, Chomitz et al. (2009) demonstrated that increased aerobic fitness and regular physical activity are associated with an enhanced academic performance in reading and mathematics. Chaddock et al. (2012) reported that children with higher physical activity levels may show better selective attention, inhibition of improper responses and maintenance of information in working memory. Similarly, another study conducted on children assessed the relationship between aerobic fitness and executive control. The conclusions suggest that fitness is associated with greater cognitive performance on an executive control task through enhanced cognitive control (Hillman et al. 2009).

Cognitive abilities usually show a gradual decline after middle adulthood, which may lead to dementia in later life (Chaddock et al. 2012). Prakash et al. (2011) verified that cardiorespiratory fitness relates to cognition in old adults being higher levels of fitness associated with better behavioral performance. Moreover, Colcombe et al. (2004) established that highly fit humans appear to have an increased functioning of the attentional network on the brain and cognitive task when compared with low-fit persons. The same authors suggested that physical activity at an early phase of life is a beneficial influence on diverse cognitive functions for many years. Also, Scarmeas et al. (2009) suggested that physical activity can reduce or prevent functional decline associated with aging and decreases the probability of Alzheimer´s emergence. As mentioned previously, older adults with greater physical activity and aerobic training levels showed decreased of cognitive impairment and dementia, as well as enhanced function and brain structure (McAuley et al. 2011, Voss et al. 2011). A study conducted with older man and women showed that midlife physical activity helps to sustain cognitive function and may decrease or delay the possibility of dementia in late life (Chang et al. 2010). Further studies suggested that greater physical activity levels and aerobic fitness in adults over the age of 65 are associated with enhanced cognitive performance (Chaddock et al. 2012). Similarly, a study with adults over the age of 65, who participated in physical activity program more than three times per week for at least 15 min showed that this group of active seniors have 34% less risk to be diagnosed

with dementia compared with those who exercised less than three times per week (Larson et al. 2006).

In general, both human and animal studies reported that physical activity and aerobic exercise training appears to have positive effects on non-pathological and pathological conditions on the aging brain (Kramer et al. 2006, Morie et al. 2010). Regular physical activity during life is associated with better school achievement and lower risk for cognitive decline, dementia and of risk for neurological disorders (Chaddock et al. 2012). Some of the mechanisms and targets by how exercise affects cognitive function will be addressed in the next sections.

2.1.1.2 Structural and morphological alterations

As previous referred, physical exercise is an important factor that positively modulates mental health throughout life (Hillman et al. 2008). Indeed, robust effects of physical activity have been reported in humans and rodents and have demonstrated that physical exercise is able to confer health protective benefits against several neurological diseases, seen at morphological level that could be translated into an increased brain function (Biedermann et al. 2012, van Praag et al. 1999). Erickson and colleagues (2011) reported that regular exercise increases hippocampus volume and induces plastic changes in specific brain structures. Exercise training seems to influence brain plasticity by hippocampus size, dendritic growth, cortical thickness and synapse formation, increased cell proliferation, increased hippocampal-dependent memory and learning and synaptic plasticity (Chaddock et al. 2010b, Pereira et al. 2007, Simpson and Kelly 2011, Voss et al. 2012). In fact, exercise has been described as a crucial factor in maintaining cerebrovascular integrity, increasing cerebral circulation, increasing dendritic connections and capillary growth and, finally, increasing the efficiency of the processing functions of the central nervous system (Radak et al. 2001a).

Chaddock et al. (2012) showed that higher-fit children present more efficient neuroelectric activation, larger brain volumes in the hippocampus and basal ganglia than less fit children. Also, higher-fit children showed greater bilateral hippocampal volumes and superior relational memory task performance compared to lower-fit children. Additionally, bilateral hippocampal volume was found to mediate the relationship between fitness cardiorespiratory level (VO_2 max) and relational memory (Voss et al. 2013, Voss et al. 2012). Similarly, it has been shown that young adults who did practice aerobic exercise for 3 months demonstrated a significantly increased hippocampal *dentate gyrus* blood volume over baseline (Ahlskog et al. 2011).

Age-related alterations in brain structure interfere with the hippocampal. However, according to Erickson and colleagues (2011) physical activity in older adults increase hippocampal perfusion, cerebral blood volume and the size of the anterior hippocampus, leading to improvements in spatial memory. Verstynen and co-workers (2012) demonstrated that aerobic exercise decreased the atrophy of several brain regions including the medial temporal lobe, basal ganglia and prefrontal cortex. This one year-long study with older adults, who participated in aerobic exercise tests and were submitted to magnetic resonance imaging assessement, concluded that higher cardiorespiratory fitness levels were associated with increased gray matter volume in the dorsal striatum and better cognitive performance.

At the same time, rodent models have demonstrated that voluntary aerobic exercise positively affects the hippocampus, increased cell proliferation, enhance hippocampal-dependent memory and learning processes, synaptic development, and angiogenesis (Chaddock et al. 2010b, Voss et al. 2012). Indeed, an adaptive response to functional and structural changes induced by chronic exercise training is angiogenesis (Roque et al. 2011). Angiogenesis is the neoformation of capillaries and a complex process requiring proliferation, migration and congregation of endothelial cells to form new vessels (Roudier et al. 2009). Huang et al. (2013) demonstrated that running exercise-induced increases of the capillaries in the female rat cortex might be one of the

structural bases for the exercise-induced improvement in the spatial learning capacity of middle-aged female.

The beneficial effects of physical exercise on brain functions suggest special attention of adult neurogenesis in cognitive and mental health (Yau et al. 2011). Thus, neurogenesis is the process by which neurons are generated from neural stem and progenitor cells. Hippocampal neurogenesis is known to be regulated by age stress, learning, seizures and exercise (Snyder et al. 2001). Yau et al. (2011) showed that exercise promotes dendritic plasticity and hippocampal neurogenesis. In elderly humans, imaging studies have shown changes in the hippocampus that may lead to cognitive decline. However, exercise training prevented or reversed these deleterious morphological and behavior consequences of aging (Snyder et al. 2001, van Praag et al. 2005). Moreover, research indicates that neurogenesis has a role in learning and memory. A study with wheel running in rodents resulted in a 3-4 fold or even larger enhance in the survival and formation of new neurons (van Praag 2009).

In summary, physical exercise has unquestionable beneficial effects on brain health (Bernardi et al. 2013). Brain structure and function appears to be closely related to the practice of regular exercise which, in turn, seems to contribute to an increased aging brain function and to counteract neurodegeneration.

2.1.1.3. Neurochemical adaptations

Exercise-induced enhanced brain function could also be explained by neurochemical alterations. Neurotrophic factors (Cotman and Berchtold 2002), stress hormones (Schoenfeld and Gould 2012), and neurotransmitters (Meeusen and De Meirleir 1995) can be influenced by physical exercise and could have an important role in the development, plasticity and brain health.

In fact, alteration in the concentration and activity of neurotransmitters, stress hormones and neurotrophic factors induced by exercise seem to modulate neurochemical alterations, including increased learning, cognition and behavior, higher adaptation to stress and anxiety situations, increased regulation of mood,

appetite and sleep, better response to inflammatory state and increased neural development (Cote et al. 2011, Goekint et al. 2012, Simpson and Kelly 2011). Additionally, some studies demonstrated that physical exercise modulates several physiological mechanisms that may increase brain resistance to aging and neurodegeneration (to refs see Marques-Aleixo et al. 2012). Table 1 summarizes some neurochemical alterations induced by physical exercise.

Table 1. Neurochemical alterations induced by exercise in healthy animals

Neurochemic factors	Main cognitive function	Role of physical exercise	References
Neurotransmitters and stress hormones			
Serotonin (5HT)	Memory; Learning ; Regulation of mood, appetite and sleep; Related with feeling well and happiness	↑ or~	Goekint et al. (2012); Dey (1994); Ishikawa et al. (2013); Rethorst et al. (2012)
Dopamine (DA)	Cognition and behavior; Motivation; Working memory; Learning; Attention; Mood; Dreaming and sleep	↑ or~	Foley et al. (2008); Goekint et al. (2012); Tsuchiya et al. (2012); Simpson et al. (2011)
Noradrenaline (NA)	Increase heart rate and blood flow to skeletal muscle at the fight-or-flight response	↑ or**~**	Goekint et al. (2012); Ishikawa et al. (2013); Madarame et al. (2013); Simpson et al. (2011)
Acetylcholine (Ach)	Memory; Learning; Lowers heart rate and force of cardiac contraction; Vasodilation	↑	Parnow et al. (2012)
Opioids	Analgesic effects, which decrease perception and reaction to pain; increase pain tolerance; Deficit respiratory; Sedation; Strong sense of euphoria	↑ or~	Jonsdottir et al. (1997); Simpson et al. (2011)

Gamma-Aminobutyric Acid (GABA)	Regulation of mood, appetite and sleep; Related with decrease anxiety; Regulation of muscle tone	↑	Urakawa et al. (2013); Ni et al. (2009); Hill et al. (2010)
Glutamate	Learning; Memory; Increase Brain Function and Mental activity; Decrease fatigue	↑	Mourzakis et al. (2002); Simpson et al. (2011)
Cortisol	Adaptation to stress; Maintain glucose levels appropriate even in fasting; Catabolism of skeletal muscle and adipose tissue; increase vasoconstriction caused by adrenaline; Decrease the use of glucose, saving it to the brain	↑,~or ↓	Zoladz et al. (2002); Dean, (2002); Kanaley et al. (2001)
Neurotrophic factors			
Brain-Derived Neurotrophic Factor (BDNF)	Memory; Learning; Long-term memory; Neural development;	↑ or~	Quirie et al. (2012); Cote et al. (2011); Boyce et al. (2007); Skup et al. (2000); Vivar et al. (2012); Goekint et al. (2012)
Nerve Growth Factor (NGF)	Development; Maintenance of peripheral sympathetic and embryonic sensory; Maturation; Response to offending stimuli; Immune system and inflammatory cells	↑	• Liu et al. (2011); Bonini et al. (2013); Vivar et al. (2012); Chae et al. (2012); Simpson et al. (2011)

Neurotrophin-3 (NT-3)	Survival and differentiation of existing neurons; Growth of new neurons and synapses;	↑	Sharma et al. (2010); Boyce et al. (2007); Skup et al. (2000); Cote et al. (2011)
Neurotrophin-4 (NT-4)	Learning; Memory; Long-term memory; Adult locomotory behaviour; Epidermis development	↑	Skup et al. (2002); Funakoshi et al. (1995); Skup et al. (2000); Cote et al. (2011)

↑, significant increase; ↓, significant decrease; ~, no significant changes.

Neurotransmitters can be catecholamines and tryptamines and are associated with emotionality, anxiety and behavior; stress hormones are necessary in “fight or flight” response and other individual stress situations and, finally, neurotrophic factors are proteins known to promote cell survival and functions particularly related to synaptic plasticity. Therefore those neurochemical parameters are intimately related with specific brain function and can be modulated by alterations induced by physical exercise.

2.2 Relevance of Mitochondria on Brain Function

As previously reported, with enhancing life expectancy, age-related neurodegenerative disorders resulting in structural and neurophysiological alterations in the brain are extremely complex. The literature shows that healthy mitochondria are crucial to the maintenance of the integrity of human systems, including brain and nervous system function (for refs see Marques-Aleixo et al. 2012). Several studies suggested a relationship between mitochondrial function and cognitive function in humans (Inoue et al. 2000). Mitochondrial respiration is fundamental to maintain the cortical and hippocampal plasticity for establishment for spatial memory (Tanaka and Watanabe 2008). Therefore, it is increasingly important to find strategies to counteract the decline of mitochondrial function as this can be a decisive contributor to the aging process and acceleration of neuropathological conditions.

Three-dimensional reconstruction indicates that mitochondria constitute a complex branched, forming a reticular network which alters in size, shape and complexity (Benard and Rossignol 2008b). The next table explains the main functions of the mitochondrial sub-structures (Table 2).

Table 2. The Mitochondria Specialized Structures

Structural components	Main functions
Outer membrane	- is needed for compartmentalization of the specific activity of mitochondria from the cytosol; - contains a great numbers of porins that permit free diffusion of small molecules;
Inner Membrane	- contains all the essential components of the electron transport chain; - compartmentalized proteins with five types of functions: 1) ATP synthase, 2) execute the redox reactions of oxidative phosphorylation, 3) specific transport proteins that control metabolite passage into and out of the mitochondria matrix, 4) protein import machinery and 5) mitochondria fusion and fission protein;
Inner Membrane Space	- space the protons (H^+) that are pumped to create the proton motive force, which drive ATP production; - contains many proteins that may play important roles in cell death like the cytochrome c and the apoptosis inducing factor;
Cristae	- are the functional internal compartments formed by the many invaginations and folding of the inner membrane; - in this structure occurs the cellular respiration and grouped to protein complexes of the respiratory chain and the proteins that are involved in the iron/sulfer group biogenesis;

Matrix	- is where enzymes catalyze the oxidation of pyruvate, the end product of glucose, which occurs in the cytosol, addicted to energy rich products that drive the electron transport chain; - in this structure are located the enzymes that carry out the ß-oxidation citric acid cycle and the mitochondrial DNA (mtDNA).

Mitochondria are the powerhouses of the cell able to generate large quantity of ATP; however, these important organelles also serve numerous other functions in addition to energy production, such as calcium regulation and cell signaling, thus determining cell survival or death.

Cellular respiration, the process of breaking down food into energy, carbon dioxide and water, is related to several interconnected pathways and steps: glycolysis, tricarboxylic acid cycle (Kreb´s Cycle), beta-oxidation, electron transport chain (ETC) and oxidative phosphorilation.

The ETC operates by a step-wise transfer of energy rich electrons from NADH and $FADH_2$ donors to inferior energy acceptor molecules (complex I-IV, ubiquinone and cytochrome c). The NADH dehydrogenase (complex I) receives electrons from NADH and succinate desydrogenase (complex II) from $FADH_2$. These protein complexes are starting points of the ETC. The rest of the ETC operates despite of which complex (I or II) initiated the chain. The remaining carrier molecules (ubiquinone and cytochrome c) and protein complexes cytochrome bc1 (complex III) and cytochrome c oxidase (complex IV) continue the electron transfer until the reduction of molecular oxygen (O_2) (Benard and Rossignol 2008b, Gilkerson et al. 2003a).

Therefore, the pathways for the electrons transferency throughout the ETC can be described as follows:

Table 3 summarizes the specific functionality of the diverse members of protein complexes of the ETC.

Table 3. Functions of the five complexes for ETC

Complexes	Function
I – NADH Dehydrogenase (Coenzima Q redutase)	Has the capacity to oxidize NADH. Proton pump to the intermembrane space of mitochondria contributes to the electrochemical gradient of protons and can guide to the generation of reactive oxygen species (ROS).
II - Succinate Desydrogenase (Ubiquinone redutase)	Catalyze the oxidation of succinate to fumarate with the creation of ubiquinol from ubiquinone.
III – Cytochrome bc1: Cytochrome c Reductase	Through ubiquinol, and simultaneously that ocurring explosion of a pair proton, an electron is transferred to the Cytochrome c.
IV – Cytochrome c Oxidase	O_2 is reduced to water and energy used to pump protons produced.

Oxidative Phosporilation (OxPhos) is the electrochemical gradient (Δp) driven process of phosphorylating (adenine diphosphate). Three to four protons are transported down their (Δp) back to the matrix through the ATP synthase – complex V to produce 1 molecule of ATP (Boyer 1997).

Another very important function related to mitochondrial network is calcium (Ca^{2+}) regulation. Calcium is a signaling molecule and a second messenger that can regulate metabolism in the cell. In physiological conditions, the extracellular concentration of free ionic calcium is 1-2 mM and cellular range are between 50 and 100 nM being levels inside mitochondria in the scale of μM (Hansford and Zorov 1998). According to Gunter et al. (2000), three dehydrogenases associated to the Kreb´s Cycle as well F_0F_1ATPase and the ANT are activated by moderate concentrations of intramitochondrial calcium concentration. Mitochondria can modulate calcium signaling by exporting additional calcium into the intracellular cytosolic space (propagating) or by sequestering even

insignificant enhances of calcium (inhibiting). The level of calcium loading into mitochondria is dependent the frequency and duration of stimuli. The capacity of mitochondria to regulate calcium with such an exact spatiotemporal control, underscores the important roles that calcium plays in the cell (Gunter et al. 2000). However, despite calcium regulates cell and even mitochondrial metabolism, large amounts of calcium influx into mitochondria may inhibit oxidative phosphorylation and directly affect the rate of ROS production. Unnecessary and excessive calcium loads can also generate assembly of the mitochondrial permeability transition pore (mPTP) (Kann and Kovacs 2007). The permeability transition pore complex is a channel that is assembled at the junctions between the inner membrane and the outer membrane and, despite some controversy is proposed to be composed and/or regulated by adenine nucleotide translocase (ANT), cyclophilin D (Cyp D), hexokinase, voltage-dependent anion channels (VDAC) (Haworth and Hunter 1979). More recently, the ATP synthase as been purposed to have an important role in the regulation of the mPTP (Bonora et al. 2013). The formation of mPTP may have devastating cellular consequences as a result of the dissipation of mitochondrial proton gradients, liberation of cytochrome c and initiation of apoptosis (Haworth and Hunter 1979).

It is also assumed that mitochondria play an important role on cell signaling, including cell survival and death pathways (Smaili et al. 2003). Cell death is an indispensable event that occurs by apoptosis and/or necrosis. Apoptosis, or planned cell death, is necessary for survival and plasticity, while necrosis is an unexpected death due to a lack of accessible ATP indicative of energy breakdown (Brown and Borutaite 2001). Cytochrome c and mitochondrial nitric oxide (mtNOS) are two crucially important molecules that play dual roles in cellular respiration and in these two cell survival and death signaling pathways. These molecules are directly or indirectly modulated by calcium levels. Cytochrome c is familiarly linked to mitochondrial respiration by transferring electrons between complex III and IV of the ETC. Ott et al. (2002) refer that apart from the cause or mechanism for cytochrome c extrusion, it is a potent incentive for initiation of apoptosis. Oxygen uptake is synchronized by mtNOS

that can reversibly inhibit cytochrome oxidase – complex IV – and the production of superoxide (O_2^-) from the electron transport chain. Under certain pathological conditions significant suppression of mitochondrial respiration and elevations of oxidative stress are observed (Smaili et al. 2003). Considering the importance of mitochondrial process in neuronal function, it has been suggested that mitochondria may possibly be a potential target for pharmacological and non-pharmacological strategies to minimize the impact of several neurodegenerative disorders (Marques-Aleixo et al. 2012). Calabrese et al. (2001) demonstrated that evidence for mitochondria susceptibility to damage in neurodegenerative disorders is, at least in part, based on decreases in respiratory chain complex activities. Such defects in respiratory complex activities, probably associated with oxidant/antioxidant balance perturbation, may have serious impact on energy metabolism contributing to cellular degeneration.

The neurons are highly differentiated cells that need large amounts of ATP for preservation of ionic gradient across the cell membranes and for neurotransmission (Kann and Kovacs 2007). Since the majority of neuronal ATP is generated by oxidative metabolism, neurons depend on mitochondrial function and oxygen supply (Ames 2000). Although the brain is a non-contractile tissue, an increase on energy metabolism seems to indirectly influence neuronal function (Dishman et al. 2006). In addition, mitochondria are intracellular organelles containing their particular genomes (mtDNA) and playing an important role in ATP production through oxidative phosphorylation (Inoue et al. 2000). Mutations on mtDNAs and deficiencies in mitochondrial respiration are associated with an extensive diversity of disorders, such as mitochondrial and neurodegenerative diseases, diabetes and aging (Tanaka et al. 2008).

2.2.1 Brain mitochondrial response to physical exercise

As mentioned previously, physical activity and particularly the regular physical exercise has been considered a decisive strategy in the therapy of several pathologies. Thus, the understanding of the underlying physiological

mechanisms that are associated with physical exercise-mediated benefits assume high relevance.

Considering different mechanisms and targets, brain mitochondrial metabolism and function seems to be highly modulated by physical exercise. The study of some metabolic brain alterations induced by physical exercise has been based on the analysis of the content and/or activity of several enzymes, particularly those implicated in aerobic energy production pathways (Ding et al. 2006). In fact, an increase in ATP synthase content may be one of the adaptations by which exercise enhances energy production in brain mitochondria. Importantly, different exercise types such as free wheel voluntary running or forced running on a treadmill have distinct impacts on hippocampal mitochondrial proteins (for refs see Marques-Aleixo et al. 2012). In fact, several studies suggested that enhanced physical activity induced brain alterations in translational efficiency or protein constancy that may compensate upstream transcriptional responses (Marques-Aleixo et al. 2012).

Physical exercise induces an increase of O_2 consumption and energy utilization which, in turn, increases ATP synthesis. This suggests that exercise induces important adaptations in the mitochondrial activity of hippocampal neurons in order to sustain metabolic demands.

2.2.1.1 Mitochondrial respiratory chain and antioxidant adaptations

As previously mentioned, mitochondria play a decisive role in animal cells and are involved in aerobic energy production, converting oxygen and nutrients into ATP (Benard and Rossignol 2008a). The mitochondrial respiratory chain is the “locus” of a spatial series of redox reactions in which electrons are transferred from a donor molecule to an acceptor molecule. Oxidative phosporilation is an important cellular process that uses oxygen and electron derived from simple sugars, fatty acids and proteins to create ATP being five inner membrane located protein/enzymatic complexes (I-V) involved in this process (Gilkerson et al. 2003b). During oxidative phosphorylation, these complexes conduct

chemical reactions that drive the production of ATP. In particular, they produce an irregular electron charge on either side of inner membrane and this disparity in electrical charge provides the proton-motive force for ATP production (Benard and Rossignol 2008a).

Some studies analyzed brain mitochondrial adaptive responses to exercise and suggested that moderate running antagonize some age-related impairments in mitochondrial function in general and particularly, at ETC level (Boveris and Navarro 2008b, Navarro and Boveris 2007). Indeed, 24 weeks of moderate exercise improved ETC flux in brain mitochondria, by enhancing the activity of complexes I, III and IV, thus contributing to prevent the age-dependent mitochondrial function decline reported in sedentary rodents (Navarro et al. 2004). Also, moderate exercise reverted the progressive decrease in enzymatic activities of brain mitochondrial complexes I and IV (Navarro and Boveris 2007). Indeed, physical exercise seems to modulate mitochondrial respiratory chain, and it has been suggested that exercise may be a valuable strategy for neuroprotection by targeting these components of mitochondrial machinery (Boveris and Navarro 2008b).

ROS (reactive oxygen species) can be produced from different enzymatic and non-enzymatic reactions and mitochondria is the major intracellular source of ROS (Chakrabarti et al. 2011). Radak et al. (2008) stated that the age-related accumulation of oxidative damage impairs brain function, and exercise due to the changes in redox homeostasis (increasing antioxidant/damage repair enzyme activity, decreasing oxidative damage and increasing resistance to oxidative stress), can attenuate the accumulation of damage, causing improved brain function. Navarro et al. (2004) described that moderate and regular exercise have an important role in the upregulation of antioxidant enzymes, the decrease in oxidative stress markers and in the increased of mitochondrial enzymatic activity. In fact, exercise appears to be elementary on brain mitochondria function through enhance antioxidant levels with consequent attenuation of ROS production (Navarro and Boveris 2007).

In studies with older humans, either favorable brain redox adaptations or incapacity to revert the increased ROS that characterized aging process were found (Jolitha et al. 2006). Furthermore, exercise combined with antioxidant supplementation appears such as a possibility to reduce the age-dependent risk of oxidative modification of brain proteins and lipids (Chakrabarti et al. 2011). The same authors refer that this combination seems to substantially stimulate the endogenous antioxidant system. Indeed, the increased of mitochondrial and tissue antioxidant capacity induced by physical exercise is not exclusive of brain. The generation of ROS during exercise may play a crucial role as regulatory mediators and signaling molecules in adaptative response (Radak et al. 2001a).

2.2.1.2. Mitochondrial permeability transition pore and apoptotic signaling

In recent years the role of mitochondria in cell death has been a subject of particular attention by the scientific community. The increased of permeability of mitochondrial membranes is pivotal in cell death signaling (Tsujimoto and Shimizu 2007).

In addition to the proposed mPTP components, including Cyp D, ANT and the VDAC, hexokinase, the phosphate carriers and more recently, ATP synthase might also be involved as potential components of mPTP (Bonora et al. 2013). Furthermore, some studies demonstrated that Cyp D seems to be the essential element of the mPTP opening (Halestrap and Brenner 2003, Kokoszka et al. 2004), but ANT and VDAC seem to be responsible for the interface with the apoptotic proteins (Crompton 2003). Additionally, it has been suggested that the mPTP state is regulated by proteins of the Bcl-2 family comprising pro-apoptotic fractions as Bax, Bak, Bok, and anti-apoptotic as Bcl-2, Bcl-XI, Bcl-w. The relative proportion of these proteins seems to have an important role in the apoptotic susceptibility (Bernardi et al. 2001, Tsujimoto 2003b).

Several factors facilitate the assembly of these specific proteins leading to the opening of the mPTP. The regulation of mPTP appears to be modulated by the decreasing of transmembrane electrical potencial (Δψ), the increasing in inorganic phosphate concentration, the decreasing of the content of adenine nucleotides (ATP and ADP) and, finally, the increasing of oxidative stress. Importantly, it has been also suggested that mPTP is a process favored by dysregulation Ca^{2+} homeostasis (Tsujimoto and Shimizu 2007). Korshunov et al. (1997) showed that transient mPTP opening may contribute to the Δψ regulation and operate as a channel for fast release of Ca^{2+}. In fact, increasing Ca^{2+} concentration continues to be one of the main factors that lead to cellular apoptosis (Richter 1997).

Cells have two essential apoptotic signaling pathways, the intrinsic and the extrinsic (Green and Evan 2002a). Regardless endoplasmic reticulum-mediated path, the commonly referred intrinsic pathway is activated by the release of different mitochondrial pro-apoptotic proteins to the cytosol (Green and Evan 2002b, Wang 2001), among which we can emphasize the cytochrome c. Once released into the cytoplasm, and in the presence of ATP, cytochrome c binds apoptosis activating factor protease (Apaf-1), enhances its oligomerization and the recruitment/activation of pro-caspase-9. The protein complex formed by cytochrome c, Apaf-1 and pro-caspase-9 is called apoptosoma (Desagher and Martinou 2000, Hill et al. 2003, Tsujimoto 2003a). Mitochondria contain other pro-apoptotic proteins in the intermembrane space such as Smac/DIABLO, HtrA2/Omi, apoptosis inducing factor (AIF), and endonuclease G (EndoG) (Green and Evan 2002b, Wang 2001). Together, Smac/DIABLO and HtrA2/Omi are able to prevent inhibitory apoptosis proteins (IAPs) to facilitate the activation of caspases. Moreover, AIF and EndoG translocase are related to an apoptotic stimulus that appear to be responsible for chromatin condensation and DNA fragmentation (Ravagnan et al. 2002).

Programmed cell death is essential in the development, morphogenesis, tissue alteration and immune regulation also being connected to several pathologies such as neurodegenerative disorders (for refs see Marques-Aleixo et al. 2012). Apoptosis is a mechanism characterized by cell contraction, chromatin

condensation, DNA disintegration, protrusions of the plasma membrane and apoptotic bodies formation (Fiers et al. 1999). Skulachev et al. (2000) suggested that apoptotic process appears to be preceded by changes in mitochondrial membranes and mediated by ROS. Thus, as previously mentioned, the antioxidants have the capacity to protect and prevent the negative consequences caused oxidative stress. Additionally, increasing evidences provide support that oxidative stress and apoptosis are closely linked physiological phenomena and are implicated in the pathophysiology of some of chronic diseases, including Alzheimer's and Parkinson's as well as ischemia of heart and brain (Calabrese et al. 2000).

In fact, inappropriate apoptotic responses are implicated in several neurodegenerative conditions, and there are some reports showing that physical exercise affords protection against deleterious stimuli and/or pathophysiological conditions (Sim et al. 2004). A study with trained animals during 21 days showed that endurance training prevented increase in Bax levels caused by chronic stress on cortical mitochondria, suggesting that exercise inhibit Bax translocation to mitochondria with resultant decrease in its proapoptotic activity (Haack et al. 2008). On the other hand, Um et al. (2008) reported a decrease of several proapoptotic proteins, including caspases 9 and 3 as well as an increased Bcl-2 protein after an exercise training program.

Therefore, and according to Murer et al. (2001), physical exercise can possibly alter the mitochondrial efficiency and apoptotic signaling with preventive and/or therapeutic implications in some pathological conditions.

2.2.1.3. Biogenesis and Dynamic

There is increasing evidence suggesting a crucial role of mitochondrial dysfunction in the aging process and in a large number of brain diseases (Chaturvedi and Flint Beal 2013). Deleterious alterations in mitochondrial biogenesis and dynamics could be negatively associated with mitochondrial function, contributing to an impaired brain function. However, stimulation of

mitochondrial biogenesis could be a compensatory adaptation against excessive fission and degradation mechanisms that characterize most of neurodegenerative pathologies (Knott et al. 2008).

It is well established that exercise training induces not only an increase in muscle mitochondria, but also in brain mitochondria (Holloszy 1967). However, little research has focused on the brain mitochondrial response to physical activity and/or exercise training. Given the important role of mitochondrial dysfunction in some pathological disorders and neurodegenerative conditions, the implications for exercise induced increase in brain mitochondrial biogenesis is potentially large (Steiner et al. 2011). Mitochondrial biogenesis can be promoted via an increase in transcriptional coactivators of Silent Information Regulator T1 (SIRT1), which interacts with deacetylates and activates the peroxisome proliferator-activated receptor- coactivator 1-alpha (PGC-1α) (Cotman et al. 2007). PGC-1α is considered the “master regulator” of mitochondrial biogenesis, antioxidant defense and was also associated with neuroprotection (Puigserver and Spiegelman 2003, Wareski et al. 2009). Increasing in rodent brains PGC-1α seem to be related to increases in exercise tolerance (Steiner et al. 2011). Same authors verified that brain adaptations to endurance training induced mitochondrial biogenesis through PGC-1α and SIRT1 mRNA overexpression and, simultaneously, increased mtDNA. Wareski et al. (2009) referred that PGC-1α overexpression in neuronal cells of the cortex, midbrain and cerebellum was related to increased mitochondrial density.

In addition to mitochondrial biogenesis, mitochondrial dynamics is equally important in brain structure and function. Neurons are also particularly sensitive to changes in mitochondrial movement and distribution (for refs see Marques-Aleixo et al. 2012). Mitochondrial dynamics is regulated by fission and fusion mechanisms, which are regulated by fission and fusion-related proteins, respectively. Fission-related proteins include dynamin-related protein 1 (Drp1) and fission 1 (Fis1), while mitofusins (Mfn1/2) and optic atrophy type 1 (Opa1) operate as fusion proteins. These fission/fusion-associated proteins are present in the neurons and are able to promote fast alterations in mitochondrial dynamics that can be significant in context of mitochondrial metabolism

(Nakamura et al. 2010). Additionally, the unbalance of mitochondrial dynamics in neurons could also contribute to the interference to Ca^{2+} homeostasis (Knott and Bossy-Wetzel 2008), mitochondria depolarization, translocation of proapoptotic mediators to mitochondria and to the release of cytochrome c leading to apoptosis (Yuan et al. 2007). Also, brain mitochondrial dysfunction can result from negative alterations in the fission-fusion machinery concomitant with neurodegenerative disorders (Chan 2006). Chen et al. (2007) reported that excessive fragmented mitochondria have been associated with inhibition of cell growth, a decrease in mitochondrial respiration and loss of mitochondrial membrane potential.

Twig et al. (2008) verified that increased fusion and decreased levels of fission inhibit mitophagy, consequently establishing a relationship between mitochondrial dynamics and mitophagy. Mitophagy is a selective autophagy of mitochondria (Lemasters 2005) and could be triggered by the alteration of K^+/H^+ activity and failure of cation homeostasis, the damage of oxidative phosphorylation and enhanced ROS system (Twig et al. 2008). Finally, dysfunction in mitochondrial dynamics, negative changes in ROS production and antioxidant capacity, disruption of Ca^{2+} homeostasis and mtDNA mutations are interrelated and can have deleterious consequences in the aging process or neurodegenerative disorders (Chan 2006, Knott and Bossy-Wetzel 2008). Despite the relevance of mitochondrial dynamic in brain mitochondrial health and on the pathogenesis of some neurodegenerative disorders, and the previous described role of physical exercise as a possible strategy for brain mitochondria protection, to our knowledge, only one study observed an increased expression of Mfn1 and Mfn2 in trained rats after 6-weeks of training (Liu and Zhou 2012).

2.3. Physical exercise: implications for brain health

2.3.1. Aging and neurodegenerative diseases – role of mitochondria, oxidative damage and apoptotic cell death

The aging process refers to a series of time-dependent changes at molecular and cellular levels leading to several characteristic features that compromise the functional fitness of the organism (Chakrabarti et al. 2011). Aging is associated with a general decline of physiological functions with a more marked effect on those that depend on central nervous system, such as behavior and cognitive performances (Navarro et al. 2004). Oxidative stress, mitochondrial dysfunction, inflammatory response, changed cell signaling and gene expressions are some of the causes of brain aging that lead to morphological ant structural alterations in the brain along with metabolic deficits and cognitive decline (Chakrabarti et al. 2011). In fact, morphological dysfunction seems to be closely related to aging and to several neurodegenerative diseases (Moreira et al. 2010). Bishop et al. (2010) reported that the decrease of mitochondrial function is related to shorter lifespan and may contribute to brain aging and, enhancing neuronal vulnerability to age-related pathologies. The central nervous system is very susceptible to aging, since it is extremely metabolically active, uses a great amount of oxygen, has an elevated content of free fatty acids that can be simply damaged by free radicals, and has limited regenerative properties (Toescu and Verkhratsky 2003). The overproduction of mitochondrial ROS could be a decisive contributor to brain senescence and neurodegeneration (Boveris and Navarro 2008a). In fact, progressive accumulation of ROS could compromise brain cell structure and its components, particularly mitochondria structure and function, and trigger apoptotic pathways that can result in neuronal death. The “free-radical theory of aging” sustains that critical cellular components are under regular attack by free radicals, contributing to age-related functional declines seen in normal aging, as well as in degenerative diseases (Harman 1956). However, this is a controversial topic, with some studies reporting higher activities of the

antioxidant enzymes, like superoxide dismutase (SOD) and catalase (CAT) in older animals, and others a decreased in SOD activity in older animals (Ochoa et al. 2011) and no differences in CAT activity between older and young rats (Meng et al. 2007). Accordingly, Ochoa et al. (2011) suggest that compensatory mechanisms of the redox systems in brain mitochondria might occur in order to counteract the enhanced oxidative stress associated with aging. When the production of ROS is significantly high, the endogenous antioxidant capacity became deficient increasing brain susceptibility (for refs see Marques-Aleixo et al. 2012). In fact, signs of oxidative damage seems to be enhanced in neurodegenerative disorders, and are normally associated by decreased levels of antioxidants (Gilmer et al. 2010a).

Mitochondrial DNA (mtDNA) is particularly vulnerable to high levels of oxidative stress suggesting that the accumulation of mtDNA mutations may play an important role in aging and in neurodegenerative diseases (Yang et al. 2008). As previously referred, damaged ETC components could contribute to mitochondrial bioenergetic deficiency resulting in elevated levels of ROS production (Ames et al. 1993). Additionally, Correia et al. (2010) suggested that mitochondrial oxidative stress and mtDNA injure also contribute to neuronal cell degradation and death through the improved susceptibility mPTP.

Regular physical exercise seems to retard the accumulation of cell damage and physiological dysfunction that is characteristic of the aging process and neurodegeneration (Navarro et al. 2004). Some studies suggested that implementing continuous exercise programs for individuals in the early stages of Parkinson´s disease has resulted in improved daily activity, motor performance and overall functional independency (Comella et al. 1994, Lau et al. 2011). Lau et al. (2011) examined the effects of treadmill exercise on movement and balance coordination, changes in dopamine neuron biomarkers, mitochondrial functions, and neurotrophic factor activities in mouse model of Parkinson´s disease with moderate neurodegeneration. These authors concluded that exercise produces neuronal and mitochondrial protection, suggesting that increased exercise activity would be a non-pharmachological neuroprotective approach for mitigating neurodegenerative diseases such as

Parkinson. Therefore, lesions on Substantia Nigra pars compacta found in mouse model of Parkinson´s disease were decreased when these animals were submitted to a chronic treadmill exercise (Lau et al. 2011). Additionally, evidences suggest that voluntary physical exercise decreases both cortical and hippocampal ß-amyloid peptide levels in a mouse model of Alzheimer´s disease (Um et al. 2008).

2.3.2. Traumatic brain injury

As previously mentioned, there is a large amount of experimental evidence that established an age-related decline in mitochondrial function (Navarro and Boveris 2004, Navarro et al. 2002). Mitochondria appear to have an increased susceptibility to perturbation with age, suggesting that the increased mitochondrial dysfunction observed following brain injury may impede recovery in aged animals (Gilmer et al. 2010b).

Traumatic brain injury (TBI) is defined by the Center for Disease Control as a blow, bump or penetrating injury to the head that disrupts the function of the brain. Experimental focal TBI results in a fast and important loss of neurons in the tissue instantly underneath the site of the impact. TBI consists of a primary insult resulting from the biomechanical forces directly damaging neuronal tissue and the depth of the insult is directly proportional to extent of neuronal tissue loss (Gilmer et al. 2009). Several of these injuries happened in young adulthood and result in significant injury on cognitive, motor, emotional functioning, main and persisting deficits to executive functioning, attention, memory, and speed of processing compromise psychosocial functioning and quality of life (Hawthorne et al. 2009). Imaging studies have also shown evidence of TBI-induced deterioration, including decreased cerebral blood flow, decreases in whole brain volume, lesion expansion, atrophy of discrete gray and white matter structures including the hippocampus, and reduced white matter integrity (Frasca et al. 2013). Nevertheless, extensive tissue loss is partially due to the disruption of mitochondrial respiration occurring fast after the insult (Xiong et al. 1997). Associated with this insult there is a large influx of cytosolic calcium that

overloads mitochondria, which results in metabolic dysfunction and increased in oxidative damage (Gilmer et al. 2010b). Same authors referred that neuronal survival is intimately related to mitochondrial homeostasis, owing to the fact that mitochondria supply the centre nervous system with a majority of ATP and regulate Ca^{2+} within the cell. As results of these two functions, mitochondrial usually operate close to their physiological peaks and are very susceptible to cellular perturbations (Fineman et al. 1993).

Several therapeutic interventions that aim stabilizing mitochondria have shown promising results by reducing overall neuronal tissue damage such as enhancing neurological outcome following TBI (Xiong et al. 1997, 2005). Finsterer et al. (2008) reviewed many drugs that might be utilized to improve aspects of mitochondrial bioenergetics in several respiratory chain diseases, some of which may be effective in TBI as well. Significant early respiration changes in mitochondrial bioenergetics may represent part of the primary injury difficult to mitigate through to pharmacologic intervention (Gilmer et al. 2010b).

Gilmer et al. (2009) performed a detailed analysis of mitochondrial bioenergetics following mild, moderate and severe injuries. These authors reported that damage to mitochondria could occur at several locations in the molecular machinery, which could be guilty for declines in respiration. Some of the possible location of damage are transporter proteins responsible for importing substrates inside mitochondria; enzymes needed to initiated the Kreb´s cycle such as pyruvate dehydrogenase; several complexes of the ETC used for production of proton motive force; or the ANT that exchanges ADP for ATP across the inner mitochondrial membrane (Gilmer et al. 2009). In fact, functional, biochemical and structural changes that occur within the mitochondria following TBI, which suggest that therapeutic intervention aimed at assisting mitochondria, need to be initiated early for possible neuroprotection (Gilmer et al. 2010b).

As a large majority of people with TBI are young and likely to survive into older age, it seems to be important find many possible ways to counteract the TBI insults that are characterized by set of biochemical cascades. To our

knowledge, there are no reports targeting mitochondrial adaptations to physical exercise as a possible strategy against TBI injuries. However, it is important to highlight that as both exercise and TBI interfere with brain mitochondrial function by different mechanisms, it is reasonable to hypothesize that these organelles may be central to explain the physiological outcome of the combination.

Indeed, people with TBI frequently present a sedentary lifestyle, require endurance and have a decline in peak aerobic capacity compared to health sedentary persons (Bhambhani et al. 2003). Increased physical activity and exercise training improves cardiorespiratory fitness in some populations with physical and cognitive impairments. Consequently, increasing the endurance and cardiorespiratory fitness of people with TBI would seem to have important preventing and treatment-related implications (Mossberg et al. 2010). The same authors reported that assessing endurance capacity and cardiorespiratory fitness early in the TBI rehabilitation is fundamental and also providing effective and available training modalities appear to be an imperative consideration for persons with TBI. On the other hand, while the relationship of physical exercise and fatigue following a TBI has not been extensively studied, fatigue is frequently reported by persons who have incurred a TBI.

Given the degree of cognitive, motor and behavioral impairments that affect TBI population, enhancing aerobic fitness may be demanding. It seems to be positive improving physical endurance and/or metabolic capacity in patients with a TBI (Bhambhani et al. 2003).

3. AIM

The aim of the present study was to analyze the effect of treadmill endurance training and free wheel voluntary physical activity on animal behavioral and brain mitochondrial bioenergetics, giving particular emphasis to the permeability transition pore opening susceptibility and to oxidative damage markers.

We can define as specific purposes of this work, to study the effects of two chronic exercise (TM and FW) models:

- On animal behavioral Y-Maze and Open field tests;
- On end-points associated with brain mitochondrial oxygen consumption;
- On the fluctuations of brain mitochondrial transmembrane electrical potential;
- On the susceptibility to calcium-induced brain mitochondrial permeability transition pore opening;
- On brain mitochondrial oxidative damage markers.

4. MATERIALS AND METHODS

4.1 Reagents

Deionized water (18.7 MΩ) from an arium®611VF system (Sartorius, Göttingen, Deutschland) was used. All other chemicals were purchased from Sigma Aldrich (Sintra, Portugal).

4.2 Animals

All experiments involving animals were conducted in accordance with the European Convention for the Protection of Vertebrate Animal Used for Experimental and Other Scientific Purposes (CETS no. 123 of 18 march 1986 and 2005 revision) and the Commission Recommendation of 18 June 2007 on guidelines for the accommodation and care of animals used for experimental and other scientific purposes (C (2007) 2525). The authors are accredited by the Federation of Laboratory Animal Science Associations (FELASA) for animal experimentation. Eighteen Sprague-Dawley male rats (aged 3 weeks) were randomly divided into three groups (n = 6 per group): sedentary (SED), treadmill endurance training (TM), free wheel voluntary physical activity (FW). Only male rats were used at this point to avoid hormone-dependent alterations in drug mitochondrial function/toxicity. During the experimental protocol, animals were housed in collective cages (two rats per cage) and were maintained in a room at normal atmosphere (21–22◦C; 50–60% humidity), receiving food (Scientific Animal Food and Engineering, A04) and water ad libitum in 12-h light/dark cycles.

4.3. Exercise protocols

The animals from SED groups were not exercised but were placed on a non-moving treadmill or in a locked free wheel five times per week (10–30 min/session), with the purpose of habituate animals to the possible environment stress induced by treadmill without promoting any physical training adaptations.

4.3.1. Endurance training protocol

The animals from TM group were exercised 5 days/week (Monday–Friday) in the morning (between 10:00 and 12:00 A.M.), for 12 weeks on a LE8700 motor driven treadmill (Panlab, Harvard, U.S.A). The treadmill speed was gradually increased over the course of the 12-week training period. The protocol included 5 days of habituation to the treadmill with 10 min of running at 15 m/min, with daily increases of 5 -10 min until 30 min was achieved (week 0). Habituation was followed by one consecutive week of continuous running (30 min/day) at 15 m/min and was gradually increased until 60 min/day on the week 1. The velocity increased gradually from 18 m/min to 30 m/min (Table 4).

Table 4. TM exercise protocol

Week		**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
TM (m/min)	**Velocity**	**15**	**18**	**20**	**22**	**24**	**25**	**25**	**27**	**27**	**28**	**28**	**30**	**30**
Exercise (min/day)	**Duration**	**30**	**60**	**60**	**60**	**60**	**60**	**60**	**60**	**60**	**60**	**60**	**60**	**60**

4.3.2. Voluntary physical activity

The animals from FW group were housed in a polyethylene cage equipped with a running wheel (perimeter=1,05m, Type 304 Stainless steel (2154F0106-1284L0106) Technicplast, Casale Litta, Italy).The rats were allowed to exercise *ad libitum* with an unlimited access to the running wheel 24h/day. Running distance was recorded using ECO 701 Hengstler (Lancashire, U.K.).

4.4. Behavioral tests

All behavioral tests were videotaped and analyzed off-line by an experimented researcher under blind conditions.

4.4.1 Y-maze

To measure spontaneous alternation of behavior and exploratory activity, a **Y-maze**, with arms 41 cm (long) by 12 cm (wide) with 14 cm walls was used. Each animal received one trial, in the course of which the animal were placed into one of the three alleys and allowed free exploration of the maze for 5 min. Alternations and total number of arm choices was recorded and movements were tracked using a digital camera (Sony® DCR-HC42E). Spontaneous alternation, expressed as a percentage, refers to that proportion of arm choices differing from the previous two choices. Thus, if an animal made the following sequence of arm choices (3,2,1,2,3,2,1,3), the total number of alternation opportunities would be six (total entries minus two) and the percentage alternation would be 67% (four of six) (King and Arendash 2002).

4.4.2 Open field

For **open-field** testing of activity and exploratory behavior, an open black box (70 X 70 cm) with 28.5 cm walls was used. The box floor was painted with lines to demarcate 16 squares (17,5 X 17,5 cm each). For the single trial, each

animal was admitted to the center of the enclosure and permitted to explore the interior for 5 min and movements were tracked using a digital camera (Sony® DCR-HC42E). For distance-covered analysis, a kinematic analysis was done for each animal using APASystem (Ariel Dynamics Inc., USA), digitizing the nose tip manually and frame by frame at a frequency of 50Hz. After the dimensional reconstruction using DLT procedure (Abdel-Aziz et al. 1971) a low pass filter of 5Hz was used (Winter et al. 1990). The total number of line crossings, central entries, rearings and grooming were analyzed (King and Arendash 2002). Additionally, the activity time, the time spent into the center of the box, the rearing and grooming duration were also recorded.

4.5 Animal sacrifice, heart and *soleus* extraction

Forty-eight hours after the last TM exercise session, non-fasted rats were euthanized by cervical dislocation between 9:00 and 10:00 AM to eliminate possible effects due to diurnal variation. After quickly opening the chest cavity, rat hearts were weighed. Right *soleus* muscle was also rapidly extracted and weighed. Portions of approximately 50 mg of one soleus muscle were separated, homogenized in homogenization buffer (200 mM Tris, 137 mM NaCl, 0.2 mM EDTA, 0.5 mM EGTA, 1% triton X-100, tissue: buffer ratio of 100 mg/mL, pH 7.4) using a Teflon pestle on a motor driven Potter-Elvehjem glass homogenizer at 0–4°C three to five times for 5 s at speed low setting, with a final burst at a higher speed setting. Homogenates were centrifuged (2 min at 3000 × g, 4°C, in order to eliminate cellular debris) and the resulting supernatants were stored at −80°C for later determinations, as detailed bellow. Protein content from *soleus* homogenates were spectrophotometrically determined by using the biuret method using bovine serum albumin as standard (Gornall et al. 1949).

4.6 Brain mitochondria isolation

Briefly, after animal decapitation, the whole brain minus the cerebellum was rapidly removed, washed, and carefully homogenized with a tightly fitted Potter–Elvehjem homogenizer and a Teflon pestle at 4°C in 10 mL of isolation medium (225 mM mannitol, 75 mM sucrose, 5 mM Hepes, 1 mM EGTA and 0.1% bovine serum albumin BSA, pH 7.4.) containing 5 mg of bacterial subtilisin protease type VIII. Single brain homogenates were brought to 30 mL and then centrifuged at 750 × g for 5 min. The resulting pellet was removed and the supernatant suspension centrifuged at 12,000 × g for 10 min. The pellet, including the fluffy synaptosomal layer, was re-suspended in 10 mL of the isolation medium containing 0.02% digitonin and centrifuged at 12,000 × g for 10 min. The brown mitochondrial pellet minus the synaptosomal layer was re-suspended again in 10 mL of isolation medium and centrifuged at 12,000 × g for 5 min. The pellet was re-suspended in 10 mL of washing medium (225 mM mannitol, 75 mM sucrose, 5 mM Hepes, pH 7.4) and centrifuged at 12,000×g for 5 min. The final mitochondrial pellet was re-suspended in 150-200 µL of the washing medium. All mitochondrial isolation procedures were performed at 0-4ºC. Mitochondrial protein concentration was spectrophotometrically determined by using the biuret method using bovine serum albumin as standard (Gornall et al. 1949). An aliquot of brain mitochondrial suspension was saved for later measurements of oxidative damage markers, as detailed bellow. The remaining mitochondrial suspension was used within 3–4 h after the excision of the brain and was maintained on ice (0-4ºC) throughout this period. There was no significant alteration of the mitochondrial respiratory control ratio between the first and the last measurements.

4.7 Mitochondrial respiratory activity

Mitochondrial respiratory function was measured polarographically, at 30ºC using a Biological Oxygen Monitor System (Hansatech Instruments) and a Clarktype oxygen electrode (Hansatech DW1, Norfolk, UK). Reactions were

conducted in a 0.75 mL closed, thermostated and magnetically stirred glass chamber containing 0.8 mg/mL of mitochondrial protein in a respiration buffer containing 100 mM KCl, 100 mM sucrose, 10 μM EGTA, 2 mM KH_2PO_4, and 5 mM Hepes, pH 7.4. After 1-min equilibration period, mitochondrial respiration was initiated by adding glutamate/ malate (G/M) to a final concentration of 5 and 2.5 mM, respectively. State 3 respiration was determined after adding ADP (150 nmol); state 4 was measured as the rate of oxygen consumption after ADP phosphorylation. The RCR (state 3/state 4) and the ADP/O ratios, the number of nmol ADP phosphorylated by natom of oxygen consumed, were calculated according to Estabrook (1967).

4.8 Mitochondrial electric transmembrane potential

Mitochondrial electric transmembrane potential ($\Delta\psi$) was monitored indirectly based on the activity of the lipophilic cation tetraphenylphosphonium (TPP+) using a TPP^+ selective electrode prepared in our laboratory as previously described (Ascensao et al. 2011). Reactions were carried out in 1 mL of reaction buffer containing 100 mM KCl, 100 mM sucrose, 10 μM EGTA, 2 mM KH2PO4, and 5 mM Hepes, pH 7.4, supplemented with 3 μM TPP^+ and 0.8 mg/mL of mitochondrial protein. For the measurements of $\Delta\psi$ with complex I-linked substrates, energization was carried out with G/M (5 mM and 2.5 mM, respectively) and ADP phosphorylation was achieved by adding 150 nmol ADP. The lag phase, which reflects the time needed to phosphorylate the added ADP, was also measured during both experiments.

4.9 Mitochondrial calcium accumulation and mPTP induction

Mitochondrial calcium (Ca^{2+}) accumulation capacity was determined by adding small pulses of Ca^{2+} (45 nmol/mg per pulse) until mPTP opening was observed as an irreversible fall in $\Delta\psi$. The reaction mixture was continuously stirred and the temperature was maintained at 30ºC. The assays were performed in 1 mL

of reaction medium (200 mM sucrose, 10 mM Tris, 10 μM EGTA and 5 mM KH_2PO_4, pH 7.4) supplemented with 3 μM rotenone and 8 mM succinate with 0.8 mg of protein/mL. A negative control was performed with cyclosporine A (1μM) to inhibit mPTP (Broekemeier et al. 1989).

4.10 Mitochondrial oxidative damage

Before analysis, mitochondrial membranes were disrupted by several freeze–thawing cycles to allow free access to substrates. The extent of lipid peroxidation in brain mitochondria was determined by measuring MDA contents by colorimetric assay, according to a modified procedure described previously (Buege and Aust 1978). Suspended mitochondria were centrifuged at 12,000 × g for 10 min and re-suspended in 150 μL of a medium containing 175 mM KCl and 10 mM Tris–HCl, pH 7.4. Subsequently, the three experimental groups were mixed with 2 volumes of trichloroacetic acid (10%) and 2 volumes of thiobarbituric acid (1%). The mixtures were heated at 80–90ºC for 10 min and re-cooled in ice for 10 min before centrifugation (4000 × g for 10 min). The supernatants were collected and the absorbance measured at 535 nm. The amount of MDA content formed was expressed as nanomoles of MDA per milligram of protein (ε535=1.56 x 10−5 M−1.cm−1).

Mitochondrial content of oxidative modified SH groups, including GSH and other SH-containing proteins, was quantified by spectrophotometric measurement according to Hu (1990). Briefly, a mitochondrial suspension containing 5 mg/mL protein was mixed with 0.25 M Tris buffer pH 8.2 and 10 mM DTNB and the volume was adjusted to 1 mL with absolute methanol. Subsequently, the samples were incubated for 30 min in the dark at room temperature and centrifuged at 3000 × g for 10 min. The colorimetric assay of supernatant was performed at 414 nm against a blank test. Total SH content was expressed in nanomoles per milligrams of mitochondrial protein (ε414=13.6 mM−1.cm−1).

4.11 Soleus citrate synthase activity

Soleus CS activity was measured using the method proposed by Coore et al. (1971). The principle of assay was to initiate the reaction of acetil-CoA with oxaloacetate and link the release of CoA-SH to 5,5-dithiobis (2-nitrobenzoate) at 412 nm.

4.12 Statistical analysis

All data are expressed as the mean±SEM (Standard Error of the Mean). Statistical analyses were performed using GraphPad Prism (version 6.0). Two-way repeated-measures ANOVA for body weight and distance cover by exercised groups was used to verify the effect of exercise over time. For all other parameters, one-way analysis of variance ANOVA was used to compare differences between groups. To determine specific group differences, the one-way ANOVA were followed by Bonferroni post-hoc tests. In all cases, the significance level was set at $p \leq 0.05$.

5. RESULTS

5.1 Characterization of animals and exercise protocols

Body weight alterations and distances covered by the animals during the entire protocol are shown in Figure 1. No significant differences in the mean body weight of the animals from the beginning of the protocol were found, however both chronic exercise types, TM and FW decreased body weight from 11th and 7th week, respectively, until the end of the protocol (p≤0.05; TM and FW vs SED) (Figure 1A). No differences were found regarding food consumption (data non shown, p≤0.05) and water consumption was higher in FW group compared with SED group (data non shown, p≤0.05).

As can be seen in Figure 1B, animals from FW group ran significantly more than TM group from the 2nd week until the end of the protocol (p≤0.05, FW vs TM).

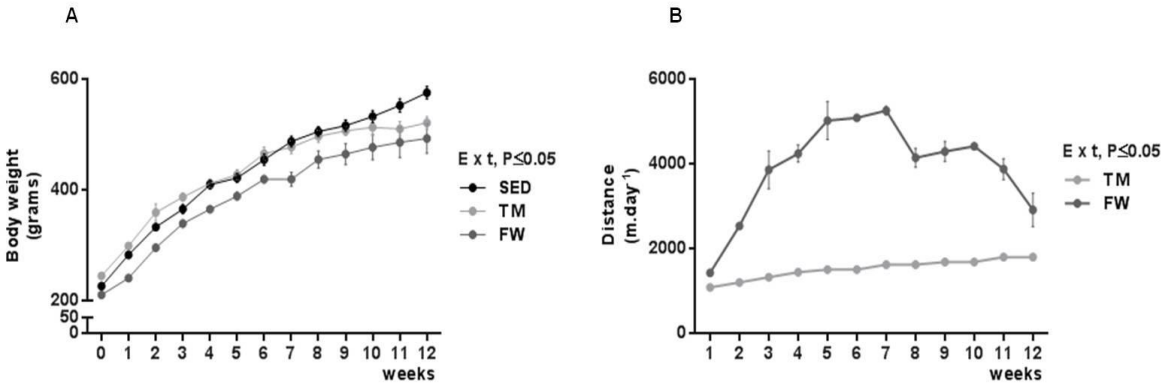

Figure 1. Effect of physical activity on body mass alterations over time (A) and distance run per day by treadmill and free wheel groups during the 12 wks of protocol (B). Significant differences (p≤0.05) are mentioned in the text. Significant (p≤0.05) effects of Exercise (E), time (t), or their interaction (E x t) are shown; Non Significant (NS, P>0.05).

Body, heart and brain absolute weights, heart and brain weight to body weight ratio, mitochondrial protein yielding as well as the activity of *soleus* citrate synthase in the three groups are shown in Table 5. Both chronic exercise types

decreased final body weight, increased heart weight and the heart and brain weight to body weight ratios (TM and FW vs SED). No significant differences between groups were observed regarding the initial body weight, brain weight and yield of mitochondria isolation. TM, but not FW, increased the activity of *soleus* citrate synthase (TM vs SED).

Table 5. Animal data and yield of mitochondrial protein isolation

	SED	TM	FW
Initial body weight (g)	207±3.91[a]	214±3.76[a]	211±1.55[a]
Final body weight (g)	598±10.58[a]	522±9.87[b]	498±5.98[b]
Heart weight (g)	1.44±0.03[a]	1.92±0.08[b]	1.85±0.10[b]
Heart weight/body weight ($mg.g^{-1}$)	2.32±0.08[a]	3.53±0.10[b]	3.68±0.12[b]
Brain weight (g)	1.28±0.04[a]	1.30±0.04[a]	1.31±0.02[a]
Brain weight/body weight ($mg.g^{-1}$)	2.13±0.05[a]	2.48±0.06[b]	2.49±0.09[b]
Brain mitochondrial yielding (mg protein/g tissue)	22.74±3.82[a]	24.76±2.86[a]	23.57±4.96[a]
Soleus citrate synthase activity ($mM.\ min^{-1}.mg^{-1}$)	10.94±2.07[a]	23.34±1.79[b]	11.22±2.65[a]

Values (mean ± SEM). Different letters are significantly different ($p \leq 0.05$).

5.2 Behavioral tests

5.2.1 Y-maze

To study alterations in working memory induced by both types of chronic physical activity, animals from SED, TM and FW groups were tested for spontaneous alternation in the Y maze (Figure 2). As it can be observed, TM significantly increased number of total entries and alteration percentage (TM vs SED).

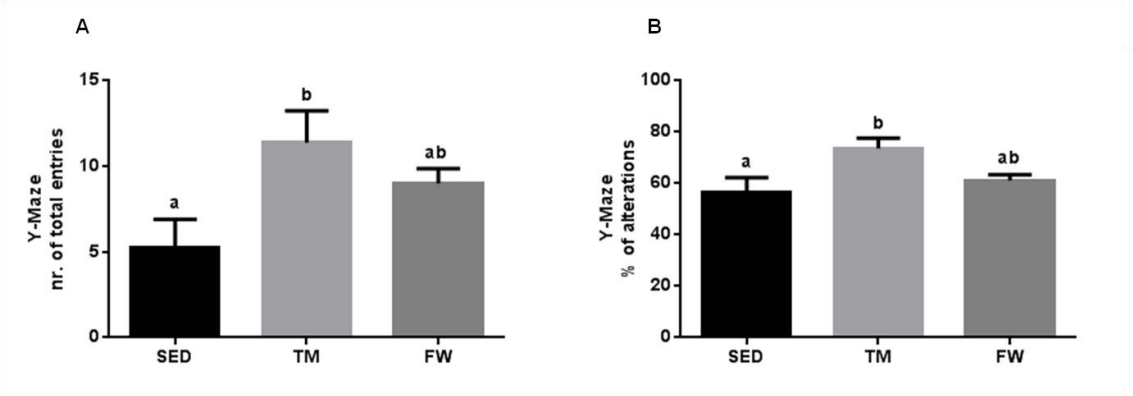

Figure 2. Effect of exercise on Y-maze behavior, number of total entries (A) and % of alterations (B). Data are means±SEM for each experimental group. Experimental details are provided in methods. Different letters are significantly different (P≤0.05).

5.2.2 Open Field

The alterations on general activity and exploratory behavior in an open field induced by both types of chronic physical activity were analyzed (Figure 3). As shown, the TM exercise increased % activity time, the locomotive distance traveled, the number of lines crossed, the number of central entries and rearings (TM vs SED). No significant alterations were observed on grooming frequency between groups.

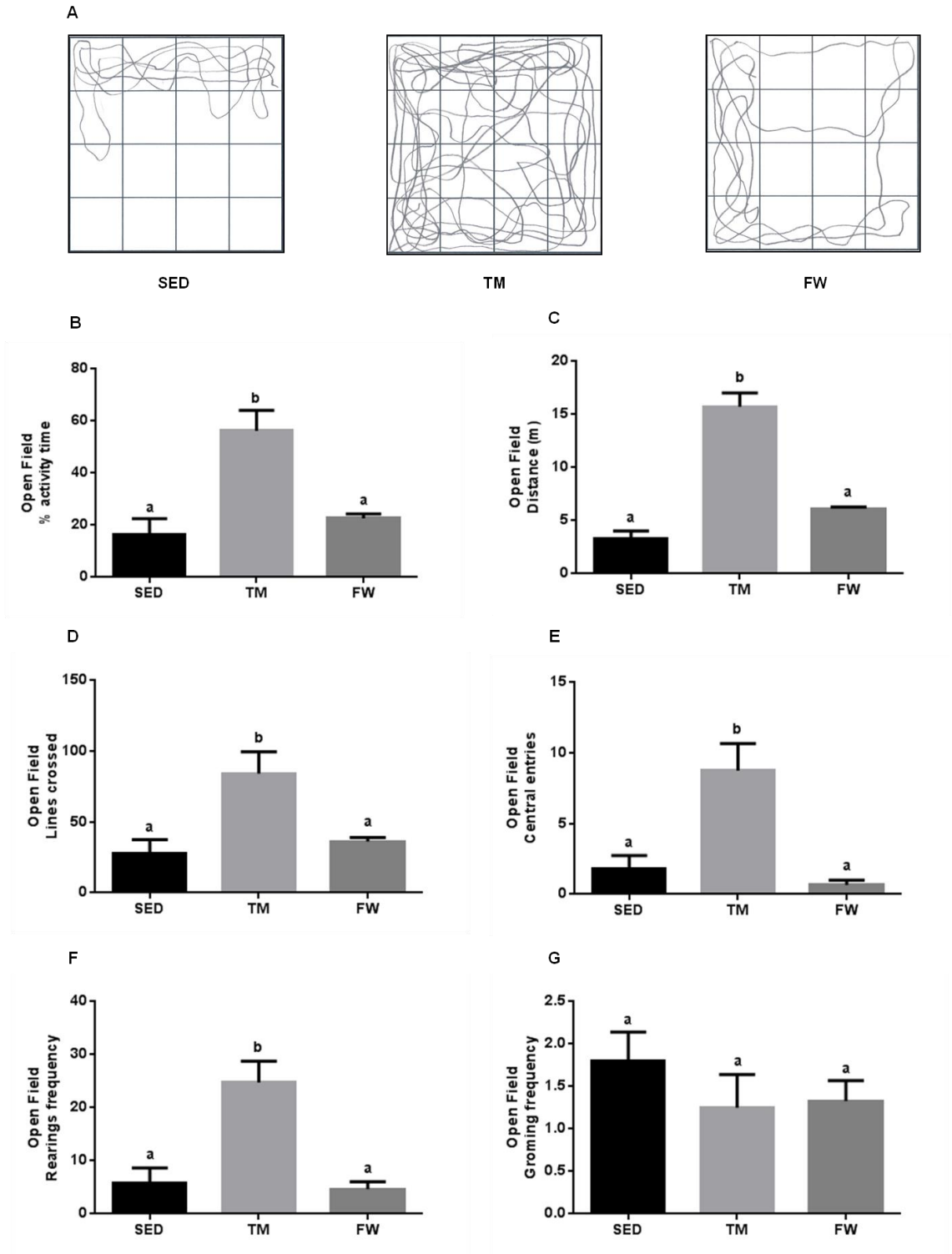

Figure 3. Effect of exercise on Open Field behavior; illustrative example of a SED, TM and FW animal travel pathway in 5 min exploration (A), % activity time (B), locomotive distance traveled (C), number of lines crossed (D), number of central entries (E), number of rearings and grooming performed (F and G). Data are means±SEM for each experimental group. Experimental details are provided in methods. Different letters are significantly different (P≤0.05).

5.3 Brain mitochondrial oxygen consumption and transmembranar electric potential

Mitochondrial respiratory activity was measured to identify exercise-dependent effects (Figure 4). Similarly, variations in maximal ∆ψ and during ADP phosphorylation cycle (Figure 5) were determined using G/M as substrates.

As it can be observed in Figure 4, TM and FW exercise increased State 3 respiration, the coupling between oxygen consumption and ADP phosphorylation (RCR) and the efficiency of ATP synthesis (ADP/O) (TM and FW vs. SED).

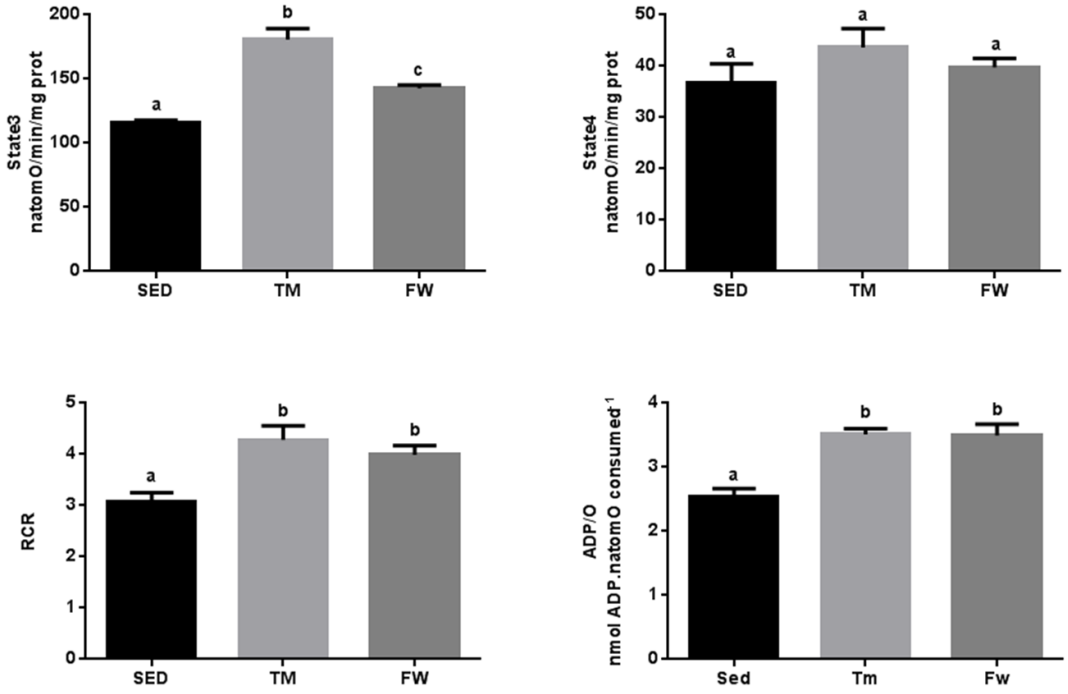

Figure 4. Effect of exercise on brain mitochondrial respiration. Data are means±SEM for brain mitochondria (0.8 mg/mL protein) obtained from different mitochondrial preparations for each experimental group. Oxidative phosphorylation was measured polarographically at 30ºC in a total volume of 0.75 mL. Respiration medium and other experimental details are provided in methods. RCR, respiratory control ratio (state 3/state 4); ADP/O, number of nmol ADP phosphorylated by atom of O_2 consumed. Different letters are significantly different (P≤0.05).

Although no alterations were observed regarding the maximal ∆ψ and ADP depolarization and repolarization between groups, TM decreased the ADP lag phase (TM vs. SED) (Figure 5).

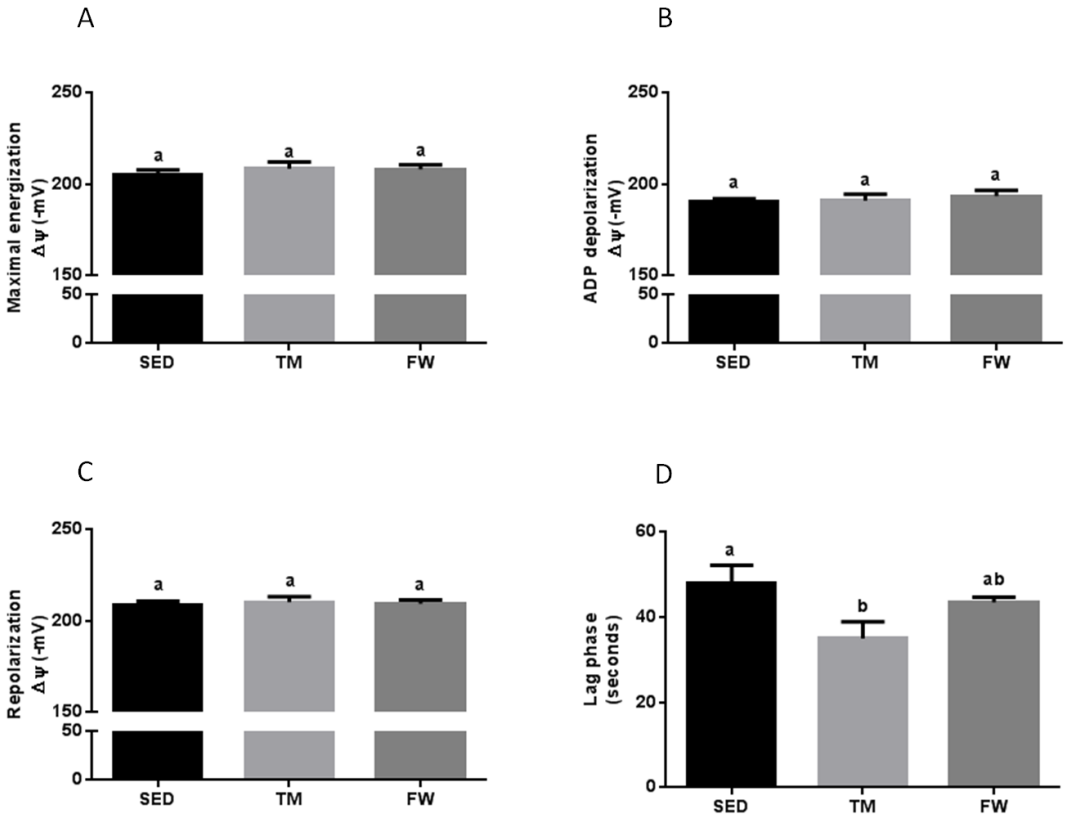

Figure 5. Effect of exercise on brain mitochondria ∆ψ fluctuations (maximal energization, ADP-induced depolarization, and repolarization) and ADP phosphorylation lag phase. Data are mean±SEM for brain mitochondria (0.8 mg/mL protein) obtained from different mitochondrial preparations for each experimental group. Figure shows the average response of maximal mitochondrial membrane potential developed with glutamate (5 mM) plus malate (2.5 mM), the decrease in membrane potential after ADP addition (depolarization), the repolarization value after ADP phosphorylation, and the lag phase. Mitochondrial transmembrane potential was measured using a TPP^+-selective electrode at 30ºC in a total volume of 1 mL. Reaction medium and other experimental details are provided in methods. Different letters are significantly different (p≤0.05).

5.4 Calcium induced mitochondrial permeability transition

We next questioned whether exercise altered the amount of calcium needed to induce the mPTP. Figure 6 shows the maximum amount of brain mitochondrial

Ca^{2+} accumulated until mPTP opening occurred in SED and in TM and FW groups. As shown, the TM group accumulated significantly more Ca^{2+} before mPTP induction than the SED group (TM vs. SED). A negative control using cyclosporine A to inhibit mPTP induction by Ca^{2+} was performed.

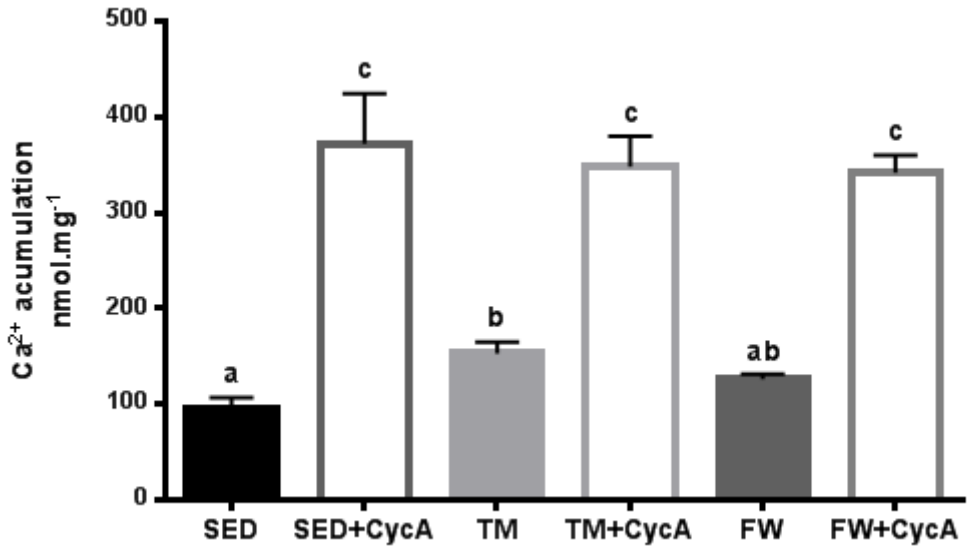

Figure 6. Effects of chronic physical activity on brain mitochondria calcium-induced membrane depolarization. Data are means± SEM for brain mitochondria (0.8 mg protein/mL) obtained from different mitochondrial preparations in each experimental group. The figure shows the amount of calcium accumulated before the irreversible fall and the respective negative control using Cyclosporin A (1 µM) to inhibit mPTP induction by Ca^{2+} measured in succinate energized mitochondria and using TPP^{+} selective electrode at 30 ºC in a total volume of 1 mL. Different letters are significantly different ($p \leq 0.05$).

5.5 Brain mitochondrial oxidative damage markers

It was also analyzed the effect of the TM and FW on the extent of brain mitochondrial oxidative damage. Figure 7 shows brain mitochondrial -SH content (A) and MDA levels (B) on brain mitochondrial in SED and in both types of exercised animals. As shown, no significant alterations were observed on mitochondrial -SH content or MDA levels within groups.

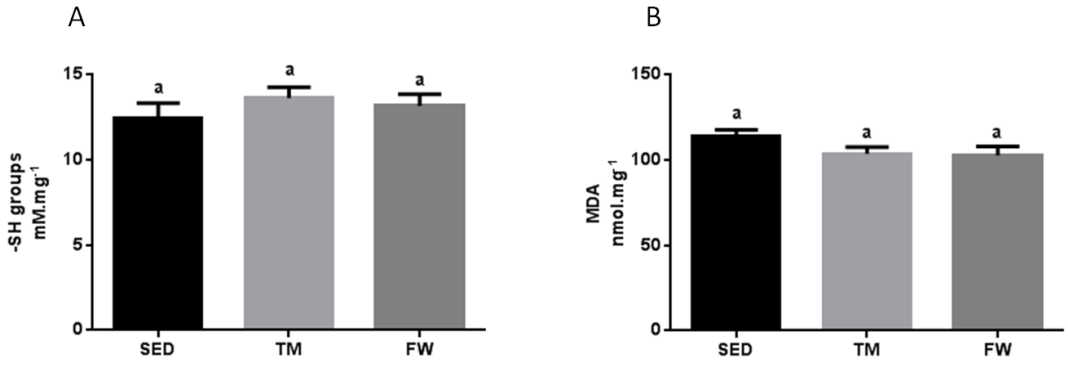

Figure 7. Effects of chronic physical activity on brain mitochondrial reduced sulfhydryl groups (A) and MDA contents (B). Data are means±SEM for brain mitochondria obtained from different mitochondrial preparations for each experimental group. Different letters are significantly different (p≤0.05).

6. DISCUSSION

6.1 Animals and exercise protocol

Several external factors, aging and neurodegenerative diseases can compromise some cognitive functions through progressive bioenergetic alterations or impairments (Pereira et al. 2012). In this regard, mitochondria appear in the center of focus due to their critical metabolic functions in cells and especially in a high energy-expensive organ such as brain. Considering the multiple mechanisms by which mitochondrial impairment can lead to dysfunction or even death of brain cells, many neuroprotective interventions have targeted mitochondria, including physical activity (Ding et al. 2006).

Based on the positive relationship between exercise and mitochondrial bioenergetic, not only in skeletal muscle but also in brain, suggested by several authors (Bayod et al. 2011, Lanza and Sreekumaran Nair 2010, Steiner et al. 2011), we used isolated mitochondria as a model of tissue dysfunction and toxicity strategy in the present study.

Long-term treadmill exercise training in a chronic model of Parkinson disease protected against impaired mitochondrial function (Lau et al. 2011), and voluntary running combined with melatonine treatment exerted a synergistic effect in the protection of the mitochondrial functionality in 3xTg Alzheimer disease mice (Garcia-Mesa et al. 2012). These works suggested that exercise can play a significant role in brain mitochondrial machinery improvements and likely serves to attenuate mitochondrial dysfunction that characterizes aging and neurodegenerative diseases. Based on these observations, we examined whether both types of chronic exercise (treadmill and freewheel running) exert similar effects on brain mitochondria from young healthy rats. Interestingly, our results showed several positive alterations in behavioral and brain mitochondrial function induced by treadmill trained rats. FW group ran significantly more than those counterparts from TM group; however the TM animals showed a regular profile of adaptation in response to training. Thereby, although the intensity of voluntary exercise (FW) may be lower than that of typical treadmill-based

exercise, the substantial duration of the exercise stimulus appears to be sufficient to also produce robust adaptive responses (Allen et al. 2001).

Our study sample included only male rats. We chose to exclude female rats since the expression and activity of mitochondrial antioxidant enzymes is higher in these animals than in male rats (Borras et al. 2003). Similarly, several authors have reported the existence of an interaction between hormonal status and mitochondrial production of ROS (Gigli and Bussmann 2001, Vina et al. 2005). Consequently, in order to control the possible effect of the estrogen hormone in this study, only resorted to male rats.

6.2. Exercise effects on cerebral, cardiac, body weights and citrate synthase activity

Physical exercise is associated with several physiological adaptations in both muscle, heart and brain (Moore 1998, Radak et al. 2001a, Salmons and Henriksson 1981). These adaptations result in a delivery, uptake and more efficient use of energy substrates required to produce and sustain physical activity. Both the treadmill training and voluntary free wheel running protocols used in our study (12wks/5days/wk with progressively increase in time and intensity and free physical activity with an unlimited access to the running wheel 24h/day, respectively) resulted in a set of adaptations related to body and heart weight and heart body weight ratio (Table 5). It is consistently shown in the literature that these are classic adaptations induced by exercise training in rats (Arcos et al. 1968, Baldwin et al. 1977, Baldwin et al. 1975, Hickson et al. 1983, Kingwell et al. 1998). In fact, body and heart weight and heart body weight ratio alterations observed in our study are similar to those related to treadmill chronic exercise found in previous studies from our group (Ascensao et al. 2006, Lumini-Oliveira et al. 2009). Studies on voluntary endurance exercise are less numerous; however seem to confirm most of our results related to body weight and heart weight adaptations. Allen et al. (2001) observed a significant increase in absolute heart weight in 4 weeks of free wheel running. Our results

demonstrated that the consequences of treadmill training are comparable to those of free wheel running regarding the above mentioned evaluated parameters.

Although there are no studies reporting a significant increase on brain weight induced by physical exercise, some authors reported that regular exercise increased hippocampus size and volume, induced plastic changes in specific brain structures and increased cell proliferation (Chaddock et al. 2010a, Erickson et al. 2011, Pereira et al. 2007, Voss et al. 2012). In the present study, although no differences were observed regarding brain weight, there was a significant increase in ratio of brain weight to body weight in both type of exercised groups (Table 5), suggesting that physical exercise during 12 weeks is able to induce alterations in the relative size of the mentioned organs.

In addition, similarly to previous results published by our group (Ascensao et al. 2006), there was an increase in skeletal muscle citrate synthase activity in treadmill-exercised rats. This improved enzymatic activity in *soleus* muscle suggests that endurance training was an efficient chronic stimulus to ameliorate muscle oxidative metabolism. Moreover, the increased skeletal muscle oxidative capacity resulting from chronic exercise is associated with cardiac hypertrophy (Houle-Leroy et al. 2000). Interestingly, and corroborating other studies (Noble et al. 1999), FW group do not exhibited a significant increase in CS activity in the *soleus.*

Given the significant differences found in the experimental groups regarding body weight and the ratio of heart and brain weights to body weight, it seems that the duration, load and intensity of freewheel and treadmill exercise protocols was sufficient to induce appropriate organic and tissue responses. Thus, the differences in the parameters studied between both types of chronic exercised and sedentary groups are due to adaptations induced by physical exercise.

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6.3. Effects of physical exercise in behavioral tests

The regular exercise is not only associated with enhanced physical health but also with an improvement in cognitive function in both human and animal models (Chang et al. 2013, Middleton et al. 2008). The results obtained in behavioral Y-Maze test demonstrated that treadmill exercise increased the number of total entries and the percentage of alterations of the path taken (Figure 2). These results seem to suggest that physical exercise, mainly TM, promoted the improvement of memory and also of spatial memory (Erickson et al. 2011, Gomes da Silva et al. 2012). In accordance, a study conducted with adult rats suffering from 3 different levels of hippocampus-lesion suggested that learning and memory, evaluated through Y-Maze, increased immediately after the treadmill-exercise protocol (Chen et al. 2006). Van der Borght et al. (2007) also showed that 14-days of free wheel running exercise promotes increased memory acquisition, memory retention and reversal learning evaluated by the spatial Y-maze test. However, our results failed to evidence significant improvements of memory and spatial memory in the animals submitted to 12 weeks-FW exercise when compared with their SED counterparts. This suggests that different types of chronic exercise possibly result in different responses measured by the Y-maze performance.

The open-field test is usually interpreted as a measure of anxiety, exploratory time, and even general activity (Careau et al. 2012). Our results demonstrated that TM animals ameliorated performance in the open-field evaluated parameters in comparison with SED and also with FW animals (Figure 3). The activity time and distance traveled increased in TM animals, which suggests that treadmill training induced improvement in hyperactivity and locomotor activities. In contrast, Fulk et al. (2004) did not observe significant changes in total activity levels between runners and non-runners animals after 10 weeks for moderate exercise training. However, Burghardt et al. (2004) reported that chronic treadmill running produced behavioral changes in the open-field test, namely those related to enhanced defensive behaviors that are likely associated to adaptations in a variety of neurochemical systems.

Animals that are more anxious tend to spend a greater proportion of the test-time around the arena, in non-central areas (for refs see Swain et al. 2012). In accordance with other studies, our results demonstrated that TM animals revealed an increased in open-field lines crosses and central entries, suggesting that treadmill training increased locomotor behavior and decreased anxiety. Mello et al. (2008) demonstrated that rats subjected to 2 weeks of forced treadmill running exhibited significantly more locomotor behavior compared to inactive controls. However, our study failed to show differences between FW and SED groups related to line crossing and central entries, suggesting that voluntary exercise did not alter anxiety levels or locomotor activity. In contrast to our results, Salam et al. (2009) observed that animals submitted to 2 weeks of voluntary wheel running spent more time in the center of the open-field test, made more crossings between the quadrants of the open-field and made fewer escape attempts when compared to sedentary animals.
Animals from the TM group also showed significantly increased rearings compared to FW and SED animals. The improvement in this open-field test related parameter seems to be associated with spatial exploration and even motor coordination (Nascimento et al. 2011), which suggest that TM exercise, but not FW, improved these behavioral parameters. A study with sedentary diabetic and trained diabetic animals demonstrated that rearings are higher in exercised animals after 8 weeks of treadmill training (Nascimento et al. 2011) than in sedentary controls.

Finally, we measured the anxiety-related behavior by grooming frequency and no alterations were observed between groups. Pietrelli et al. (2012) investigated in rats the effect of different ages of aerobic exercise on cognitive function and anxiety-related behavior through grooming, and concluded that it is not affected either by age or exercise. In contrast, 8 weeks of treadmill exercise significantly increased anxiety measured by gromming frequency (Leasure and Jones 2008).

6.4. Effects of physical exercise on brain mitochondrial respiratory activity and membrane potential

The exercise training induces several physiological adaptations on mitochondrial machinery. Our next step was to analyze whether improved behavioral parameters caused by exercise were also observed at sub-celular level, particularly in mitochondria (Tonkonogi and Sahlin 2002). In order to confirm that physical exercise promotes improvements in brain mitochondrial function, we measured the respiratory activity associated with brain mitochondrial membrane potential and ADP-induced phosphorylation cycle (Figure 4 and Figure 5). Our data suggest that both types of chronic physical exercise induced beneficial effects on parameters associated with the functionality of the respiratory chain, specifically the increase in the RCR, state 3 and ADP/O, through the increased functionality of the phosphorylative system.

Benard and Rossignol (2008b) suggest that ADP is an important stimulator of oxidative phosphorilation and glycolytic pathways. Therefore, to understand the mitochondrial functionality and its oxidative phosphorylation capacity, it is important to analyze the respiratory indices, state 3, state 4, RCR and ADP/O (Figure 4). State 3 is a parameter associated with oxygen consumption after ADP addition, whereas state 4 is the measurement of oxygen consumption after the complete ADP phosphorylation (Estabrook 1967). Moreover, the respiratory control ratio (state3/ state4) is considered a respiratory parameter associated with the mitochondria structural integrity and functionality, and so is a measure of respiratory rate dependence of ATP synthesis (Tonkonogi et al. 2000). Additionally, another qualitative parameter of mitochondrial function evaluated was the ADP/O, which rely on the number of molecules of ATP produced by an atom of oxygen consumed (Estabrook 1967) and expresses the phosphorylation efficiency.

Nulton-Persson and Szweda (2001) proposed that the increased state 3 is associated with enhanced respiratory chain function through up-regulation of intrinsic oxido-reductases and/or increased of reduced equivalents and

consequently, increased electrons supplied to the ETC. This, associated with increased activity of protein complexes of ETC after chronic exercise, suggests that oxidative capacity increased with physical activity (Holloszy et al. 1970). In fact, Campos et al. (2012) reported that moderate exercise training (over 8 weeks) increased mitochondrial state 3 respiration and RCR in rats with heart failure. Lau et al. (2011) observed that 18 weeks of treadmill exercise with chronic parkinsonian mice increased state 3 respiration and RCR compared to sedentary mouse. These findings agree with the results of our study.

In addition, both types of exercise (FW and TM) increased ADP/O compared to SED group, which means that the exercised groups need less oxygen to phosphorylate the same amount of ADP compared to SED animals.

The improvements described above regarding state 3 respiration were accompanied by a reduction in lag phase, using malate and pyruvate as substrates. The lag phase reflects the ability of mitochondria to rapidly repolarize after ADP addition. The reduction in lag phase found in TM group suggest a decrease in the time required to restore the dissipated membrane potential after ADP addition, and consequently phosphorylation in brain mitochondria compared SED animals. To our knowledge, the effect of physical activity on this mitochondrial-associated parameter has never been measured in the brain.

The additional study of membrane potential appears to be essential for an integrated analysis of mitochondrial function, since it reflects the basic energy relations in maintaining cellular homeostasis. The electrochemical gradient due to the pumping of protons across the inner membrane is essential (Murphy and Brand 1988), among other things, for the phosphoriyation of ADP (Stock et al. 1999). According to the results of the present study, both exercise protocols did not affect the maximum $\Delta\psi$ using pyruvate+malate and neither fluctuations associated with phosphorylation cycle were observed.

6.5. Effects of physical exercise on calcium-induced brain mitochondrial permeability transition

Among other factors, mitochondrial dysfunction and the opening of the mitochondrial permeability transition pore (mPTP) induced by stress conditions, are intimately related. As mentioned before i) it has been described that mitochondria interfere with the dynamics of cellular Ca^{2+}, in particular the regulation of cytosolic Ca^{2+} levels, this being considered as the main driver for the opening of mPTP (Zoratti and Szabo 1995); ii) under conditions of oxidative stress and/or increased phosphate levels, one of the factors that contributes to the susceptibility of the mPTP opening seems to be Ca^{2+} overload in the matrix (Bernardi and Petronilli 1996, Tsujimoto and Shimizu 2007); iii) it is recognized that the mPTP opening is directly involved in cell death either by apoptosis or necrosis (Skulachev 2000), and iv) exercise has been recommended as an effective strategy to prevent and/or restore several mitochondrial dysfunctions in several tissues including some related mPTP (Lumini et al. 2008).

The mPTP is assembled from a group of preexisting proteins in the mitochondrial inner and outer membranes, with Ca^{2+} binding sites on the matrix side of the inner membrane believed to regulate this pore activity. Generally, the mPTP opening is due to mitochondrial Ca^{2+} overload and can result in pathological conditions, and ultimately to cell death (Brookes et al. 2004).

Although the importance of this topic, to our knowledge, the effects of physical exercise on the susceptibility to mPTP opening on brain mitochondria, has not been yet investigated. In the present study, it was determined the maximum amount of Ca^{2+} accumulated by brain mitochondria until mPTP opening. Results showed that brain mitochondria from TM group accumulated higher amounts of Ca^{2+} compared to SED, whereas no differences were found between FW and SED group (Figure 6).

The mPTP opening and the release of proapoptotic proteins have been associated with intrinsic mitochondrial-driven apoptotic pathways leading the activation of caspase cascades (Hengartner 2000). At this purpose, Siu et al.

(2004) reported a reduction of apoptotic markers on muscle mitochondria after exercise training during 8 weeks, suggesting a decrease in susceptibility of mPTP opening. The mPTP and Ca^{2+} overload are familiarly related with some alterations in respiratory mitochondria such as Δψ, mitochondrial production of ROS, antioxidants, pro-and anti-apoptotic levels, endogenous Ca^{2+} in mitochondrial matrix, and several other hypothetical component proteins that regulate the pore, VDAC, ANT, hexokinases, phosphate carrier and Cyp D (Adhihetty et al. 2008, Crompton et al. 1999, Halestrap and Brenner 2003). Lumini-Oliveira et al. (2011) analyzed the effects of endurance training (14 weeks of treadmill running) against cardiac mitochondrial dysfunction induced by streptozotocin. Endurance training reverted the hyperglycemia-induced CypD elevation and attenuating decrease of ANT and VDAC.

Although we did not asses measure the expression of these proteins mPTP regulation, the probable increased functionality of the phosphoryation system in general and of the ETC in particular, resulting from the 12 weeks of TM may had have some implications on the ability of Ca^{2+} uptake. Therefore, we can speculate that one of the possible reasons why trained mitochondria accumulate more Ca^{2+} might be related to the effect of these adaptations on mPTP protein machinery.

6.6. Effect of physical exercise on brain mitochondria oxidative stress markers

Oxidative damage has been associated with physiological dysfunction of the brain. Radak et al. (2001b) suggested that limb immobilization induces oxidative damage to the hippocampus, and this damage is associated with impairment of cognitive function. The same authors refer an inverse relationship between the decreased oxidative modification of proteins resulting from regular exercise and brain function (Radak et al. 2001a). Although ROS are a normal product of aerobic metabolism and at certain concentrations crucial activators of stress responses and gene expression for a wide range of proteins, ROS production

above the cell tolerance level, can induce significant oxidative damage to macromolecules ultimately compromising cell viability (for refs see Radak et al. (2007).

The electron transport associated with the mitochondrial respiratory chain is considered one of the major processes leading to ROS production at rest but also during physical exercise (Di Meo and Venditti 2001). Theoretically, during exercise, the increased electron flow through the mitochondrial ETC leads to an increased rate of ROS production. Thus, if mitochondrial production of ROS supplies a notable contribution to exercise-induced oxidative stress, mitochondria should be a primary target of oxidative damage (Venditti et al. 2007). ROS can cause damage to mitochondrial membranes and cytoplasmic structures through the oxidation of phospholipids, proteins, and nucleic acids, which compromises mitochondrial and brain function (Liu et al. 2000). However, similarly to other reports (for refs see Marques-Aleixo et al. 2012), our results showed no alterations on two hallmarks of oxidative damage to proteins and lipids, respectively sulfhydryl proteins content and malondialdehyde levels, on brain mitochondria. Data suggest that both types of exercise modulated ROS production and antioxidants systems in a way that did not translate into variations in -SH and MDA levels in brain mitochondria. Liu et al. (2000) referred that the brain utilizes 20% of the total oxygen consumed by the entire body at rest. The oxygen consumption rate increases during exercise; nevertheless, the brain oxygen consumption is known to be constant during exercise, suggesting that is unlikely that exercise causes increased oxidative stress to brain. However, several authors refer that exercise impacts brain mitochondrial function, including oxidative stress modulation (for refs see Marques-Aleixo et al. 2012).

In the present study, the levels of MDA in brain did not change after TM or FW exercise. The main outcomes on the training effects on brain lipid peroxidation are as follows: Coskun et al. (2005) reported that brain TBARS levels did not change after 6.5-week running exercise; Devi et al. (2004) demonstrated no significant change in hippocampal and cerebral cortex TBARS levels with 12-week swimming; Radak et al. (2001a) did not find any change in TBARS levels

after 9-week of regular swimming exercise. Navarro and Boveris (2004) found lower TBARS levels in mice brain after 24-week running exercise. Liu et al. (2000) demonstrated a significant decrease in brain homogenate and mitochondrial MDA levels after 8-week running exercise.

Thiols (-SH) have numerous functions, including a central role in coordinating the antioxidant defense network. Thiols, act as reducing agents, as electron acceptors, neutralizing ROS into a less toxic byproducts and getting oxidized to a disulfide (-S-S-). Therefore, sulfhydryl residues are highly susceptible to oxidative damage. Regarding the level of oxidativelly modified proteins, in our study, we did not find differences on sulphydryl groups, including glutathione (GSH) and other SH-containing proteins between SED, TM and FW groups. Somani et al. (1995) found an increase in GSH content in cortex and brain strem after 7.5 weeks of treadmill running, Somani and Husain (1997) and Liu et al. (2000) did not find differences in brain GSH levels after 6.5 weeks and 8 week treadmill running, respectively, compared to control animals. However, controversial results can be found in literature regarding the effects of exercise on brain MDA levels and GSH content, and no information is known about the specific effects of voluntary FW running. These data could suggest that mitochondrial adaptations may be influenced by the bioenergetic nature and duration of exercise programs. Moreover, age and animal characteristics may also contribute to the experimental variability, and therefore explain the controversial results found regarding brain oxidative stress and physical exercise (Marques-Aleixo et al. 2012).

In our study, we did not evaluated the antioxidants levels on brain mitochondria in addition to -SH levels; however some studies reported that exercise induces favorable redox adaptations with consequent attenuation of oxidative damage markers (for refs see, Marques-Aleixo et al. 2012). Importantly, in the current study, we used young and healthy rats and the effects of regular physical exercise in favorable brain mitochondrial redox balance seem to be even more efficient reversing the increased oxidative stress associated with aging, neurodegenerative diseases or other oxidative stress associated pathophysiological conditions (Marques-Aleixo et al. 2012). It is accepted that

ROS and the changes in redox homeostasis could play a role in the complex mechanism by which physical exercise benefits the brain (for refs see Radak et al. (2007). Indeed, the most accepted explanation is that ROS can modulate the activity of redox sensitive transcriptions factors, increasing the brain antioxidant capacity and preventing redox alterations. This feedback mechanism could be imperative to prevent the extent of oxidative damage and apoptosis ultimately leading to neuroprotection.

Therefore, we can hypothesize that the possible increase in ROS production induced by both types of physical exercise, was balanced by the increase in the antioxidant capacity, and therefore minimize the effect of oxidative stress in brain and protect this organ against further oxidative pathophysiological conditions.

7. CONCLUSIONS

The present study provides additional information about the mechanisms by which treadmill endurance training and free wheel voluntary physical activity interacts with brain mitochondrial bioenergetics and cognitive function.

The analysis of the effects of treadmill endurance training and free wheel voluntary physical activity on animal behavioral, brain mitochondrial bioenergetics and particularly susceptibility to mPTP opening and oxidative stress markers resulted in the following conclusions:

- Treadmill endurance training induces a significant increase in memory, learning, spatial memory (Y-Maze), locomotor and exploratory activity (Open field);
- The two types of exercise induced improvement in parameters associated with brain mitochondrial oxygen consumption;
- No alterations were observed regarding the maximal Δψ and ADP depolarization and repolarization in all groups, but only treadmill exercised group decreased the ADP lag phase;
- The treadmill endurance training accumulated significantly more calcium before mPTP induction and, consequently, decreases the susceptibility to calcium induced brain mPTP opening;
- No alterations were observed on brain mitochondrial oxidative stress.

Brain behavioral tests performance agree with most brain mitochondrial functional data, being treadmill exercise the most effective strategy for brain protection.

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