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Research Centre in Physical Activity, Health and Leisure

Physical exercise as a preventive and therapeutic strategy against behavioral dysfunction in Alzheimer's disease

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Supervisors: Professor José Fernando Magalhães Pinto Pereira

Professor António Alexandre Moreira Ribeiro de Ascensão

Telma Bernardo

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PALAVRAS-CHAVE: DEMENTIA, MITOCHONDRIAL FITNESS, ENDURANCE TRAINING, COGNITION IMPROVEMENT, ANIMAL MODEL

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‘The roots of education are bitter,

but the fruit is sweet.’- Aristoteles

To my daughter.

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Resumo

O exercício físico tem sido associado à proteção neuronal contra alterações relacionadas com o envelhecimento, sendo recomendado como estratégia preventiva e terapêutica não farmacológica a pacientes com doenças neurodegenerativas, incluindo a doença de Alzheimer (AD). Dada a sua importância crucial em inúmeros mecanismos da biologia celular e o reconhecimento do seu papel enquanto organelos mediadores da proteção celular induzida pelo exercício físico face a inúmeras patologias, é expectável que as mitocôndrias possam ter um envolvimento importante nos fenómenos de tolerância cruzada entre exercício e AD. Na perspetiva de estabelecer o "estado da arte" e acrescentar conhecimento em torno desta problemática, a presente dissertação compreende uma revisão de literatura e um estudo experimental, realizado com animais, que foi desenvolvido utilizando um modelo esporádico já estabelecido da AD, a forma mais representativa na população, em oposição à designada familiar. Aqui, pretendemos analisar os efeitos do exercício físico crónico, utilizado tanto como estratégia preventiva (antes da indução da doença), como terapêutica (durante e após a doença) nas alterações estruturais e funcionais a nível mitocondrial no córtex cerebral e no hipocampo de um modelo de AD esporádico. Também pretendemos avaliar se, um programa crónico de treino de endurance combinado com um estilo de vida ativo (mimetizado aqui pelo acesso à roda-livre), poderia retardar ou mesmo impedir a disfunção comportamental intrínseca à AD. Nos nossos trabalhos, foram utilizados parâmetros determinados *in vitro* em mitocôndrias isoladas, associados ao consumo de oxigênio mitocondrial, análise histofotométrica decorrente de microscopia eletrónica e análise comportamental. Os resultados sugerem que o exercício físico melhorou a orientação espacial e os défices de memória e mitigou as alterações morfológicas ultra-estruturais deletérias que caracterizam a AD, incluindo a diminuição do número de sinapses e elevado dano mitocondrial. Foram observadas adaptações benéficas nos parâmetros de consumo de oxigênio mitocondrial, incluindo no estado 3, estado 4 e no índice de controlo respiratório, utilizando substratos para os complexos I e II, em mitocôndrias de sinaptossomas e extra-sinaptossomais. Adicionalmente, os

nossos resultados também sugerem que a atividade física voluntária, isoladamente, não foi capaz de neutralizar os efeitos deletérios da AD, embora quando acompanhada pela realização crónica de corrida de endurance em tapete rolante (ao longo da vida ou numa fase mais tardia) pode ser capaz de prevenir ou reverter défices cognitivos e fenotípicos associados à doença. Em suma, estas observações contribuirão com conhecimento adicional para melhor compreender os efeitos de diferentes tipos e timings de exercício ao longo da vida na progressão da doença neste modelo específico, reforçando o papel fundamental das adaptações mitocondriais nesse processo.

PALAVRAS-CHAVE: DEMÊNCIA, FITNESS MITOCONDRIAL, TREINO ENDURANCE, MELHORIA DA APRENDIZAGEM, MODELO ANIMAL

Abstract

Physical exercise has been associated with neuronal protection against age-related alterations and is recommended as a preventive and therapeutic non-pharmacological strategy in the management of patients with neurodegenerative disorders, including Alzheimer's disease (AD). Due to their central role in several biological cell mechanisms and their role as key players against several pathological constrains, it is known that mitochondria may have an important association with the cross-tolerance phenomena between exercise and AD, particularly. In order to contribute to establish the state of the art and increase the scientific knowledge in this issue, the present dissertation comprises one literature review and one animal-based experimental study that were developed using an already established sporadic model of AD, the most prevalent and representative form of the disease, in opposition to the so called familiar. Herein, we intended to analyze the effects of chronic exercise, used as both preventive (before disease induction) and therapeutic (throughout and after the presence of the disease) strategies on brain cortex and hippocampus mitochondrial structural and functional alterations in a sporadic AD-like model. We also intended to evaluate, whether a chronic-endurance training program combined with an active lifestyle (mimicked here by the free access to wheel running) could delay or even prevent AD behavioral phenotyping progression. Therefore, we used *in vitro* mitochondrial oxygen consumption endpoints, histophotometric analysis from electron microscopy, and behavioral analysis to address our question. Our data suggest that chronic exercise improved spatial learning and memory deficits and mitigated deleterious morphological alterations characterizing AD, including diminished synapse number, severe damage scores, and injured mitochondria. Positive alterations were observed in mitochondrial oxygen consumption endpoints, including state 3, state 4, and respiratory control ratio (either using complex I or II-driven substrates) in synaptosomal and non-synaptosomal mitochondria. Moreover, our data also suggest that voluntary physical activity *per se* was not able to counteract the deleterious AD effects, although when accompanied by chronic endurance treadmill running (either lifelong or later-life) may be able to prevent or reverse cognitive and phenotypic impairments

associated with AD. Ultimately, these findings will contribute to better understand the effects of different exercise types and timings throughout life span in the progression of the disease in this specific model, reinforcing the key role of mitochondrial adaptations in this process.

KEYWORDS: DEMENTIA, MITOCHONDRIAL FITNESS, ENDURANCE TRAINING, COGNITION IMPROVEMENT, ANIMAL MODEL

List of Abbreviations

ABAD – Amyloid-binding alcohol dehydrogenase

Aβ – Amyloid beta peptide

$A\beta_{1–40}$ – Amyloid beta peptide 1-40

$A\beta_{1–42}$ – Amyloid beta peptide 1-42

$A\beta_{25-35}$ – Amyloid beta peptide 25-35

AD – Alzheimer's disease

ADP – Adenosine diphosphate

AMPK – Adenosine 5′ monophosphate-activated protein kinase

APLP – Amyloid precursor-like protein

APP – Amyloid precursor protein

APP23 – Alzheimer's disease transgenic mouse model

APPsw – Swedish-type mutant APP

APP/PS1 – Double-transgenic mice expressing a chimeric mouse/human amyloid precursor protein and a mutant human presenilin 1

ATP – Adenosine triphosphate

Bax – Bcl-2-associated X protein

Bcl-2 – B-cell Lymphoma 2

BDNF – Brain derived neurotrophic factor

BSA – Bovine serum albumin

CA1 – Hippocampal cornu ammonis 1

Ca^{2+} – Calcium ion

CAT – Catalase

COX – Cytochrome *c* oxidase

CRP – C-reactive protein

CSF – Cerebrospinal fluids

Cu/Zn-SOD – Copper/zinc- dependent superoxide dismutase

DNA – Deoxyribonucleic acid

Drp1 – Dynamin-related protein 1

EDTA – Ethylenediamine tetraacetic acid

EGTA – Ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid

ER – Endoplasmic reticulum

ET – Endurance training

ETC – Electron transport chain

fAD – Familial form of Alzheimer's disease

G/M – Glutamate/malate

GPx – Glutathione peroxidase

GR – Glutathione reductase

GSH – Reduced glutathione

GSSG – Oxidized glutathione

H_2O_2 – Hydrogen peroxide

HEK – Human embryonic kidney 293 cells

HEPES – 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid

HSPs – Heat shock proteins

HT22 – Mouse hippocampal neuronal cell line

HTRA2 – High temperature requirement protein A2

Icv – Intracerebroventricular

IcvSTZ – Intracerebroventricular administration of Streptozotocin

IFNγ – Interferon gamma

IGF-1 – Insulin-like growth factor-1

IL-6 – Interleukin-6

KCl – Potassium chloride

KH_2PO_4 – Potassium dihydrogen phosphate

LPO – Lipid peroxidation

MAM – Mitochondria-associated endoplasmic reticulum membranes

MAPs – Microtubule associated proteins

Mfn1 – Mitofusin 1

MIP-1α – macrophage inflammatory protein 1-alpha

Mn-SOD – Manganese-dependent superoxide dismutase

mPTP – Mitochondrial permeability transition pore

mtDNA – Mitochondrial deoxyribonucleic acid

NFT – Neurofibrillary tangles

NRF1/2 – Nuclear respiratory factors1/2

NSE/APPsw – Neuron-specific enolase /Swedish mutation of amyloid precursor protein mice model

$^{\bullet}OH$ – Hydroxyl radical

$O_2^{\bullet-}$ – Superoxide anion

OGG1 – 8-oxoguanine DNA glycosylase-1

OxD – Oxidative damage

OXPHOS – Oxidative phosphorylation system

p-CaMKII – Phosphorylated calcium /calmodulin-dependent protein kinase II

PDH – Pyruvate dehydrogenase

PET – Positron emission tomography

PGC-1α – Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PPAR-γ – Peroxisome proliferator-activated receptor gamma

PP2B – Calcineurin

PUFA – Polyunsaturated fatty acids

RAGEs – Receptor for advanced glycation end products

RCR – Respiratory control ratio

RNA – Ribonucleic acid

ROS – Reactive oxygen species

RUN – Running wheel

sAD – Sporadic form of Alzheimer's disease

Sed – Sedentary

SIRT1 – Silent information regulator 1

SIRT3 – Silent information regulator 3

SOD – superoxide dismutase

SP – Senile plaques

STZ – Streptozotocin

3xTg-AD – Triple transgenic mice for Alzheimer's disease

TCA – Tricarboxylic acid

TEM – Transmission electron microscopy

TFAM – Mitochondrial transcription factor A

Tg2576 – Alzheimer's disease transgenic mouse model

TIM – Translocase of the inner membrane

TNF-α – Tumor necrosis factor alpha

TOM – Translocase of the outer membrane

VDAC – Voltage-dependent anion channel

VEGF – Vascular endothelial growth factor

VPA – Voluntary physical activity

VPA+ET – Voluntary physical activity + endurance treadmill training

VPA+ET-post – Voluntary physical activity + endurance treadmill training after the surgery

CHAPTER 1. [Introduction]

Introduction

Alzheimer's disease (AD) is the most common form of dementia, accounting for 60–70% of all dementia cases (Choi et al., 2018; Graham, Bonito-Oliva, & Sakmar, 2017). Currently, there are ~47 million people with dementia worldwide, and the number is expected to triple by 2050 (Murphy, Xu, & Kochanek, 2013; Prince et al., 2013), which led the World Health Organization (WHO) to label dementia as a public health priority (Wortmann, 2012). From a neuropathological point of view, AD hallmarks include extracellular deposition of senile plaques and intracellular deposition of neurofibrillary tangles (Ravelli, Rosario, Vasconcelos, et al., 2017; Rostami, Javan, Moghimi, Haddad-Mashadrizeh, & Fereidoni, 2017). The AD brain is further characterized by a progressive loss of neurons and synapses number in specific brain regions, especially in the hippocampus (Llorens-Martin et al., 2014; West, Coleman, Flood, & Troncoso, 1994). AD clinical features comprised loss of memory, cognitive impairment, and decline of the language function (Bernardo et al., 2016), but there are also some neurobehavioral symptoms associated, namely apathy, depression, irritability, disorientation, delusions, and mood instability (Abd El-Kader & Al-Jiffri, 2016). Consequently, AD represents a considerable challenge to patients, their family, caregivers, and for the health system (van Alphen et al., 2016).

There is no disease-modifying intervention for AD and dementia up to date, and the symptomatic pharmacological treatments have limited efficacy and considerable side effects (Song, Yu, Li, & Lei, 2018; Zucchella et al., 2018). Diabetes, midlife hypertension, midlife obesity, depression, and physical inactivity are among the main modifiable AD risk factors (Norton, Matthews, Barnes, Yaffe, & Brayne, 2014). Indeed, physical inactivity and a sedentary lifestyle are associated with metabolic alterations and other adverse health conditions known to be involved in the early onset and progression of dementia (Falck, Davis, & Liu-Ambrose, 2017; Wheeler et al., 2017). Increased sedentary behavior is also linked with diminished cognitive function (Bankoski et al., 2011; Falck et al., 2017). Moreover, dementia patients are more sedentary and perform less physical activity than their cognitive counterpart, further aggravating this

feedback loop (Hartman, Karssemeijer, van Diepen, Olde Rikkert, & Thijssen, 2018). Thus, physical exercise emerged as both preventive and therapeutic non-pharmacological strategy against many established types of dementia, including AD (Groot et al., 2016). Besides fewer side effects and better adherence levels compared to medications (Strohle et al., 2015), physical exercise prompt improvements in cognitive function, decreased neuropsychiatric symptoms, and delays daily-living activities decline in AD patients (Buchman et al., 2012). However, this protective relationship is not always consistent across studies (Z. Du et al., 2018). The heterogeneity of the physical exercise intervention type, time, intensity and frequency, kind of used cognitive tests, short-term follow-up time, control conditions, small sample sizes, heterogeneity in terms of study design, as well as uncertainty about the clinical significance of outcomes limits the overall conclusions in AD patients (Cammisuli, Innocenti, Fusi, Franzoni, & Pruneti, 2018; Frederiksen, Gjerum, Waldemar, & Hasselbalch, 2018; Zucchella et al., 2018). Moreover, most of the animal-based studies use distinct models of AD and/or different physical exercise regimens, which often results in different outcomes regarding this cross-tolerance effect.

Although the cellular and sub-cellular mechanisms associated with AD pathogenesis are not yet completely understood, there is accumulating evidence implicating mitochondrial dysfunction in its onset and progression (Grimm, Friedland, & Eckert, 2015; Itoh, Nakamura, Iijima, & Sesaki, 2013; Picone, Nuzzo, Caruana, Scafidi, & Di Carlo, 2014). The mitochondrial network has been particularly implicated not only in providing most of the energy for cells, but also in other vital processes including redox modulation, regulation of calcium and other ions homeostasis, activation of cell fate through death mechanisms, and interfering in distinct signaling pathways (Norambuena et al., 2018). Physical exercise prompts mitochondrial biogenesis, namely in the hippocampus, through the activation of specific genes (Vina et al., 2014; Wrann et al., 2013). It also modulates mitochondrial metabolism, dynamics and redox balance in the brain (I. Marques-Aleixo, Oliveira, Moreira, Magalhaes, & Ascensao, 2012), which is of particular importance as these processes are impaired in AD conditions

(Bernardo et al., 2016; Gusdon et al., 2017; B. Sheng et al., 2012; Xinglong Wang et al., 2009).

Generally speaking, AD is classified into late-onset sporadic AD (sAD) and early-onset familial AD (FAD). Most AD cases (about 95 %) are sporadic and for this reason, many experimental models have been developed in the last two decades for investigation of the underlying mechanisms behind the disease (Martin-Maestro et al., 2017; Ravelli, Rosario, Camarini, Hernandes, & Britto, 2017). The intracerebroventricular (icv) injection of streptozotocin (STZ) results in a well-established animal model presenting many particular features of sAD. Once implemented, this sporadic AD model induces chronic brain dysfunction with long-term and progressive deficits in learning, memory and cognitive behavior as main phenotypic features (Bao et al., 2017; Ravelli, Rosario, Camarini, et al., 2017). The icv-STZ AD brains are further characterized by insulin resistance, neuroinflammation, cholinergic deficits, β-amyloid and tau proteins accumulation, and enhanced oxidative stress (Biasibetti et al., 2017; Sharma & Gupta, 2001; Wu et al., 2018). Indeed, based on the close analogy between AD patients and rats that underwent icv-STZ administrations regarding brain metabolic and behavioral imbalance, this model has been used extensively as a rodent experimental model for sporadic AD studies (Lu et al., 2017; Nitsch & Hoyer, 1991; Salkovic-Petrisic, Knezovic, Hoyer, & Riederer, 2013). Behavioral, neurochemical and structural features resembling those found in sporadic AD patients were already well described in this icv-STZ animal model (Correia et al., 2013; Grieb, 2016; Salkovic-Petrisic et al., 2013). However, the impact of chronic physical exercise on mitochondrial structure and function in sAD condition, presenting features of most AD cases, has not been analyzed so far. A deep and further understanding of the brain mitochondrial machinery adaptations induced by physical exercise will allow the development of new and more effective preventive and therapeutic strategies against AD pathology.

To our knowledge, a scarce number of studies reported the effect of the exercise as a preventive rather than a therapeutic non-pharmacological tool against the sporadic manifestation of AD. Besides, although forced running and voluntary running are the two most commonly used chronic exercise protocols in rodent

models, whether and how they exert differential effects on the brain function are often controversial (Y. F. Liu et al., 2009). Therefore, the present thesis comprises an extensive descriptive and narrative review on the effects of physical exercise on brain mitochondrial fitness, focusing on the possible mechanisms against Alzheimer's disease, and an experimental work aiming to analyze the protective and therapeutic role of distinct chronic physical exercise types on a plethora of complementary behavioral features, on brain cortex and hippocampus ultrastructural alterations and on mitochondrial function in a rat model of sporadic AD (icv-STZ administration).

CHAPTER 2. [Aims]

Aims

Considering the tremendous impact of the Alzheimer's disease nowadays, and the recognition of physical exercise as a non-pharmacological tool able to delay or even prevent several health-deleterious conditions, the main purpose of this dissertation was to analyze the impact of different physical exercise models (voluntary physical activity [VPA] or VPA+endurance training [VPA+ET]), introduced at different times of life (lifelong or later life), against sporadic AD pathology induced in a rat model. This general purpose encompasses specific objectives presented below:

- To investigate whether a chronic-endurance training program combined with an active lifestyle (mimicked here by the free access to wheel running) could delay or even prevent deleterious-associated behavioral phenotyping progression in a rat model of sporadic AD.

- To analyze the effects of chronic physical exercise combined with an active lifestyle, used both as preventive (before disease induction) or therapeutic (throughout and after the presence of the disease) strategies on brain cortex and hippocampus mitochondrial structural and functional alterations in a rat model of sporadic AD.

CHAPTER 3. [Theoretical Background]

Review paper

Physical Exercise and Brain Mitochondrial Fitness: The Possible Role Against Alzheimer's Disease

Bernardo, TC[1], Marques-Aleixo I[1], Beleza, J[1], Oliveira PJ[2], Ascensão A[1], Magalhães J[1],

[1]CIAFEL - Research Centre in Physical Activity, Health and Leisure, Faculty of Sport, University of Porto, Portugal

[2]CNC - Centre for Neuroscience and Cell Biology, UC-Biotech, Biocant Park, University of Coimbra, Portugal

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ABSTRACT

Exercise is one of the most effective strategies to maintain a healthy body and mind, with particular beneficial effects of exercise on promoting brain plasticity, increasing cognition and reducing the risk of cognitive decline and dementia in later life. Moreover, the beneficial effects resulting from increased physical activity occur at different levels of cellular organization, mitochondria being preferential target organelles. The relevance of this review paper relies on the need to integrate the current knowledge of proposed mechanisms, focus mitochondria, to explain the protective effects of exercise that might underlie neuroplasticity and seeks to synthesize these data in the context of exploring exercise as a feasible intervention to delay cognitive impairment associated with neurodegenerative conditions, particularly Alzheimer's disease.

Keywords: Exercise; Mitochondria; Alzheimer's disease

3.1. Introduction

Alzheimer Disease (AD), the most common neurodegenerative disorder in which the nervous system progressively and irreversibly deteriorates, affects millions of people worldwide. The etiology of AD has a genetic background in 1-5% of the population (familial early-onset <60 years), while the majority, accounting for more than 95%, represent sporadic cases (late-onset >60 years) (Reitz & Mayeux, 2014). As AD is mainly a late-onset age-dependent disorder, it is estimated that its prevalence will increase along with the increase in life expectancy, exacerbating the societal and economic impact in the coming years. AD clearly is associated with systemic manifestations that extend beyond the central nervous system. In fact, triggered by environmental and endogenous factors, the risk for brain dysfunction and AD is augmented by obesity, diabetes, hypertension, hypercholesterolemia and chronic inflammation (J. K. Morris, Honea, Vidoni, Swerdlow, & Burns, 2014).

The symptoms of AD appear several years after the disease initiation and are characterized by a progressive cognitive decline, mostly related with memory and thinking language impairment, confusion and disorientation (Gimenez-Llort, Mate, Manassra, Vida, & De la Fuente, 2012). Additionally, AD is related with neurobehavioral disarrangements, including apathy, depression, agitation and anxiety (Gimenez-Llort et al., 2012). The AD brain is further characterized by decreased neuronal cell proliferation (A. Hamilton & Holscher, 2012; L. K. Hamilton et al., 2010), survival (Krezymon et al., 2013; Shruster & Offen, 2014) and differentiation (Ben-Menachem-Zidon, Ben-Menahem, Ben-Hur, & Yirmiya, 2014; Cotel, Jawhar, Christensen, Bayer, & Wirths, 2012), and a progressive loss of neurons and synapses number in specific brain regions, particularly in the hippocampus, followed by changes in the cortical and subcortical structures and complexity (Biscaro, Lindvall, Tesco, Ekdahl, & Nitsch, 2012; Correia et al., 2013; Krezymon et al., 2013).

From a histopathological point of view, extracellular deposition of senile plaques (SP) mainly composed of amyloid β peptide (Aβ) and intracellular deposition of neurofibrillary tangles (NFT), comprised of hyperphosphorylated tau are common

features of AD clinical diagnosis stage. In addition, an early intracellular accumulation of Aβ, preceding the formation of extracellular Aβ deposits and NFT formation in the brains of AD patients (Golde & Janus, 2005; Gomez-Ramos & Asuncion Moran, 2007; F. M. LaFerla, Green, & Oddo, 2007; P. I. Moreira et al., 2004) and of AD mouse models, may be a key factor in the induction of neuronal stress characterizing the progression of AD (Pigino et al., 2009; Rebeck, Hoe, & Moussa, 2010; Tomiyama et al., 2010; Umeda et al., 2015; Umeda et al., 2011; Wirths et al., 2001).

Mitochondria have a central role in cellular energy metabolism; however, these organelles are also involved in several other important cellular tasks, such as intracellular calcium regulation and redox and apoptotic signaling. The progressively accumulation of Aβ within mitochondria has also been associated with the onset and progression of AD neuronal homeostasis perturbation. Accordingly, Swerdlow and Khan (Swerdlow & Khan, 2004) proposed the "mitochondrial cascade hypothesis" to explain sporadic late-onset AD. Briefly, this hypothesis suggests that mitochondrial deregulation is the primary event in AD sporadic pathology leading to SN and NFT deposition. The precise mechanism behind this is still elusive, although some studies indicate that gradual accumulation of Aβ within mitochondria may be the link for mitochondria-mediated toxicity (Caspersen et al., 2005; Hansson Petersen et al., 2008). Moreover, mitochondrial dysfunction comprises an increased production of reactive oxygen species (ROS) namely superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (•OH), which further enhances Aβ production closing a vicious circle (K. Leuner et al., 2012).

Physical exercise has been associated with neuronal protection against aging associated alterations and is recommended as a preventive and therapeutic non-pharmacological strategy in the management of patients with neurodegenerative disorders (J Eric Ahlskog, Yonas E Geda, Neill R Graff-Radford, & Ronald C Petersen, 2011; Kirk-Sanchez & McGough, 2014; I. Marques-Aleixo et al., 2012). Although this concept is nowadays accepted, the precise molecular mechanism by which physical exercise protects the brain against the onset and the progression of AD has not been fully understood so far. Exercise induces a

myriad of cellular and subcellular adaptations, being probably one of the most important the modulation of mitochondrial network (I. Marques-Aleixo, Santos-Alves, Mariani, et al., 2015; Peeri & Amiri, 2015). In fact, given the pivotal role of mitochondria providing the energy required for metabolism, it is not surprising that modulation in these organelles even in non-contractile tissue, such as brain, have been associated with physical exercise. Therefore, it is expected that mitochondria may have a central role to explain the cross-tolerance phenomena between exercise and AD (**Figure 3.1**).

The present review discusses the potentiality of physical exercise as a mediator of neuroprotection against AD. Particularly, this work highlights the role of mitochondria as critical organelles responsible for adaptive responses with potential beneficial neurological outcomes. It is our belief that the understanding of the potential interaction between physical exercise and mitochondrial-related paths are essential to fully ascertain the safety and efficient application of exercise models as examples of active life-styles and supportive interventions to delay and antagonize AD side effects.

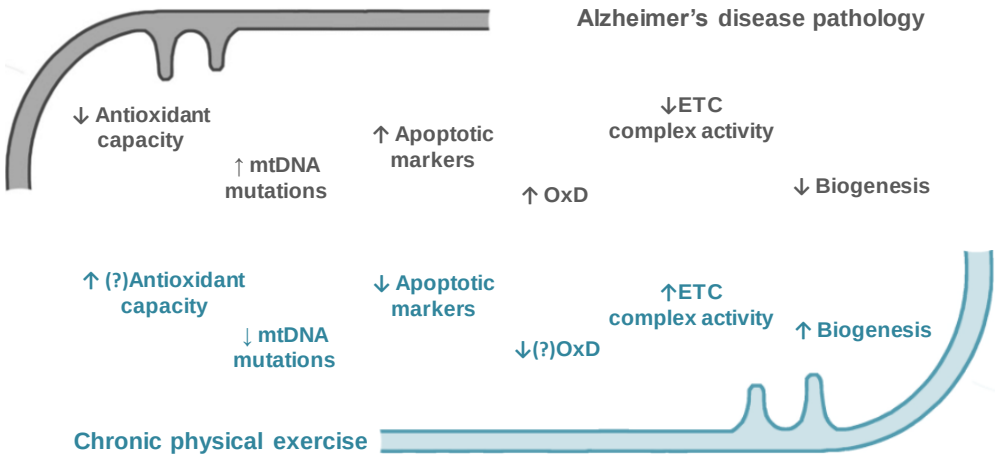


Figure 3.1: Schematic summary of described adaptations triggered by chronic physical exercise against brain mitochondrial dysfunction in Alzheimer's disease pathology (Adapted from (I. Marques-Aleixo et al., 2012) with permission). mtDNA, mitochondrial DNA, OxD, oxidative damage; ETC, electron transport chain; ↑, increase; ↓, decrease; ?, no consensual information.

3.2. The relevance of mitochondria for a healthy brain function

High metabolic energy demands are required by the human brain for its normal function. Despite its small size, brain consumes about 20% of the body's total oxygen. In fact, since neurons have a limited glycolytic capacity, they are highly dependent on mitochondrial energy production. Approximately 90% of cerebral adenosine triphosphate (ATP) production occurs in the mitochondria (Rolfe & Brown, 1997) . This energy is essential to support several cellular processes, such as synthesis, secretion and recycling of neurotransmitters and neuronal membrane potential (Magistretti & Allaman, 2015). However, together with energy metabolism, mitochondria also play a pivotal role in cell survival and death-related mechanisms maintaining cellular redox potential, regulating apoptotic pathways and contributing to the regulation of synaptic plasticity (Mark P Mattson, Marc Gleichmann, & Aiwu Cheng, 2008). Moreover, mitochondria are also involved in intracellular calcium (Ca^{2+}) homeostasis (Jacobson & Duchen, 2004). For instance, synaptic terminal mitochondria accumulate or release excess intracellular Ca^{2+} to maintain homeostasis (Y.-g. Tang & Zucker, 1997). Being synaptic mitochondria involved in the regulation of neurotransmission (Billups & Forsythe, 2002), mitochondrial function impairment leads to cellular alterations that range from subtle changes in neuronal function to neuronal death and degeneration.

Mitochondria are the major producer of reactive oxygen species (ROS) and at the same time a target of ROS toxicity (Eckert, Schmitt, & Götz, 2011). Under normal physiological conditions, ROS are produced by electron leakage as a by-product of OXPHOS; however an efficient antioxidant network counteracts its harmful effects (Turrens, 1997). Moreover, ROS are also involved in intracellular signaling pathways acting as second messengers (Allen & Tresini, 2000), thus subtle rise of the steady-state ROS concentration has been considered to have a fundamental physiological role (Dröge, 2002). However, when mitochondrial function is compromised, an imbalance in the redox steady-state may occur either by an increased ROS production or by a decreased antioxidant defense capability. In fact, a supra-physiological production of mitochondrial ROS linked to a defective scavenging system is associated with aging and age-associated

diseases, such as AD (Bernardo et al., 2013; Nunomura et al., 2006). Neurons increased susceptibility to oxidation due to its polyunsaturated fatty acids (PUFA) enriched membranes, low activity of antioxidant enzymes, (Picklo Sr & Montine, 2007; Santos et al., 2001) and its high content in transition metals (Jomova, Vondrakova, Lawson, & Valko, 2010; Kann & Kovács, 2007; Migliore & Coppedè, 2009), causes irreversible alterations to surrounding macromolecules and ultimately facilitates neuronal degeneration and death.

Neurons are elongated cells in which energy-dependent mechanisms must spatially match energy production to local energy usage. Therefore, mitochondria have a ubiquitous distribution along the cells with a major presence in synapses, where the demand for ATP is critical for synaptic transmission. In axons, mitochondrial movement is driven by two oppositely directed motor proteins throughout the microtubules, kinesins and dyneins, (Bereiter-Hahn & Jendrach, 2010; Hollenbeck & Saxton, 2005). Tightly bind to microtubules, the microtubule associated proteins (MAPs), including tau, regulate their function and also ensure the transport cargo (Maday, Twelvetrees, Moughamian, & Holzbaur, 2014; Vershinin, Carter, Razafsky, King, & Gross, 2007). This organized railroad to transport mitochondria from soma to nerve terminals (providing a localized ATP supply and Ca^{2+}-buffering capacity) or back to soma in a retrograde way (for recycling damaged mitochondria by mitophagic processes) is essential for neuron survival. However, besides mitochondrial rapid bidirectional transport, the crosstalk between mitochondrial quality-control systems and autophagy are also central for the maintenance of a precisely distributed and healthy/functional mitochondrial network. Mitochondrial dynamics is governed by fission and fusion events in which small spheres or tubular-like structures are respectively generated, being these process intimately connected with biogenesis and selective degradation (Twig, Hyde, & Shirihai, 2008). Generally, dysfunctional mitochondria can be repaired by fusion with healthy mitochondria allowing the mixture of its contents, whereas severely damaged mitochondria are segregated by fission, ultimately leading to their elimination by mitophagy (a cargo-selective autophagic mechanism that degrades mitochondria within lysosomes after their transport back to the soma) (Chen & Chan, 2009; Detmer & Chan, 2007;

Westermann, 2010; Youle & Narendra, 2011). These dynamic processes regulate mitochondrial function by enabling the recruitment of healthy mitochondria to subcellular compartments with high demands for ATP, the content exchange between mitochondria and the mitochondrial shape control and mitochondrial turnover via mitophagy (Cai & Tammineni, 2016; Chen & Chan, 2009; Z.-H. Sheng & Cai, 2012). Therefore, these mechanisms constitute a closely coordinated and reciprocal elaborate system of mitochondrial quality control, constantly monitored in order to maintain a healthy mitochondrial population and consequent neurons viability. Disruptions in any of these processes lead to mitochondrial dysfunction, cellular failure and neurological defects (Chen & Chan, 2009; Z.-H. Sheng & Cai, 2012).

Considering the importance of mitochondrial machinery in neuronal function, it is not surprising that mitochondrial dysfunction has been recognized as an early event in AD pathology, preceding and inducing neurodegeneration and memory loss. The mechanisms behind the interaction between the hallmarks of AD and mitochondrial dysfunction will be addressed to better comprehend mitochondria has a therapeutic target against the onset and progression of AD.

3.3. Hallmarks of AD-induced mitochondrial dysfunction

3.3.1 APP and Aβ

In mammals, the Amyloid-β protein precursor (APP) gene family comprises, besides APP, the two amyloid precursor-like proteins APLP1 and APLP2. These are type 1 membrane proteins with a long extracellular N-terminal and a short intracellular C-terminal domain, harboring several protein interaction (Klevanski et al., 2014). The APP family members are highly expressed in neurons and have been localized in somata, axons, and dendrites. Although the physiological role of APP and its processing products in the central nervous system is controversial, it has been proposed that this protein undergoes fast axonal transport and all three APP family members have been identified as constituents of the presynaptic active zone of central nervous system neurons (Laßek et al., 2013).

Even though mice lacking single APP family members are fully viable, they exhibit severe metabolic abnormalities and behavioral deficits (Heber et al., 2000; Ring et al., 2007). In contrast, double knockout mice lacking APLP1/ APLP2 or APP/APLP2 die within the first day after birth (Heber et al., 2000; Von Koch et al., 1997), suggesting a determinant physiological role of these proteins, particularly APLP2. The APP localized in the plasma membrane (Kinoshita et al., 2003) has been suggested to have important roles in cell adhesion (Breen, Bruce, & Anderton, 1991) and cell movement (Sabo, Ikin, Buxbaum, & Greengard, 2001). However, APP can still be found in cellular organelles, including the trans-Golgi network (H. Xu, Greengard, & Gandy, 1995), the endoplasmic reticulum (ER), endosomal, lysosomal (Kinoshita et al., 2003) and mitochondrial membranes (Mizuguchi, Ikeda, & Kim, 1992).

Currently, it has been proposed that the accumulation of intraneuronal Aβ is an early event in the progression of AD, preceding the formation of extracellular Aβ deposits. In fact, a link between intracellular and extracellular Aβ has been explored suggesting that extracellular Aβ may originate from intraneuronal pools and a delicate dynamic equilibrium exists between these two Aβ pools (reviewed in: F. M. LaFerla et al., 2007). Moreover, it has been also proposed that Aβ can be re-accumulated from the extracellular medium by receptor-dependent mechanisms (Takuma et al., 2009) or endossomal/lysosomal pathway (Petersen et al., 2008) and/or it can be generated within the ER and Golgi system and secreted as part of the constitutive secretory pathway (Cook et al., 1997; Greenfield et al., 1999; Hartmann et al., 1997).

Numerous studies report AD pathology associated with abnormal mitochondrial dysfunction and with the mitochondrial localization of Aβ and APP even before extracellular deposition of SP (Ofman et al., 2003; J.-Z. Wang & Liu, 2008; Y. Yan et al., 2007), which highlights the particular role of mitochondrial APP as an early feature for AD pathogenesis. Although under normal circumstances is targeted to the ER and reach their final destination in the plasma membrane through a secretory pathway, it has also been found to be associated with mitochondrial translocation channels under APP overexpression conditions in cell cultures or in the brains of transgenic mice overexpressing APP (Anandatheerthavarada,

Biswas, Robin, & Avadhani, 2003), forming a super-complex with Tom40 and Tim23 subunits (Milton, Mayor, & Rawlinson, 2001). This accumulation of APP in mitochondrial import channels is negatively associated with the ability to import nuclear-encoded proteins, eventually resulting in the collapse of mitochondrial function and ultimately in neuronal cell death in AD-affected brain regions (Devi, Prabhu, Galati, Avadhani, & Anandatheerthavarada, 2006). Nevertheless, a mechanism involving the cleavage of APP by serine protease HTRA2 in mitochondrial intermembrane space seems to prevent mitochondrial dysfunction caused by the accumulation of APP (H.-J. Park et al., 2006). Moreover, the protease Omi, involved in mitochondrial quality control by degrading unfolded or misfolded proteins (Radke et al., 2008) is also involved in mitochondria-associated APP cleavage (Pavlov et al., 2011). However, it remains to be clarified if the inhibition of APP accumulation in mitochondrial import channels or increased proteolysis of APP would protect neurons from mitochondria-mediated injury and potentially decelerate AD progression (Pavlov, Petersen, Glaser, & Ankarcrona, 2009).

Studies on *post-mortem* AD brains, patients with cortical plaques and transgenic APP mice have shown that Aβ accumulates within mitochondria, resulting in mitochondrial dysfunction (mtDNA defects, abnormalities in mitochondrial dynamic and trafficking) (Caspersen et al., 2005; Devi et al., 2006; Heng Du et al., 2008; Lustbader et al., 2004; Manczak et al., 2006). Although the exact mechanisms are not fully understood, accumulating evidence suggest that Aβ is imported to mitochondria. In fact, since active γ-secretase was found to be particularly abundant in the contact sites connecting mitochondria and ER (Area-Gomez et al., 2009), it is possible that under pathological AD conditions, significant amounts of Aβ can be produced in the mitochondria surrounding area, resulting in Aβ-mediated mitochondrial dysfunction. Nevertheless, the understanding of how Aβ can reach mitochondria has been particularly challenging. Several authors proposed complex pathways of Aβ traffic-mediating mitochondrial and neuronal dysfunction. Hanson and coworkers (Petersen et al., 2008) suggested that Aβ is accumulated by mitochondria through the TOM/TIM complex and accumulates in mitochondrial cristae. Both $A\beta_{1-40}$ and $A\beta_{1-42}$ import

were prevented by pre-treatment with proteinase K to remove receptors from the outer mitochondrial membrane, Tom20, Tom22, and Tom70. Moreover, Takuma and coworkers (Takuma et al., 2009), suggested that the receptor for advanced glycation end-products (RAGEs) is also a binding receptor for Aβ, and therefore mediates the intraneuronal transport of Aβ and the consequent mitochondrial and neuronal dysfunction. However, Cha and collaborators (2012) reported that blocking RAGEs in HT22 cell line failed to rescue the $A\beta_{1-42}$-mediated mitochondrial disruption of both morphology and function, which suggests that RAGE-Aβ engagement is not involved in the process of mitochondrial disruption by Aβ. Mitochondrial Aβ accumulation also can derive from the ER/Golgi. As previously stated, several subcellular compartments, including trans-Golgi network, lysosomes and ER, may participate in APP amyloidogenic processing and Aβ generation within the cells. The ER and mitochondria are organelles functionally and morphologically connected via mitochondria-associated ER membranes (MAM), and involved in crucial cellular metabolic processes, such as calcium signaling, glucose, phospholipid and cholesterol metabolism, and the regulation of apoptosis (de Brito & Scorrano, 2010; Hayashi, Rizzuto, Hajnoczky, & Su, 2009). Impairment of the communication between ER and mitochondria may represent a common hit in AD as these processes seem to be significantly altered early during AD pathogenesis (Bezprozvanny & Mattson, 2008; Martins et al., 2009; Stefani & Liguri, 2009). In fact, an abnormal expression of some MAM-associated proteins in *post-mortem* AD brains and AD transgenic mice models was also shown (Hedskog et al., 2013). The enzymes of the amyloidogenic pathway have been shown to localize in MAM (reviewed elsewhere: Pinho, Teixeira, & Glaser, 2014), which makes it a site of Aβ production in close proximity to mitochondria (Schreiner, Hedskog, Wiehager, & Ankarcrona, 2015). Moreover, besides being a possible source of mitochondria-localized Aβ (Area-Gomez et al., 2009), it has been suggested that this pathway affects the metabolic and signaling pathways ruled by this site (Schreiner et al., 2015).

3.3.2 Tau and intracellular neurofibrillary lesions

Along with Aβ, tau hyperphosphorylation and aggregation are major proximal causes of neuron loss in AD pathogenesis (Bloom, 2014). Tau is an important microtubule-associated protein abundant in the central nervous system (Binder, Frankfurter, & Rebhun, 1985). Under physiological conditions, tau is a soluble protein that promotes microtubule assembly and stabilization and affects the dynamics of microtubules in neurons (reviwed in: Avila, Lucas, Perez, & Hernandez, 2004; Iqbal et al., 2005; F. M. LaFerla et al., 2007; Lee, Goedert, & Trojanowski, 2001). The phosphorylation of tau regulates microtubule binding and assembly (J.-Z. Wang & Liu, 2008); however, under pathologic conditions, tau overexpression and hyperphosphorylation at certain residues appears to impair axonal transport of organelles through microtubules, including mitochondria, causing synapse “starvation” and depletion of ATP (Alonso et al., 2010; Brandt, Hundelt, & Shahani, 2005; Mandelkow, Stamer, Vogel, Thies, & Mandelkow, 2003; Sapir, Frotscher, Levy, Mandelkow, & Reiner, 2012; Shahpasand et al., 2012). Under this pathological condition, tau undergoes a series of post-translational changes including abnormal phosphorylation, glycosylation, glycation, and truncation (reviwed in: G Amadoro et al., 2004), which may render tau more prone to form NFT, a major hallmark of AD. Following aggregation, microtubules disintegrate, impairing the neuronal transport system and eventually leading to cell death. Hyperphosphorylation is believed to be an early event in the pathway that leads from soluble to insoluble and filamentous tau protein (Braak, Braak, & Mandelkow, 1994), resulting in the formation of the potently cytotoxic filamentous structures (Frank M LaFerla, 2010). Factors affecting tau hyperphosphorylation are not fully understood and in fact, it has been suggested that tau pathology can be triggered by different mechanisms, dependent and/or independent of Aβ.

Being aging the main risk factor for late-onset AD and brain aging marked by mitochondrial bioenergetic deficits, defect in ROS scavenging and increase in ROS production (reviewed in: Leuner, Müller, & Reichert, 2012; I. Marques-Aleixo et al., 2012), chronic oxidative stress has been suggested as a critical factor for

hyperphosphorylation of tau in AD neurons. In fact, this mechanistically links mitochondrial oxidative stress with AD onset and progression.

As referred above, tau overexpression and phosphorylation have been linked to the inhibition of mitochondrial transport along the microtubules (Dubey, Chaudhury, Kabiru, & Shea, 2008; Shahpasand et al., 2012; Stamer, Vogel, Thies, Mandelkow, & Mandelkow, 2002; Stoothoff et al., 2009). In fact, tau inhibits the transport of APP into axons and dendrites, and this inhibition causes the accumulation of APP in the cell body (Mandelkow et al., 2003; Stamer et al., 2002). Moreover, besides abnormal distribution of mitochondria in AD neurons, it has been suggested that Aβ and tau, in an independent and/or synergistic way(s), lead to pathological deterioration of mitochondria in AD by impairing multiple mitochondrial-related pathways, including bioenergetics and quality control (reviwed in:Eckert et al., 2011; Reddy, 2011). Accordingly, N-terminal truncation of tau has been detected in cellular and animal AD models, as well as in synaptic mitochondria and cerebrospinal fluids (CSF) from human AD subjects (Giuseppina Amadoro et al., 2014; Giuseppina Amadoro et al., 2010; Corsetti et al., 2008; S.-Y. Park & Ferreira, 2005; Reifert, Hartung-Cranston, & Feinstein, 2011; Rohn et al., 2002). This fragment is neurotoxic in primary cultured neurons (G Amadoro et al., 2004), compromising mitochondrial respiratory and energy-generating systems (Atlante et al., 2008; Lasagna-Reeves et al., 2011; Rhein et al., 2009) and leading to mitochondrial potential loss and oxidative stress (Quintanilla, Dolan, Jin, & Johnson, 2012). Moreover, tau oligomers also seem to participate in the activation of the mitochondrial apoptotic pathway (Lasagna-Reeves et al., 2011). Concomitantly to bioenergetic deficits and synaptic damage, mitochondrial dynamics abnormalities toward a fragmented profile (Manczak & Reddy, 2012; Quintanilla et al., 2012) and an extensive autophagic clearance of mitochondria (mitophagy) (Corsetti et al., 2015) also seem to be associated with tau pathologies.

Overall, hallmarks of AD seem to interfere with mitochondrial function, impairing, at least in part, neuronal energetic and respiratory chain and contributing to the supra physiological generation of ROS, oxidative damage and activation of neuronal apoptosis. Moreover, mitochondrial dysfunction also seems to

exacerbate AD hallmarks in a vicious circle. Specific alterations of mitochondrial bioenergetics and consequent raise in ROS production, as well as the disruption of mitochondrial quality control systems in primary neuron culture, brain tissue from AD patients and transgenic AD models will be detailed below.

3.3.3 Energy-metabolism, mitochondrial dysfunction and oxidative stress in AD

Decreased brain metabolism is one of the earliest features of AD. In fact, the degree of cognitive impairment in AD brains has been linked to the extent of mitochondrial dysfunction (Dragicevic et al., 2010) and several reviews have already addressed the involvement of mitochondrial dysfunction in the pathogenesis of AD (Benek et al., 2015; Cabezas-Opazo et al., 2015; Cadonic, Sabbir, & Albensi, 2015; Cavallucci, Ferraina, & D'Amelio, 2013; Correia et al., 2012; P. Coskun et al., 2012; Friedland-Leuner, Stockburger, Denzer, Eckert, & Muller, 2014; Garcia-Escudero, Martin-Maestro, Perry, & Avila, 2013; Grimm et al., 2015; Hroudová, Singh, & Fišar, 2014). Generically, it is clear the emerging relevance of these organelles as important mediators of AD pathological events and as attractive targets to pharmacological and non-pharmacological interventions against neurodegeneration.

Several mitochondrial mechanisms are affected in AD. Briefly, AD transgenic models and *post-mortem* AD brain human studies reported several defects in mitochondrial bioenergetics in AD that culminate in decreased ATP synthesis, namely impairments in the enzymatic activity of the protein complexes of the ETC, Ca^{2+} deregulation, alterations in antioxidant enzymatic activity and increased ROS production (even before the appearance of Aβ and tau tangles) (Grimm et al., 2015; Perry et al., 2000; Schmitt et al., 2012; Yao et al., 2009; Yin, Boveris, & Cadenas, 2014). In this context, Yao et al. (2009) found clear signs of mitochondrial dysfunction evidenced by the decline in OXPHOS activity, pyruvate dehydrogenase (PDH) and cytochrome *c* oxidase (COX) regulatory enzymes in a female triple transgenic Alzheimer's mice (3xTg-AD) model. Moreover, these were accompanied by increased H_2O_2 production and lipid peroxidation. Similar

results were obtained by Correia and coworkers (Correia et al., 2013) in Wistar male rats after 5 wks of intracerebroventricular streptozotocin injection. Altered expression and activity of PDH, COX, isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase were also found in fibroblasts from AD patients and *post-mortem* AD brain tissue (Chaturvedi & Flint Beal, 2013; J. K. Morris et al., 2014). Also, the voltage dependent anion channel VDAC, a major component of the outer mitochondrial membrane that regulates ion fluxes and metabolites, seems to be impaired due to oxidative damage in different AD models (Ferrer, 2009). Moreover, evidence from AD *post-mortem* brains (Bosetti et al., 2002; Carvalho, Correia, Perry, Castellani, & Moreira, 2015) showed a decrease in ATP levels and reduced respiratory chain complexes I, III, IV and V activity and content. Studies from different independent groups also highlighted mitochondrial impairment and oxidative damage in cell lines expressing mutant APP (X. Wang et al., 2009; X. Wang et al., 2008), Aβ treated mammalian cells (H. Du et al., 2010) and primary neurons from AD transgenic mice (Calkins, Manczak, Mao, Shirendeb, & Reddy, 2011; Calkins & Reddy, 2011a).

Furthermore, Völgyi and collaborators (2016) evaluated proteome changes in synaptic and non-synaptic mitochondria of APP/PS1 mice (model of APP overproduction and Aβ accumulation) at different ages. These authors reported significant changes of 60 different mitochondrial proteins, the majority related to energy metabolism (ETC and TCA cycle), oxidative stress response and apoptosis.

Importantly, the increased level of mitochondrial Aβ binding to alcohol dehydrogenase (ABAD) in 3xTg-AD female mice correlated to increased generation of mitochondrial free radicals (S. D. Yan & Stern, 2005). Manczak and colleagues (Manczak et al., 2006) found an increase in H_2O_2 and a decrease in COX activity in an young APP transgenic mice model (Tg2576) prior to the appearance of Aβ plaques suggesting that oxidative stress is an early event in AD pathophysiology. Collectively, the data suggest that significant mitochondrial bioenergetics dysfunction coupled with increased oxidative stress contributes to the overproduction of Aβ and early AD pathogenesis. In line, Resende et al. (2008) reported, in an *in vivo* model of AD, decreased levels of glutathione and

vitamin E and increased activity of the antioxidant enzymes glutathione peroxidase (GPx) and superoxide dismutase (SOD) concomitant with increased lipid peroxidation. These alterations were reported during the Aβ oligomerization period, before the appearance of Aβ plaques and NFTs, suggesting that oxidative stress occurs early in AD development. By using a model of sporadic AD not associated with overexpression of familial AD-associated mutant genes, Melov and coworkers (Melov et al., 2007) showed that a deficiency in manganese-dependent superoxide dismutase (Mn-SOD) exacerbated the amyloid burden and increased the levels of phosphorylated tau, which suggest that oxidative stress from mitochondrial dysfunction promotes AD-like pathology. In fact, cumulative oxidative lesions in mtDNA occur during the aging process and are also a prominent feature in AD. *Post-mortem* AD brains exhibited a loss of integrity, including a decreased in mtDNA copy number and increased number of deletions and mutations (P. E. Coskun, Beal, & Wallace, 2004; Lin, Simon, Ahn, Kim, & Beal, 2002). This AD-associated mtDNA damage can affect protein expression, including mitochondrial-encoded genes of OXPHOS complexes leading to impairments in mitochondrial metabolism and oxidative stress (reviwed in:Reddy, 2011). Concomitantly, AD-associated mitochondrial dysfunction and increased oxidative stress likely cause further loss of calcium homeostasis and the activation of the intrinsic apoptotic pathway, mitochondrial fragmentation and abnormal distribution within the neurons (reviewed in: X. Wang et al., 2014). The gradual and chronic accumulation of oxidation products can compromise brain cell structure and its constituents, particularly mitochondrial structure and function, which ultimately result in neuronal death (Paula I Moreira et al., 2010).

Importantly, Grimm and coworkers (Grimm et al., 2015) pointed out that the vast majority of studies have been performed on cellular and animal models based on mutation found in familial AD cases. Bearing in mind that, at least among the oldest people, dementia severity is dissociated to Aβ and tau neuropathology, the ‘‘Inverse Warburg hypothesis’’ postulate that sporadic AD is a metabolic disease initiated by an age-related mitochondrial dysregulation (L. A. Demetrius & Driver, 2013; Lloyd A Demetrius, Magistretti, & Pellerin, 2014; Lloyd A Demetrius & Simon, 2012). Moreover, Demetrius and Driver (2015) highlighted that

understanding sporadic AD as a metabolic disease will help to promote effective metabolic-based therapeutic interventions, including exercise and healthy dietary habits.

3.3.4 Mitochondrial quality control alterations in AD

Mitochondrial dynamics is critical for the maintenance of mitochondrial integrity. Fission enables the renewal, redistribution and proliferation of mitochondria, whereas fusion events allow the interaction, communication and mtDNA exchange between organelles (D. C. Chan, 2006). Therefore, mitochondrial dynamics has been proposed as an important mechanism able to attenuate mtDNA mutation within the mitochondrial population. In fact, disruption in mitochondrial dynamics and abnormal mitophagy has been extensible reported in AD brains (Chen & Chan, 2009).

Altered expression of mitochondrial fission and fusion-related genes, which in turn leads to an increase in mitochondrial fragmentation and abnormal mitochondrial dynamics were observed in mouse neuroblastoma cells incubated with Aβ peptide, AD transgenic mice and *post-mortem* brain specimens from AD patients (Calkins et al., 2011; Manczak, Calkins, & Reddy, 2011; Manczak & Reddy, 2012). Moreover, evidence from cells harboring mutant human APP showed increased mitochondrial network fragmentation and abnormal distribution within the cells (Stockburger et al., 2014; X. Wang et al., 2009). Similarly, in HEK and M17 neuroblastoma cells overexpressing Swedish-type mutant APP (APPsw), mitochondria were extensively fragmented and abnormally distributed (K. Leuner et al., 2012; X. Wang et al., 2008). These alterations were, at least in part, mediated by altered expression of dynamin-related protein 1 (Drp1), a regulator of mitochondrial fission and distribution, due to elevated oxidative and/or Aβ-induced stress (X. Wang, Su, Fujioka, & Zhu, 2008). Importantly, it has been also suggested that AD disturbed mitochondrial fission-fusion dynamics may contribute to impaired mitochondrial transport within the neurons (Ishihara et al., 2009; Varadi et al., 2004; Xinglong Wang et al., 2009),

which could lead to mitochondrial depletion from axons and dendrites and, subsequently, synaptic loss.

Generally, disturbed mitochondrial fusion/fission activity observed *in vitro* and in AD patient's brains leads mitochondria to a fragmented phenotype and likely interferes with mitochondrial motility and mitophagy, thereby compromising mitochondrial quality control. Aberrant mitochondrial shape, integrity, and distribution unequivocally affect the bioenergetic role of these organelles. Furthermore, mitochondrial bioenergetics deficits are linked with the supra-physiological production of ROS and these products are known to augment even more mitochondrial pathology in AD brains.

Although mitochondrial dynamics changes may contribute to alterations in mitochondrial density and mass, changes in mitochondrial biogenesis can also impact these mitochondrial parameters. Shaerzadeh and co-workers (2014) showed that after an Aβ injection in hippocampal CA1 area, not only mitochondrial fission process increased but also mitochondrial biogenesis was severely affected. In fact, expression levels of mitochondrial biogenesis-related proteins, such as PPARγ coactivator 1α (PGC-1α), nuclear respiratory factors1/ 2 (NRF1/2) and mitochondrial transcription factor A (TFAM) were significantly decreased in both AD hippocampal tissue and APPsw M17 cells (X. Wang et al., 2008). Decreased mitochondrial biogenesis was also found in Aβ transgenic AD mice (Calkins & Reddy, 2011b). These findings suggest that impaired mitochondrial biogenesis may be, at least in part, related to mitochondrial dysfunction in AD (Qin et al., 2009; B. Sheng et al., 2012). In this context, St-Pierre (St-Pierre et al., 2006) suggested that the apparent ability of PGC-1α to increase mitochondrial ETC activity while stimulating antioxidant enzymes induction makes this an almost ideal protein to control or limit the damage that has been associated with the defective mitochondrial function seen in AD.

3.4. The neuroprotective effect of physical exercise against AD

Evidence has repeatedly demonstrated that physical exercise, besides improving general health, has a specific positive impact on brain health (J. K. Morris et al., 2014) and can be an effective strategy to prevent and counteract neurodegenerative diseases (J. E. Ahlskog, Y. E. Geda, N. R. Graff-Radford, & R. C. Petersen, 2011; Hamer & Chida, 2009; Yau & Gil-Mohapel, 2014). In fact, it is well established that physical exercise improves cognitive function (Creer, Romberg, Saksida, van Praag, & Bussey, 2010; Neeper, Gomez-Pinilla, Choi, & Cotman, 1995; Nokia et al., 2016; Rasberry et al., 2011; Vina et al., 2014; Yau & Gil-Mohapel, 2014), as well as attention, memory, reaction time, language, visual-spatial, and executive function (Snowden et al., 2011). Moreover, physical exercise has been linked with a lower risk of cognitive impairment and is generally associated with behavioral-related improvements in patients with neurodegenerative diseases (I. Marques-Aleixo et al., 2012).

Exercise-induced alterations in cognition are potentially important in the context of AD improving patients' quality of life. The general improvements in brain function promoted by physical exercise seem to be related to alterations in brain structure (Åberg et al., 2009; Erickson et al., 2011; Rasberry et al., 2011). These adaptations induced by exercise include the increased hippocampal neurogenesis (Clark et al., 2011) and volume (Erickson et al., 2011; Pereira et al., 2007), the increase of synaptic plasticity (Colcombe, Kramer, McAuley, Erickson, & Scalf, 2004; Ratey & Loehr, 2011), the increase of brain blood flow (Loprinzi, Herod, Cardinal, & Noakes, 2013) and the decrease of age-related atrophy in different brain areas (Colcombe et al., 2004). Furthermore, some of the neurobiological mechanisms responsible for the beneficial effects of physical exercise in brain performance appear to be related to alterations at cellular level. Physical exercise induces an increase in the synthesis and release of neurotrophins and growth factors (Loprinzi et al., 2013), which include brain derived neurotrophic factor (BDNF) (Eggermont, Swaab, Luiten, & Scherder, 2006), insulin-like growth factor-1 (IGF-1) and vascular endothelial growth factor (VEGF) (Ratey & Loehr, 2011). The modulation of these neuromediators seems

to improve the survival and growth of different neuronal subtypes (K. L. Chan, Tong, & Yip, 2008; Heyn, Abreu, & Ottenbacher, 2004).

Besides these positive effects in brain function, physical exercise recently emerged as the most effective way to prevent and counteract neurodegenerative diseases (J. E. Ahlskog et al., 2011), including AD (Pareja-Galeano et al., 2012). However, the volume, intensity and duration of physical exercise are still unclear and under debate. Physical exercise has a positive effect in some of the most characteristic signs of AD, generally through the regulation of oxidative stress-related mechanisms on the brain (I. Marques-Aleixo, Santos-Alves, Mariani, et al., 2015; Zsolt Radak, Marton, Nagy, Koltai, & Goto, 2013; Z. Radak, Taylor, Ohno, & Goto, 2001), increased blood flow and metabolism (de la Torre, 2002), and especially decrease of cortical formation and accumulation of Aβ (Adlard, Perreau, Pop, & Cotman, 2005; Zsolt Radak et al., 2013; Z. Radak et al., 2001; Tortosa-Martinez & Clow, 2012). Moreover, some of the mechanisms through which exercise has a positive impact in AD appear to involve improvement of mitochondrial function. Due to the relevance of mitochondria on brain metabolism and its involvement on AD pathology, mitochondria has been considered a central target for pharmacological and non-pharmacological interventions against the onset and progression of AD, including physical exercise. Nevertheless, the role of physical exercise against AD-related mitochondrial dysfunction is not fully understood. The next topic will highlight existing studies approaching the role of mitochondria on the cross-tolerance phenomena between exercise and AD.

3.4.1 Oxidative stress-related adaptations induced by physical exercise

There are a number of ROS sources in neuronal cells; however, the mitochondrial ETC is one of the most important. The damaging potential of mitochondrial ROS is the core of the “mitochondrial free radical theory of aging”. Briefly, it is suggested that the accumulation of molecular damage over time induced by mitochondrial ROS lead to impairment function of respiratory chain, which in turn triggers further accumulation of ROS. This process leads to energy depletion and ultimately to cellular degeneration and death. However, accumulating evidence

has revealed that ROS are not simply by-products of mitochondrial metabolism. Instead, moderate levels of ROS have been associated with important redox signaling pathways in the healthy cell (Lapointe & Hekimi, 2010). Although this renewed perspective of ROS physiology has been increasingly recognized, many studies have clearly demonstrated that excessive amounts of ROS are also related to several mitochondrial diseases and neurodegenerative disorders, including AD.

Physical exercise can significantly increase the metabolic rate and is accompanied by ROS generation. Therefore, a simple question comes up: how ROS produced during physical exercise can be associated with exercise-induced health-promoting benefits? Bearing this in mind, it is important to distinguish between pathological ROS accumulation (reported during the aging process or AD pathology) and moderate/short-term ROS levels associated to physical exercise. Several reports, elegantly reviewed by Radak and coworkers (2016), suggested that regular exercise promotes increased brain function and protection by activating a wide range of redox signaling pathways. As described above, neuronal cells are very sensitive to oxidative stress because of their high metabolic rate, high content of oxidizable substrates and low antioxidant capacity. It is however accepted that redox-sensitive signaling pathways triggered by chronic exercise can selectively regulate the activity of brain mitochondrial antioxidant (for review see Z. Radak et al., 2016). Improvements in brain enzymatic antioxidant system and consequent modulation of oxidative damage can delay or even prevent redox alterations linked to aging and neurodegenerative disorders, such as AD (Z. Radak et al., 2016). This can be achieved not only by the induction of the antioxidant defense system, which reduces ROS levels or severity, but also because long-term adaptations to exercise can directly diminish ROS production (Z. Radak et al., 2010). Nevertheless, physical exercise triggers a complex adaptive response that can be controversial. In fact, the high heterogeneity among exercise protocols with distinct intensity, volume and duration along with different age and animal characteristics leads to different physiological, biochemical and functional adaptations, namely in the brain (I. Marques-Aleixo et al., 2012). Moreover,

gender-specific adaptations related with exercise can be found (Garcia-Mesa et al., 2011; Gimenez-Llort et al., 2010; H. Liu, Harrell, Shenvi, Hagen, & Liu, 2005), which may explain distinct exercise modulatory profiles on oxidative stress markers in the same organ.

Mechanisms behind exercise-dependent regulation of the brain redox state pointed out that regular moderate exercise may activate specific redox-sensitive transcription factors, culminating in an overall adaptive response with direct consequences on oxidative damage (Camiletti-Moiron, Aparicio, Aranda, & Radak, 2013; I. S.-A. Marques-Aleixo, E.; Moreira, P.; Oliveira, P.; Magalhães, J.; Ascensão, A., 2015; Z. Radak, Chung, & Goto, 2008; Stranahan, Martin, & Maudsley, 2012).

Knowing that, as previously mentioned, disturbances in mitochondrial machinery and redox signaling are critical events leading to AD pathology, the ability of endurance exercise to increase antioxidant potential highlights the possible role of regular exercise as an important countermeasure to mitigate many neuropathophysiological conditions. Therefore, despite the diversity in methodology, the vast majority of exercise studies dealing with AD models report significant improvements in the antioxidant capacity. **Table 1** provides a list of recent studies and summarizes major findings.

Table 1: Effects of physical exercise on antioxidant capacity and oxidative stress-induced damage in AD animal models.CAT – catalase, GPx – glutathione peroxidase, GR – glutathione reductase, GSH – reduced glutathione, GSSG- oxidized glutathione, icv – intracerebroventricular, LPO – lipid peroxidation, ROS – reactive oxygen species, RUN – running wheel, SOD – superoxide dismutase, ↓ – decrease, ↑ – increase.

Tg line/Strain	Sex	Tissue	Intervention	Main changes in antioxidant system promoted by exercise	Reference
NSE/APPsw	Not shown	Brain	Treadmill 16 wks, 13.2m/min, 1h/day, 5 days/wk	↑ Cu/Zn-SOD, ↑ CAT	Um et al. (2008)
Tg-NSE/htau23	♀♂	Brain	Treadmill 3mo, 12 or 19m/min, 1h/day, 5 days/wk	↑ Cu/Zn-SOD, ↓ Mn-SOD ↑ CAT	Leem et al. (2009)
NSE/APPsw	Not shown	Brain	Treadmill 16 wks, 13.2m/min, 1h/day, 5 days/wk	↑ Cu/Zn-SOD, ↑ CAT	J. Y. Cho et al. (2010)
3xTg-AD	♀♂	Cortex	Treadmill 5 wks, maximum: 4.2m/min, 30min/day, 5 days/wk	♀: ↓ GSH, ↓ GSSG, ~ GPX, ~ Cu/Zn-SOD, ~ Mn-SOD, ~ LPO ♂: ~GSH, ~GSSG, ~ GPx, ~ Cu/Zn-SOD, ~ Mn-SOD, ↓ LPO	Gimenez-Llort et al. (2010)
Tg-NSE/hPS2m	Not shown	Hippocampus	Treadmill 12 wks, 12m/min, 1h/day, 5 days/wk	↑ Cu/Zn-SOD , ↑ Mn-SOD	Um et al. (2011)
3xTg-AD	♀♂	Cortex	RUN 1 and 6 mo	♀:↓ LPO, ~GSSG, ~GSH, ↑ GPx ~ Cu/Zn-SOD, ~ Mn-SOD ♂:~ LPO, ↑ GSSG, ↓ GSH, ↑ GPx, ↓ Cu/Zn-SOD, ~ Mn-SOD	Garcia-Mesa et al. (2011)
3xTg-AD	♂	Cortex	RUN 6mo	↑ GSH, ~GSSG, ↑ GPx, ~ Gr, ~ Cu/Zn-SOD, ↑ Mn-SOD, ↓ LPO	Garcia-Mesa et al. (2012)

C57bl/6 (Aβ25-35 icv injection)	♂	Hippocampus	RUN, 12 days	↓ Oxidative stress marker, ↓ Antioxidant stress marker	Q. Wang et al. (2013)
APP/PS1	Not shown	Hippocampus	RUN 6 wks	~ Oxidative stress marker, ~ Antioxidant stress marker	Z. Q. Xu et al. (2013)
3xTg-AD	♀	Hippocampus	RUN 3 mo	~ Mn-SOD, ~ Catalase, ~ GPx	Garcia-Mesa et al. (2014)
APP/PS1	♂	Hippocampus	Treadmill 20 wks, 11m/min, 30min/day, 5days/wk	↓ ROS, ↑ Mn-SOD (activity), ↑ GPx (activity)	Bo et al. (2014)
Tg601	♀	Brain	Treadmill 3 wks, 10 m/min, 30 min/day, 5 days/wk	↑ LPO	Elahi et al. (2016)
3xTg-AD	♀	Cortex	RUN 3 mo	↑ Cu/Zn-SOD, ~ Mn-SOD ↓ GSSH, ↓ GPx, ↓GR, ↓ LPO	Garcia-Mesa et al. (2016)

Concerning antioxidant enzymes, SOD and catalase activities are reduced in the brains of AD animal models and AD brains patients (Leem et al., 2009; Schuessel et al., 2005; Um et al., 2008). However, some studies have highlighted that regular exercise increases such antioxidant enzymes in young rat and mice brains (Garcia-Mesa et al., 2012). Upregulation of both expression and activity of SOD and catalase were also reported in AD mice models submitted to chronic exercise (J. Y. Cho et al., 2010; Leem et al., 2009; Um et al., 2011; Um et al., 2008). Moreover, an improvement in the antioxidant defense system associated with physical exercise was further noticeable when combined with antioxidant supplementation in transgenic mice models of AD (J. Y. Cho et al., 2010; Garcia-Mesa et al., 2012).

On the other hand, data suggest that the overall levels of oxidative damage to lipids, proteins and DNA are elevated in AD brains (Guo, Sun, Chen, & Zhang, 2013). In fact, it is well established that oxidative stress-related impairment increases with the mouse age and the AD-like pathology severity (Garcia-Mesa et al., 2014). Brain tissue analysis from 12 month old 3xTg-AD mice showed higher levels of oxidative damage than those from their younger healthy counterparts (Garcia-Mesa et al., 2012). In contrast, a bulk of studies documented that endurance exercise interventions decrease lipid peroxidation in rat brain (Z. Radak et al., 2010). However, inconsistent effects of physical exercise regarding lipid peroxidation have been observed in AD models. Moreover, Elahi et al. (2016) did not find a decrease in lipid peroxidation levels in aged mice after a short-term treadmill running. As several redox-dependent signaling pathways and physiological adaptations are activated by a mild increase in ROS generation (Mancuso, Coppede, Migliore, Siciliano, & Murri, 2006; Z. Radak, Kumagai, Taylor, Naito, & Goto, 2007), redox imbalance during a short-term exercise program seems to be beneficial and prepare the cellular environment for the subsequent stimulus. Therefore, repetitive moderate levels of lipid peroxidation could be an important signal to remodel cellular membranes despite the limited repair of lipid peroxidation (Z. Radak, Zhao, Koltai, Ohno, & Atalay, 2013). As stated before, mitochondrial DNA is localized near ROS production sites, which may cause mtDNA mutations and delections.

Furthermore, mtDNA has a limited capacity for DNA repair and also lacks histones. Therefore, cumulative oxidative stress-related lesions in mtDNA are reported during the aging process and are a prominent feature in AD. *Post-mortem* analysis of AD patient's brains exhibit decreased mtDNA copy number (P. E. Coskun et al., 2010) and increased number of delections and mutations that are correlated with mitochondrial impairment (P. E. Coskun et al., 2004). Interestingly, endurance training has been shown to attenuate or mitigate several metabolic alterations in the cortex of mtDNA mutator mouse, an animal model that mimics physiological aging (Clark-Matott et al., 2015). Also, after a long-term treadmill exercise training program, mtDNA levels were preserved in 3xTg-AD mice, which suggest that mitochondria are adequately protected against ROS-induced mtDNA depletion (Garcia-Mesa et al., 2012).

The activities of oxidative damage enzymes can be considered as a second line of antioxidant defense. Mitochondrial 8-oxoguanine DNA glycosylase-1 (OGG1) activity, an enzyme responsible for the removal of oxidatively damaged bases from mtDNA, was found to be decreased in some regions of AD brains (Shao et al., 2008). Consistently, physical exercise was able to increase mtDNA repair by increasing mtOGG1 content and function in APP/SP1 animals, a transgenic mice model expressing AD phenotypes, including Aβ deposits and behavioral deficits (Bo et al., 2014). These findings suggest that exercise training was able to upregulate mtDNA repair capacity, which in turn attenuates AD-related mitochondrial impairment and phenotypic degradation (Bo et al., 2014). It has been suggested that OGG1 expression and activity may be influenced by intracellular redox status. Reduced mitochondrial glutathione is thought to be important as a posttranslational mechanism to maintain mitochondrial OGG1 active (Circu, Moyer, Harrison, & Aw, 2009). Moreover, a protein-protein interaction between MnSOD and OGG1 have been suggested for OGG1 DNA repairing activity (Bonatto, 2007). In this context, Bo, H. and collaborators (2014) showed that 20 weeks of endurance training increased MnSOD and GPX activities, being this ameliorated modulation of mitochondrial redox status, a potential mechanism involved in exercise-induced mitochondrial OGG1 activity.

Severe altered redox status and mitochondrial malfunction are also believed to interfere with calcium homeostasis, which leads to increased mitochondrial susceptibility to calcium induced mitochondrial permeability transition pore (mPTP) and depolarization, and ultimately to the release of signaling proteins from mitochondria to the cytoplasm that in turn activate apoptotic cell death (Norenberg & Rao, 2007; Toman & Fiskum, 2011). Chronic endurance training augmented the resistance to calcium-induced mPTP opening and decreased apoptotic markers in mouse brain cortex highlighting the relevant protective effect of exercise on events leading to mitochondrial degeneration and cell death (I. Marques-Aleixo, Santos-Alves, Balca, et al., 2015). Accordingly, a decrease in some pro-apoptotic proteins, including Bax, cytochrome c, caspase 3 and 9 was reported in NSE/APPsw transgenic mice model of AD after endurance training (J. Y. Cho et al., 2010; Um et al., 2008). Moreover, decreased levels of caspase 3 and 9 and increased Bcl-2 protein content were also reported in NSE/PS2m transgenic mice model of AD engaged in an endurance training regimen (Um et al., 2011). As extensive neuron loss due to apoptosis is a common feature in AD brain, exercise might be an effective strategy for extending AD neuron survival. In addition, endurance training also upregulates the levels of chaperones in brain tissue, particularly heat shock proteins (HSPs) facilitating protein import, folding and assembly. In fact, an increase in HSP-70 content was found after exercise in AD models (Um et al., 2008). HSP-70s are highly conserved proteins that protect brain cells against excitotoxic and oxidative injury and it is likely that exercise-induced HSP overexpression is mediated by redox-signaling pathways (Fittipaldi, Dimauro, Mercatelli, & Caporossi, 2014). These results suggest that exercise training can mitigate neuronal cell apoptosis implicated in the pathogenesis of AD.

3.4.2 Mitochondrial bioenergetics modulation induced by physical exercise

Mitochondrial adaptations are crucial in exercise-induced neuroprotection. In opposition to contractile-dependent effects on skeletal and cardiac muscles,

physical exercise adaptations in brain tissue are associated to systemic alterations during and after exercise.

Despite some inconsistencies related with the characteristics of the exercise protocols (Stranahan and collaborators (2012), reduced levels of intracellular Aβ and tau phosphorylation have been reported after an exercise intervention in mouse models of AD (reviewed in Stranahan et al., 2012), which suggest that physical exercise can regulate basic mechanisms underlying AD.

The BDNF have been positively associated with structural and functional plasticity of the central nervous system. In fact, BDNF signaling have been linked with healthy brain mitochondria by several distinct mechanisms, namely: i) improving glucose transport and respiratory coupling efficiency of synaptic mitochondria, ii) upregulating antioxidant enzymes iii) mediating PGC-1α-induce mitochondrial biogenesis, and iv) preventing neuronal apoptosis (reviewed in Marosi & Mattson, 2014). Moreover, mitochondrial transport and distribution seems to play an essential role in BDNF-mediated synaptic transmission (Su, Ji, Sun, Liu, & Chen, 2014). However, the expression of this neurothrophin seems to be modulated both by physical activity and by AD in opposite directions (Marosi & Mattson, 2014). Bearing this in mind, it is important to highlight that BDNF plasma levels were positively associated with physical activity levels of AD patients and that an acute bout of aerobic exercise seems to be sufficient to increase BDNF plasma levels in patients with AD (Coelho et al., 2014). Therefore, BDNF-associated mechanisms for improving neuronal bioenergetics might explain, at least in part, the effectives of exercise counteracting AD mitochondrial frailties.

Mitochondrial mechanisms underlying the protective phenotype induced by exercise in brain physiology are still elusive. Nevertheless, some studies report that exercise-induced brain mitochondrial bioenergetics adaptations include increased content and/or activity of several enzymes involved in aerobic energy production (Dietrich, Andrews, & Horvath, 2008; Ding, Vaynman, Souda, Whitelegge, & Gomez-Pinilla, 2006; Kirchner et al., 2008), increased activity or content of OXPHOS complexes (I. Marques-Aleixo, Santos-Alves, Balca, et al.,

2015; Navarro, Gomez, Lopez-Cepero, & Boveris, 2004) and improved mitochondrial ability to produce energy (I. Marques-Aleixo, Santos-Alves, Balca, et al., 2015). These are important metabolic adaptations on the mitochondrial oxidative phosphorylation system that can result in improved ability to oxidize substrates and increased rate of mitochondrial ATP synthesis. On the other hand, AD has been associated with defects in mitochondrial electron transport chain enzymes. In fact, reductions in mitochondrial complex I and complex IV efficiency have been described in AD (Manczak, Park, Jung, & Reddy, 2004; Maurer, Zierz, & Moller, 2000). Since exercise modulates both these mitochondrial respiratory chain components (Navarro et al., 2004), this might also contribute to counteract the development and symptoms of this pathology (I. Marques-Aleixo et al., 2012). Hai Bo and coworkers (2014) showed an increase in mitochondrial complex I, IV and V activities in the APP/SP1 mice model of AD after an endurance training regimen. Although García-Mesa et al. (2012) did not found an increase in protein complexes content with exercise, OXPHOS complexes levels recovered to levels of non-transgenic mice with the combined treatment of exercise plus antioxidant supplementation. Still, these authors did not report complexes activity but rather expression levels, which might explain, at least in part, the results.

Sirtuin-3 (SIRT3) is a mitochondrial deacetylase that modulates the activity of several mitochondrial proteins involved in metabolism. SIRT3 participates in the regulation of mitochondrial energy homeostasis and biogenesis. Additionally, SIRT3 deacetylates and directly activates Mn-SOD and increases NADPH levels, which also increases the pool of available GSH (Bell & Guarente, 2011; Sack, 2011). Moreover, upregulation of SIRT3 can reduce ROS production and therefore reduces apoptosis (or increase neuronal survival) and mitochondrial permeability transition pore (mPTP) induction (H. Du & Yan, 2010). In the context of neurodegenerative diseases, SIRT3 levels have been shown to be depleted in APP/PS1 models, suggesting a role of this protein in the development of AD via mitochondrial dysfunction (W. Yang et al., 2015). In contrast, an increase in SIRT3 protein content and in oxidative phosphorylation coupling was observed in the hippocampus of APP/SP1 AD mice model after endurance training (Bo et al., 2014).

3.4.3 Impact of physical exercise in mitochondrial biogenesis and quality control

Even though some studies were already published on the impact of physical exercise and AD *per se* on mitochondrial biogenesis and quality control, the possible role of exercise against these AD-related mechanisms remains unclear. The proper balance of mitochondrial biogenesis and clearance/renewal is a key determinant to maintain the overall mitochondrial physiology within the neurons (Ghavami et al., 2014). Therefore, the regulation and stimulation of these processes by exercise models may translate into positive outcomes on AD brain mitochondrial metabolic activity. Physical exercise, particularly endurance training, has been suggested to stimulate mitochondrial biogenesis through activation of silent information regulator 1 (SIRT 1) and PGC-1α in the hippocampus (Steiner, Murphy, McClellan, Carmichael, & Davis, 2011; Vina et al., 2009). This is particularly relevant because both genes are downregulated in AD conditions (Intlekofer & Cotman, 2013). Additionally, an improvement in brain cortex mitochondrial function, accompanied by increased biogenesis, fusion of healthy mitochondria and segregation of damaged mitochondria was reported after endurance training (I. Marques-Aleixo, Santos-Alves, Balca, et al., 2015). By increasing the healthy mitochondrial network, exercise possibly interferes on mitochondrial signaling pathways preventing the migration of damaged components into more fit mitochondria. These adaptations reinforce the idea that exercise may improve overall mitochondrial function, thus contributing to mitigate the development and symptoms associated with AD neurodegenerative process.

3.5. Concluding remarks

With the increase in life expectancy, neurodegenerative diseases and other common chronic diseases are becoming considerable prevalent in elderly people. Therefore, AD is a critical issue to be addressed in the context of science and research. Although research continues to make outstanding progress in the understanding of AD etiology, effective pharmacological interventions failed to be identified. Through the modulation of multiple mechanisms related with brain

health, exercise has emerged as a possible non-pharmacological strategy, likely to contribute to a protective phenotype against AD. At least in part, mitochondria are an important target organelle involved in physical exercise-related adaptations. Through the interaction with mitochondrial physiological processes, including redox modulation bioenergetics improvement, increased resistance to mPTP and decreased apoptotic signaling, activation of mitochondrial biogenesis, and the modulation of dynamics and autophagy, the role of physical exercise has been reinforced as a preventive and/or therapeutic strategy to attenuate the negative effects of AD.

CHAPTER 4. [Materials and Methods]

Materials and Methods

4.1. Chemicals

Streptozotocin (S0130), citric acid (C83155) and sodium citrate (W302600) - for citrate buffer preparation, tris-base (T1503), EDTA (E5134), sucrose (84100), BSA (A7030), KCl (P3911), KH_2PO_4 (P5379), HEPES (H3375), ADP (A2754), succinate (S3674), glutamate (G8415), malate (M7397), rotenone (R8875), glutaraldehyde (G5882), sodium cacodylate (C0250), propylene oxide (110205), and epoxy-embedding kit (45359) were purchased at Sigma (St. Louis, Missouri, EUA). Digitonin (300410) and osmium tetroxide (124505) were purchased from Merck Millipore (Burlington, Massachusetts, EUA). Uranyless EM stain (11000) and lead citrate (11300) were from Delta Microscopies (Mauressac, France). Percoll (17-0891-01) was from GE Healthcare (Chicago, Illinois, EUA) and Absolute Ethanol (E/0650DF/C17) from Fisher Scientific (Hampton, New Hampshire, EUA). Xylazine (Rompun® 2%) was from Bayer (Leverkusen, Germany), ketamine (Imalgene 1000) from Merial (Lyon, France), and isoflurane (Isofluo®) was purchased from Esteve (Barcelona, Spain). All compounds were used as received and kept according to the recommendations.

4.2. Animals

Fifty-six Wistar rats (6 weeks old; Charles River Laboratories, L'Arbresle, France) were used in this study. Mean weighing rats was 253.5 ± 13.1g at the beginning of the experiments. To avoid social isolation the animals were not confined to individual housing (Mumtaz, Khan, Zubair, & Dehpour, 2018). Instead, they were group-housed in collective cages (two rats/cage) and maintained on a reverse 12/12 light-dark cycle, temperature (23–24 °C) and humidity (50–60 %). All animals were allowed a*d libitum* access to food (4RF25 Mucedola, Milan, Italy) and acidified tap water (pH ≅ 3.5-4 to avoid potential bacterial contamination). A single animal was considered an experimental unit. Only male rats were used in this study to avoid potential hormone-dependent alterations. Animals were weekly weighed and tracked closely during the procedures. Before the beginning

of the protocol, the animals were acclimatized for 2 weeks. The division of the animals into the different groups was made in a random-manner way as follows (either intracerebroventricular streptozotocin [STZ] or saline [Sal]): sedentary (Sed), voluntary physical activity (VPA), VPA+endurance treadmill training (VPA+ET) and VPA+ET only after the injection (VPA+ET-post) (**Figure 4.1**). Distance covered inside of each running wheel cage was daily recorded to determine the weekly average of voluntary physical activity performed by each rat. Prior to the injection (either Sal or STZ), the running wheel distance from the animals that experienced VPA and VPA+ET during the entire protocol were also recorded.

All experimental procedures were performed in accordance with the European Directive 2010/63/EU, transposed into the National Law (DL n° 113/2013), which concerns the protection of animals used for scientific purposes, and were locally approved by the Ethics Committee of the Research Center in Physical Activity, Health and Leisure (Faculty of Sport, University of Porto), and by the Direção-Geral de Alimentação de Veterinária (DGAV) the national competent authority under project license number 023346. The researchers were accredited by the Federation of Laboratory Animal Science Associations (FELASA) for animal experimentation (class c).

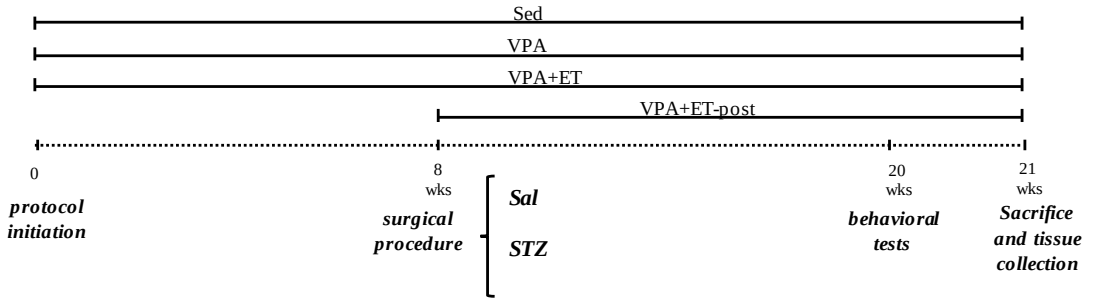


Figure 4.1: General organization of the experimental setup. Random division into groups was performed as follows (either saline [Sal] or streptozotocin [STZ]): sedentary (Sed), voluntary physical activity (VPA), VPA+endurance treadmill training (VPA+ET) and VPA+ET only after the injection (VPA+ET-post). Surgical procedure, behavioral tests and sacrifice occurred 8, 20, and 21 weeks after the protocol initiation, respectively.

4.3. Exercise protocols

Animals from VPA+ET and VPA+ET-post groups were exercised 5 days/week (Monday–Friday) between 9:00 and 12:00 AM (8 weeks prior to the icv injection and/or 12 weeks after, respectively) on a LE8700 motor driven five-lane treadmill (Panlab, Harvard, USA). Treadmill speed and exercise duration were gradually increased over the course of the training period. Briefly, ET protocol included 5 days of treadmill familiarization with 10 min of running at 15 m/min and 0 % grade with daily increases of 5 to 10 min until 30 min was achieved (week 0). Habituation period was followed by continuous running (60 min/d) with 3-min cool-down at a speed of 5 m/min.

The belt speed was gradually increased over the program until 25m/min was reached (week 2). The VPA and ET groups were housed in polyethylene cages equipped with a running wheel (Type 304 Stainless steel (2154F0106-1284L0106) Technicplast, Casale Litta, Italy), with unlimited access. Running wheel distance was recorded using ECO 701 from Hengstler (Lancashire, UK) and records were daily taken.

4.4. Surgical procedure

Rats were submitted to deep general anesthesia by an intraperitoneal (*ip*) injection of ketamine (100 mg/kg)/xylazine (5 mg/kg) and maintained with 1-2 % isoflurane through a nasal cone. Animals from Sal and STZ subgroups (6 to 8 animals *per* group) received a single bilateral icv injection of vehicle solution (0.05 M citrate buffer) or STZ, respectively. All the animals were positioned in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA) and burr holes were drilled in the skull on both sides over the lateral ventricles to perform the icv injection using the following coordinates: 0.8 mm posterior to bregma; 1.5 mm lateral to sagittal suture; 3.6 mm ventral from the surface of the brain (Noble, Wurtman, & Axelrod, 1967). The STZ solution was prepared immediately prior to the administration at the dosage of 3 mg/kg. Both, STZ and vehicle solutions (3 µL) were slowly infused into each cerebral ventricle using a Hamilton

microsyringe. The microsyringe was removed 3 min after the end of the injection and the scalp was saturated. Animals returned to their home cages the day after the surgery and ET animals resumed their respective exercise programs 7 days after the procedure.

4.5. Behavioral analysis

Behavioral tests were performed at 28 weeks of age, one week prior to the end of the protocol. White noise was used to avoid possible stressful interference. Behavioral assessments were videotaped by an overhead camera, and a blind analysis was performed later. Spatial learning and memory, general activity and alternation behavior were assessed in each animal. All the behavioral devices were cleaned with 70 % ethanol and aired between each session.

4.5.1 Morris water maze test

To analyze the spatial and long-term memory, the Morris water maze task was employed (R. Morris, 1984). A swimming-based model in which animals learn to escape a circular pool of water (200 cm in diameter, 50 cm in height, 30 cm in depth with a water temperature of 24 ± 1 °C) by reaching a hidden platform, was used. The indiscernible platform was submerged approximately 1.5 cm below the water surface in a fixed location. The animals (n = 6–8 per group) were trained daily in a 4-trial water maze task for 4 consecutive days. During daily testing, the animals were admitted successively into each of the quadrants in a semi-randomly manner and were allowed to swim. Each trial terminated as soon as the rat reached the hidden platform or after 120 seconds, followed by 15 seconds of rest on the platform. If it failed to find the platform within the 120 seconds, then it was gently guided by a wooden stick until there. The inter-trial interval was 10 min. The maze was in a well-lit white place with several visual stimuli hanging on the walls to provide spatial cues. The latency to find the platform during each trial was measured as an indicator of learning. Twenty-four hours after, a single probe test (60 seconds) without the platform was performed on the fifth day and the time

spent in the target quadrant was measured as an indicator of memory retention (Vorhees & Williams, 2006).

4.5.2 Y-Maze test

Each animal was placed in the center of the Y-maze (3 arms of 41x12x14cm) for 5 min. Testing comprised 1 single session, acquired between 12-16 PM. Herein, they could move freely, and one arm entry was considered when all four paws had entered the arm. Alternations were established as 3 different consecutive arm choices and the alternation behavior percentage was calculated by summing the number of alternations registered and dividing by the total number of arm entries minus 2, times 100 (King & Arendash, 2002). Number and pattern of arm choices were recorded for each animal and analyzed as a measure of general activity and spatial working memory, respectively.

4.5.3 Open field test

Testing comprised 1 single session, acquired between 12-16 PM. An open field apparatus (70x70x28.5 cm) virtually divided into 9 equal squares was used. Animals were placed in the center of the arena and allowed to explore it for 5 min. Crossed lines during sessions, total distance and activity time were counted as a measure of general activity. Rearing time and time spent in the center area was considered a measure of exploration habits. Additionally, anxiety levels were assessed by the freezing time. Line crossing was considered when the rat crosses one of the grid lines which separate the squares with all four paws. Rearing was considered the period leaning against the walls of the maze or standing on hind legs.

4.6. Animal euthanasia and tissues extraction

Forty-eight hours after the end of the endurance training protocol (21 weeks plus 2 days), 12 h fasted rats were anesthetized with an *ip* injection of ketamine (100

mg/kg)/xylazine (5 mg/kg). Brains (not perfused) were quickly removed, and the hippocampus and cerebral cortex were dissected out on ice, washed and weighed. Approximately half of the fresh cerebral cortex was used for mitochondrial respiratory assays. A piece of both prefrontal cortex and hippocampus was processed as described below for further morphological studies.

4.7. Cerebral cortex mitochondrial isolation

Using a Percoll density gradient, rat brain cortical mitochondria were isolated according to the method proposed by Sims *et al.* (Sims & Anderson, 2008) with slight modifications. In brief, after animal decapitation, cerebral cortex was immediately separated and gently homogenized at 4 °C with a 7 mL Dounce Type Homogenizer (8 times with loose pestle and 4 times with tight pestle) in ≅ 3 mL of ice-cold isolation buffer (10 mM Tris-base, 1 mM EDTA, 320 mM sucrose, pH = 7.4). After homogenization the volume was completed until 7 mL with isolation buffer and then centrifuged at 1,300 *g*, for 3 min at 4 °C. Supernatants were then carefully decanted and retained on ice. Pellets were again homogenized with the tight pestle and centrifuged as described above. Resulting supernatants were collected and centrifuged along with the initial supernatants at 15,300 *g*, at 4 °C for 20 min. Meanwhile, different percentages of Percoll solution were prepared for the next step: 40 % (vol/vol) Percoll solution was prepared with isolation medium stock, 3x concentrated (30 mM Tris-base, 3 mM EDTA and 960 mM sucrose, pH = 7.4). The 23 % and 15 % (vol/vol) Percoll solution was prepared from 40 % of Percoll solution and isolation medium, respectively. Supernatants were discarded, and pellets were resuspended in 8 mL of ice-cold 15 % of Percoll solution by gently homogenization with the loose pestle. Using a plastic Pasteur pipette, 40, 23 and 15 % of Percoll solutions were layered at 2, 3, and 2 mL, respectively inside the polycarbonate ultracentrifugation tubes, and the homogenized pellet was added on the top of these layers (2 mL). Tubes were then centrifuged at 30,700 *g* (Beckman Coulter, OPTIMA XL-90, Indianapolis, USA) during 10 min at 4 °C. Non-synaptosomal mitochondria were directly

collected from the 40-23 % transition phase and synaptosomes from 23-15 % transition phase. Synaptosomal fraction, containing synaptosomal mitochondria, were washed with isolation medium (to remove Percoll) supplemented with 0.02 % of digitonin (to release mitochondria) and centrifuged at 15,300 *g*, for 10 min at 4 °C. The supernatant was discarded, 0.5 mg of bovine serum albumin (BSA) was added to the pellet and a last centrifugation of 6,900 *g*, at 4 °C for 10 min was performed. Final synaptosomal mitochondrial pellet was resuspended in the isolation medium and the protein amount was determined using the biuret method calibrated with BSA (Gornall, Bardawill, & David, 1949). Non-synaptosomal mitochondria were obtained as described for synaptosomal mitochondria, without digitonin.

4.8. Measurement of brain cortex mitochondrial oxygen consumption

Brain mitochondrial respiratory function was polarographically measured at 30ºC using a Biological Oxygen Monitor System (Hansatech Instruments, Norfolk, UK). Reactions were conducted in a closed, controlled-temperature and magnetically stirred glass chamber containing 0.25 mg of mitochondrial protein within 0.5 mL of respiration buffer (100 mM KCl, 100 mM sucrose, 2 mM KH_2PO_4, 5 mM HEPES and 10 µM EDTA, pH = 7.4). Both respiratory substrates, glutamate plus malate (10 mM/5 mM) or succinate (5 mM) plus rotenone (3 µM), were added to the medium to energize mitochondria, while ADP (120 nmol/mg protein) was used to induce state 3 respiration. The respiratory control ratio (RCR), which represents a measure of oxidative phosphorylation coupling, was calculated as the ratio between mitochondrial respiration state 3 (oxygen consumption in the presence of substrate and ADP) and state 4 (basal oxygen consumption after ADP has been consumed). The ADP/O ratio, an indicator of oxidative phosphorylation efficiency, was calculated by the ratio between the amount of ADP added and the oxygen consumed during the respiratory state 3. Both indexes were determined according to Chance and Williams (Chance & Williams, 1956). Respiration rates

were calculated considering an air saturated water oxygen concentration at 30°C (236 µM).

4.9. Transmission electron microscopy

To assess brain cortex and hippocampus morphology, tissues were processed for transmission electron microscopy (TEM). Cortex and hippocampus were cut in 1 mm^3 blocks, fixed in 2.5 % glutaraldehyde in 0.2 M sodium cacodylate buffer (pH = 7.4) and then post-fixed in 2 % osmium tetroxide in 0.2 M sodium cacodylate buffer (pH = 7.4) for 2 h. Tissues were then dehydrated in a graded ethanol series (75, 95, and 100 %) and 100 % propylene oxide as a transitional solvent. After dehydration steps, the sample were embedded in Epoxy medium. Ultrathin sections (50–70 nm) were contrasted with Uranyless followed by lead citrate and were afterward analyzed with a Jeol Jem-1400 (JEOL Ltd, Tokyo, Japan) electron microscope operated at 80 kV. The images obtained were collected at original magnifications of 15,000x. The percentage of abnormal mitochondria were analyzed according to the criteria previously established (Ascensao et al., 2005) as follows: 1) no alterations; 2) mitochondria were considered as abnormal only if presented mild focal loss of cristae density, and 3) mitochondria evidencing extensive degeneration or even loss of cristae, intramitochondrial vacuoles, and mitochondria swelling. The total number of mitochondria per photomicrograph was also recorded as well as the total number of synapses (region with a synaptic cleft, pre and postsynaptic density). For this analysis, 5 images *per* animal were taken from two consecutive sections. Regarding lipofuscin granules, the mean area (μm^2) of 10 photomicrographs in each sample was calculated. ImageJ (National Institutes of Health, Bethesda, USA) software package was used to perform the analyzes.

4.10. Statistical analysis

All statistical analyses were performed using GraphPad Prism 7.03 Software (GraphPad Software, San Diego, CA, USA) or Statistical Package for the Social

Sciences version 24 (SPSS Inc., Chicago, Illinois, USA), with statistical significance a priori set at $p<0.05$. Statistical comparisons were performed using a two-way analysis of variance (ANOVA), except for the weekly average distance in the running-wheel, where a repeated-measures ANOVA was used, and for the escape latency profile in the Morris water maze test in which a three-way ANOVA was employed. These comparisons were followed by Bonferroni post-hoc test to compare differences between groups. Quantitative data are given as mean ± standard error of the mean (SEM).

CHAPTER 5. [Results]

Results

5.1 Characterization of the animals

Considering the VPA profile during the first 7 weeks of protocol, the animals that performed endurance training (VPA+ET) also presented higher VPA patterns than those that only had access to the running-wheel (**Figure 5.1**), whereas no significant alterations were found between groups after the surgical procedure.

Figure 2 shows the effects of both STZ treatment and VPA and VPA+ET on body and brain weights alterations. No alterations were observed in the initial and final body weights, excepting on Sal VPA+ET-post *vs*. Sal Sed and VPA counterparts (**Figure 5.2 A, B**). Compared to their saline counterpart, the STZ induced a decrease in final brain weight in Sed group, which was not observed in VPA, VPA+ET, and VPA+ET-post. Additionally, STZ VPA+ET-post animals revealed higher brain weights than STZ Sed animals (**Figure 5.2 C**). Despite no significant differences in final brain-to-final body weight ratio were observed between Sal and STZ animals maintained in Sed conditions, VPA, VPA+ET, and VPA+ET-post increased this ratio in all STZ conditions, and in VPA+ET-post vs. VPA saline group (**Figure 5.2 D**). Exercise effects were observed for final body and brain weight as well as for final brain-to-final body weight ratio. The disease (STZ-AD) effects were noted for brain weight.

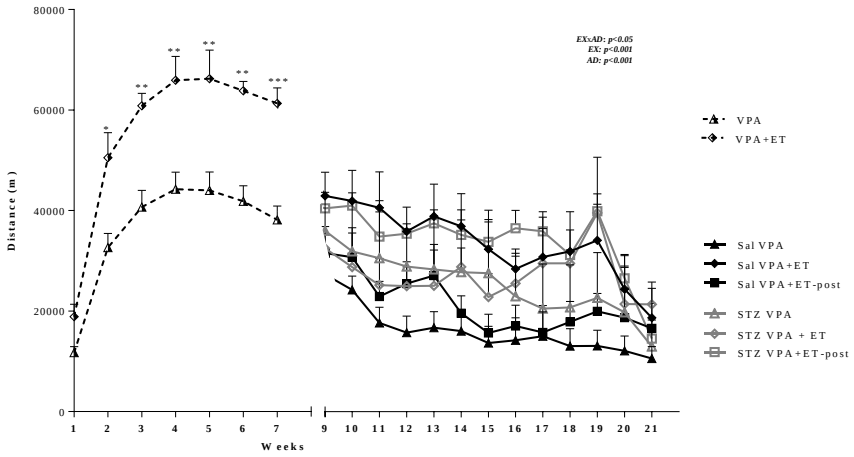


Figure 5.1: Weekly average distance (m) performed inside the activity cage before and after the surgical procedure (week 8). Sal, Saline; STZ, streptozotocin; VPA, voluntary physical activity; VPA+ET, VPA+endurance treadmill training; VPA+ET-post, VPA+ET only after the injection. Data are the means ± SEM; n=6-8 per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Significant exercise (EX), Time (T) and interaction (EX x T) effects are shown, along with their respective *p* values.

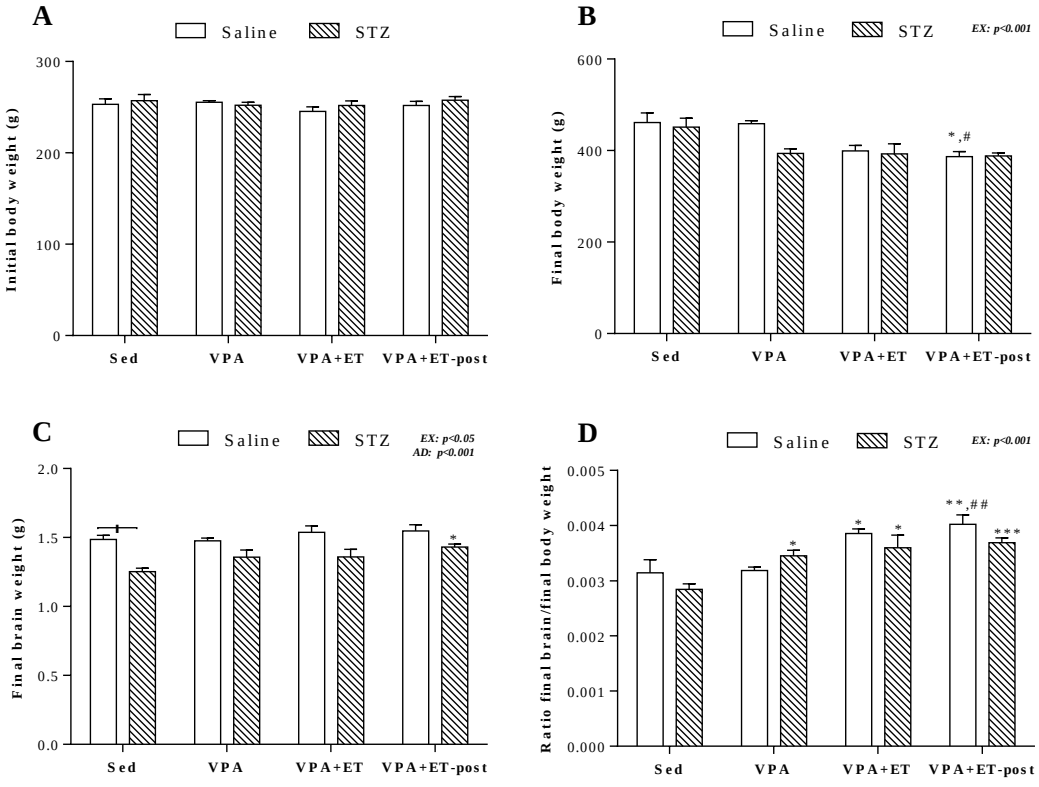


Figure 5.2: Characterization of the experimental groups. (A) initial body weight; (B) final body weight; (C) final brain weight; (D) final brain-to-final body weight ratio. STZ, icv STZ administration; Sed, sedentary; VPA, voluntary physical activity; VPA+ET, VPA+endurance treadmill training; VPA+ET-post, VPA+ET only after the injection. Data are the means ± SEM; n=6-8 per group. *) *vs*. Sed counterpart; #) *vs*. VPA counterpart. One, two, and three repeated symbols correspond to $p<0.05$, $p<0.01$, and $p<0.001$, respectively. Significant exercise (EX) and/or disease (AD) effects are indicated when applicable, along with their respective *p* values.

5.2. Behavioral function

5.2.1 Open-field test

Potential differences in general activity patterns, exploratory habits, and anxiety were assessed near the end of the protocol training using the open-field test (**Figure 5.3**). The STZ induced a decrease in the activity time in Sed, VPA and VPA+ET-post groups, which was not observed in VPA+ET group (**Figure 5.3 A**). Additionally, STZ VPA+ET group revealed higher activity patterns than its Sed counterpart. Rearing frequency results were lower in STZ Sed and STZ VPA groups compared to their Sal homologs, but that condition was counteracted by both ET programs (VPA+ET and VPA+ET-post) (**Figure 5.3 B**). Despite no significant differences were observed in Sed conditions for the time spent on the field central area, less time was spent by STZ VPA vs. Sal counterpart (**Figure 5.3 C**). In addition, more time was spent in the central area for STZ+ET animals when compared to Sed and VPA homologs. Similarly, STZ VPA+ET-post animals spent more time in the central area than their VPA counterparts. Analogous results were obtained for the covered distance and the total number of lines crossed (**Figure 5.3 D, E**). For these behavior-related parameters, no differences were observed within Sed groups, although STZ induced a decrease in both tests (distance and lines crossed) in VPA groups. The STZ VPA+ET and STZ VPA+ET-post animals performed higher distances than their Sed and VPA counterparts, showing similar results compared to their Sal homologs. As depicted in **Figure 5.3 F**, the freezing time was decreased by the endurance training programs (VPA+ET and VPA+ET-post), either for Sal and STZ conditions, although VPA alone was not able to revert the pathological stimulus triggered by STZ. An interaction between exercise and disease effects were observed for all the parameters assessed at the open-field arena (**Figure 5.3 A-F**), while a disease effect alone was observed for activity time, rearing and freezing time (**Figure 5.3 A, B, F**).

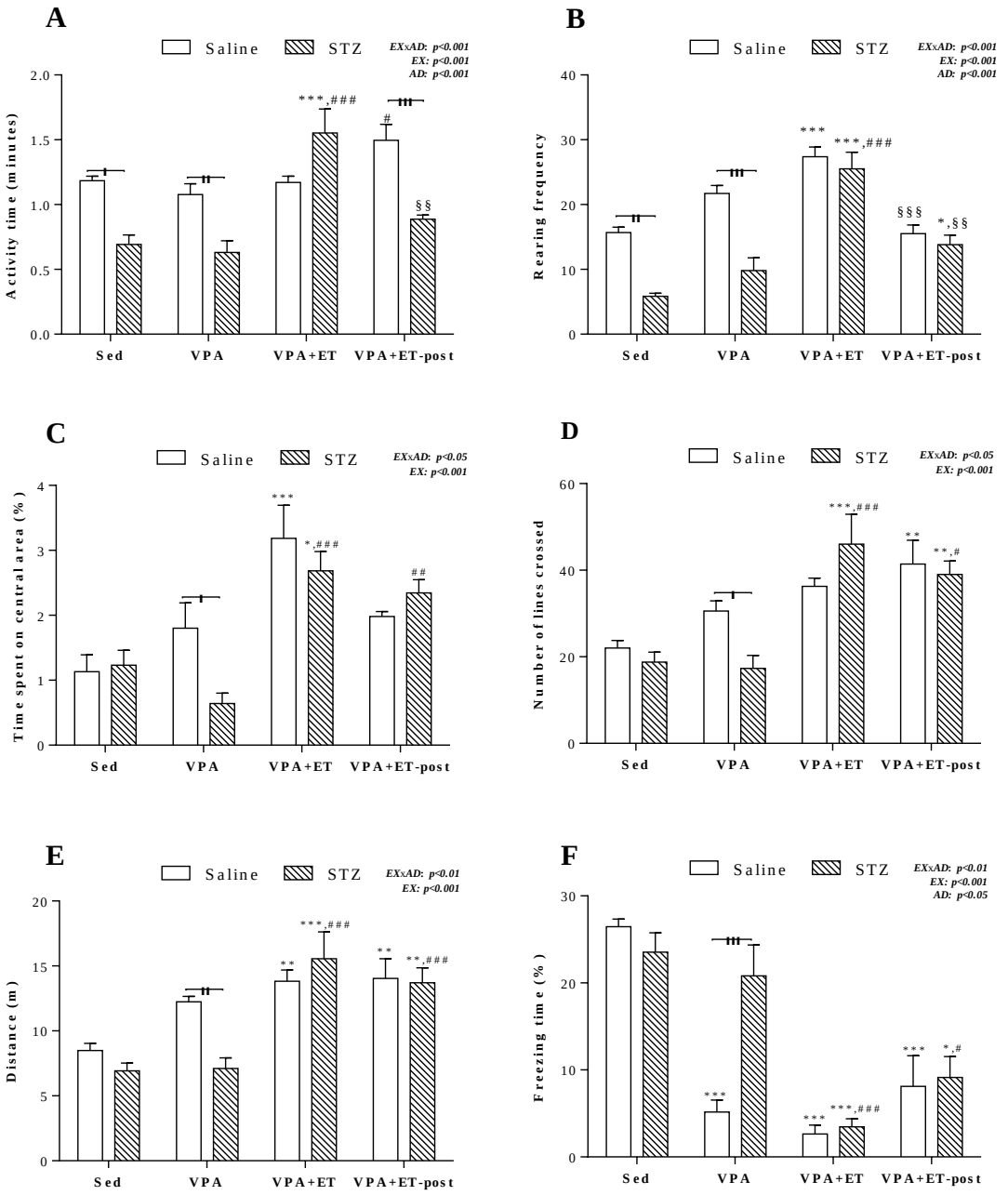


Figure 5.3: Effect of exercise and icvSTZ administration on exploratory behavior, general activity and anxiety – Open field test. (A) activity time, (B) rearing frequency, (C) % time spent on the central area, (D) the number of lines crossed, (E) distance, (F) freezing time. Experimental details are provided in methods. Presented values are means ± SEM; n=6-8 per group. STZ, streptozotocin; Sed, sedentary; VPA, voluntary physical activity; VPA+ET, VPA+endurance treadmill training; VPA+ET-post, VPA+ET only after the injection. *) *vs*. Sed counterpart; #) *vs*. VPA counterpart; §) *vs*. VPA+ET counterpart. One, two, and three repeated symbols correspond to $p<0.05$, $p<0.01$, and $p<0.001$, respectively. Significant exercise (EX), disease (AD) and/or interaction (EX x AD) effects are shown when applicable, along with their respective *p* values.

5.2.2 Y-Maze test

Spatial memory performance, assessed by the Y-maze test, showed a consistent exercise effect regarding the total number of arm entries. In this parameter, both VPA+ET and VPA+ET-post groups (either Sal or STZ) presented a higher number of arm entries compared with Sed counterparts. Moreover, STZ animals from the endurance training groups (VPA+ET and VPA+ET-post) also registered more entries than their STZ VPA homolog (**Figure 5.4 A**). The STZ triggered a decrease in the percentage of spontaneous alternations in both Sed and VPA groups, which were not observed within VPA+ET and VPA+ET-post groups (**Figure 5.4 B**). Indeed, those ET exercised groups (along with VPA) showed a percentage of spontaneous alternations similar to their saline homologs, suggesting that the pathological stimulus induced by the STZ was counteracted by both models of ET, but not by VPA alone.

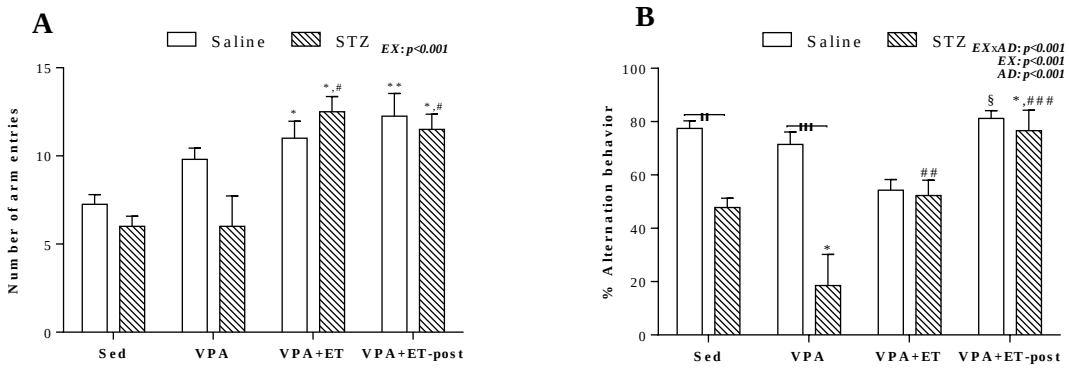
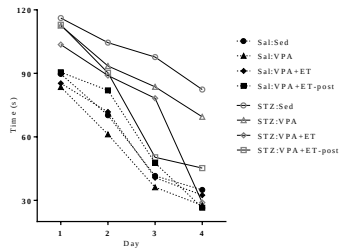


Figure 5.4: Effect of exercise and icvSTZ administration on exploratory behavior and spatial memory – Y-maze test. (A) total number of arm entries, (B) percentage of spontaneous alternations based in arms entries. Experimental details are provided in the methods. Presented values are means ± SEM; n=6-8 per group. STZ, streptozotocin; Sed, sedentary; VPA, voluntary physical activity; VPA+ET, VPA+endurance treadmill training; VPA+ET-post, VPA+ET only after the injection. *) *vs*. Sed counterpart; #) *vs*. VPA counterpart; §) *vs*. VPA+ET counterpart. One, two, and three repeated symbols correspond to $p<0.05$, $p<0.01$, and $p<0.001$, respectively. Significant exercise (EX), disease (AD) and/or interaction (EX x AD) effects are shown when applicable, along with their respective *p* values.

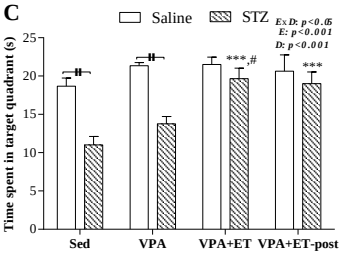
A



B

		Day 1	Day 2	Day 3	Day 4
Sal	**Sed**	89.7 ± 7.5	70.2 ± 8.1	41.6 ± 5.3 [a,b]	35.0 ± 5.1 [a,b]
	VPA	83.6 ± 7.0	61.2 ± 9.0	36.2 ± 6.2 [a,b]	27.7 ± 4.1 [a,b]
	VPA+ET	83.6 ± 10.2	71.8 ± 5.3	40.7 ± 3.7 [a,b]	32.5 ± 6.0 [a,b]
	VPA+ET-post	90.6 ± 7.8	82.0 ± 4.6	47.7 ± 8.1 [a,b]	26.5 ± 5.6 [a,b,c]
STZ	**Sed**	116.1 ± 3.9 [£]	104.6 ± 5.1 [£]	97.7 ± 4.2 [£,‡]	82.4 ± 7.5 [£,$,‡, a]
	VPA	112.6 ± 3.7 [£]	93.5 ± 8.0 [£]	83.6 ± 5.6 [£,‡, a]	69.5 ± 6.5 [£,$, a,b]
	VPA+ET	103.6 ± 3.6	89.0 ± 8.1	78.3 ± 2.8 [£,‡, a]	29.0 ± 8.3 [a,b,c]
	VPA+ET-post	113.0 ± 3.7 [£]	90.3 ± 7.2	50.4 ± 7.5 [a,b]	45.3 ± 9.2 [a,b]

C



D

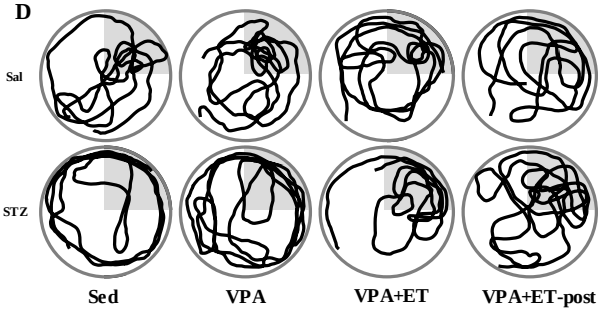


Figure 5.5: Effect of exercise and icvSTZ administration on cognitive performance. Rodents capacity to learn and retrieve was evaluated by the Morris water maze test. (A) Escape latency profile during 4 consecutive days – mean values; (B) average values ± SEM with statistical significances presented for escape latency test; (C) time spent in the area were the platform was previously, after 4 days acquisition trials; (D) illustrative example of the animal's swimming path for 60 seconds probe trial, after 4 days of trials; gray area corresponds to the quadrant where the platform was previously. Values are means of 6 to 8 animals from each condition. Experimental details are provided in the Materials and Methods section. Sal, icvSaline administration, STZ, streptozotocin; Sed, sedentary; VPA, voluntary physical activity; VPA+ET, VPA+endurance treadmill training; VPA+ET-post, VPA+ET only after the injection. £) *vs*. Sal counterpart in the same day; ‡) *vs*. VPA+ET-post in the same day; $ *vs*. STZ:VPA+ET in the same day; a) *vs*. day 1, b) *vs*. day 2; c) *vs*. day 3. *) *vs*. its Sed counterpart, #) *vs*. its VPA counterpart. One, two, and three repeated symbols correspond to $p<0.05$, $p<0.01$, and $p<0.001$, respectively. Significant exercise (EX), disease (AD) and/or interaction (EX x AD) effects are shown when applicable, along with their respective *p* values.

5.2.3 Water-maze test

Evaluating the escape latency through the water-maze test under the present cross-tolerance conditions allowed to assess the exercise and disease effects in spatial memory and long-term memory. During 4-days of acquisition sessions, STZ Sed and STZ VPA animals consecutively took longer times to perform the test compared to the other endurance trained groups (**Figure 5.5 A, B**). This suggests that STZ VPA+ET and STZ VPA+ET-post animals behaved similarly to saline animals, performing the same task in a shorter period. Considering the occupancy in the target quadrant, the decreased time spent in this space by the STZ Sed and STZ VPA groups was reverted by both VPA+ET and VPA+ET-post

(**Figure 5.5 C**). Here, a common aspect to all groups, with the exception of both STZ Sed and STZ VPA, was that they were floating in the quadrant area searching for the platform, while the other two groups (STZ Sed and STZ VPA) spent most of the time swimming aimlessly around the pool (**Figure 5.5 D**). Significant exercise and disease effects, as well as their interaction effects, were observed in this behavioral test.

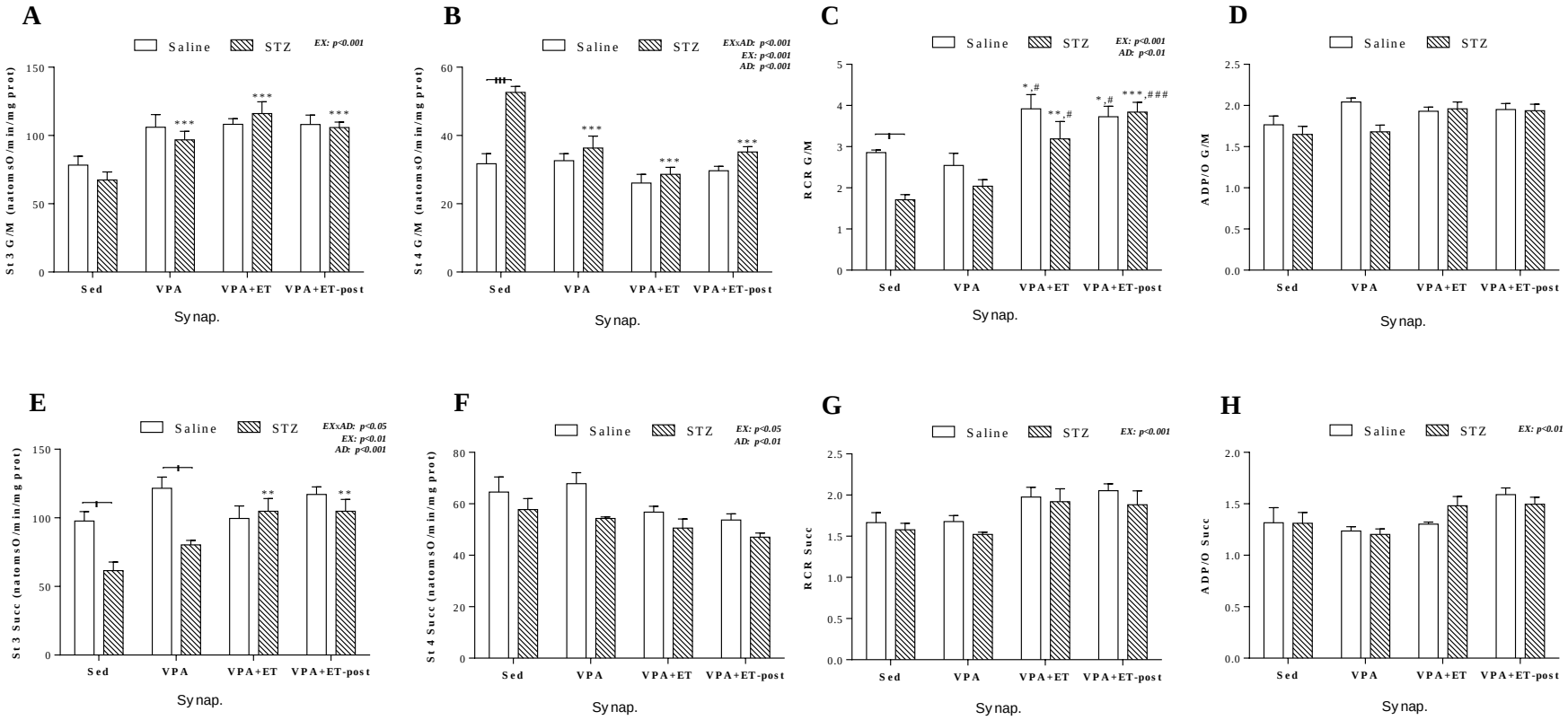


Figure 5.6: Effect of exercise and icv STZ administration on cerebral cortex synaptosomal mitochondrial respiratory parameters with 10 mM glutamate plus 5 mM malate (upper panel; A-D) or 5 mM succinate (bottom panel; E-H). (A) complex I state 3; (B) complex I state 4; (C) complex I respiratory control ratio (RCR); (D) complex I ADP/O ratio; (E) complex II state 3; (F) complex II state 4; (G) complex II RCR; (H) complex II ADP/O ratio. Mitochondria were incubated in 0.5 mL respiration medium (see Materials and Methods section). ADP (30 nmol) was added to induce state 3 respiration. The RCR was calculated as the ratio between sate 3 and sate 4 respiration. The ADP/O ratio was calculated as the number of nmol ADP phosphorylated by natoms of O consumed during ADP phosphorylation. Data are the means ± SEM obtained from individual mitochondrial preparations (0.5 mg/mL protein); n=6-8 per group. STZ, icv STZ administration; Sed, sedentary; VPA, voluntary physical activity; VPA+ET, VPA+endurance treadmill training; VPA+ET-post, VPA+ET only after the injection. *) *vs*. Sed counterpart; #) *vs*. VPA counterpart; One, two, and three repeated symbols correspond to $p<0.05$, $p<0.01$, and $p<0.001$, respectively. Significant exercise (EX), disease (AD) and/or interaction (EX x AD) effects are shown when applicable, along with their respective *p* values.

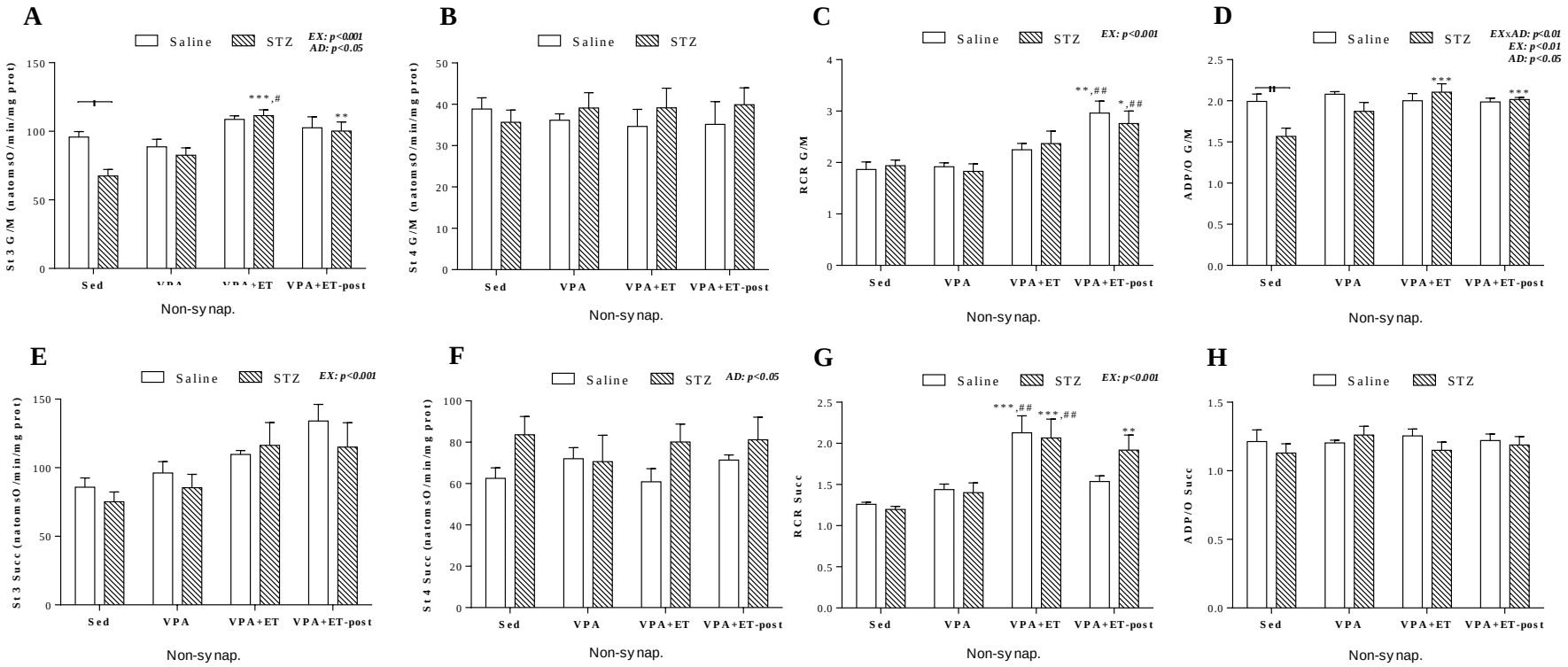


Figure 5.7: Effect of exercise and icv STZ administration on cerebral cortex non-synaptosomal mitochondrial respiratory parameters with 10 mM glutamate plus 5 mM malate (upper panel; A-D) or 5 mM succinate (bottom panel; E-H). (A) complex I state 3; (B) complex I state 4; (C) complex I respiratory control ratio (RCR); (D) complex I ADP/O ratio; (E) complex II state 3; (F) complex II state 4; (G) complex II RCR; (H) complex II ADP/O ratio. Mitochondria were incubated in 0.5 mL respiration medium (see Materials and Methods section). ADP (30 nmol) was added to induce state 3 respiration. The RCR was calculated as the ratio between sate 3 and sate 4 respiration. The ADP/O ratio was calculated as the number of nmol ADP phosphorylated by natoms of O consumed during ADP phosphorylation. Data are the means ± SEM obtained from individual mitochondrial preparations (0.5 mg/mL protein); n=6-8 per group. STZ, icv STZ administration; Sed, sedentary; VPA, voluntary physical activity; VPA+ET, VPA+endurance treadmill training; VPA+ET-post, VPA+ET only after the injection. *) *vs*. Sed counterpart; #) *vs*. VPA counterpart; One, two, and three repeated symbols correspond to $p<0.05$, $p<0.01$, and $p<0.001$, respectively. Significant exercise (EX), disease (AD) and/or interaction (EX x AD) effects are shown when applicable, along with their respective *p* values.

5.3. Mitochondrial oxygen consumption

To evaluate the effect of both physical exercise and icv STZ-induced sporadic AD-related phenotype on the mitochondrial bioenergetic profile, *in vitro* oxygen consumption rates were determined in freshly isolated brain cortex synaptosomal and non-synaptosomal mitochondria, using mitochondrial complex I and complex II energizations substrates, glutamate/malate (G/M) and succinate, respectively.

Synaptosomal mitochondrial respiration parameters with G/M-energizing substrates showed an increased state 4 in Sed STZ compared with Sed Sal group (**Figure 5.6 B**). Moreover, VPA and ET (before and after STZ treatment, combined and isolated) promoted increased state 3 and decreased state 4 in STZ groups (**Figure 5.6 A, B**). These alterations caused by STZ-induced AD phenotype resulted in a compromised RCR (**Figure 5.6 C**), which was not observed in any of the exercised groups. No significant alterations were observed for the ADP/O index in G/M energized synaptosomal mitochondria (**Figure 5.6 D**). As depicted in **Figure 5.6 E**, STZ Sed and STZ VPA animals presented a lower respiratory rate in state 3 when compared to their saline counterparts. Both STZ VPA+ET and STZ VPA+ET-post groups presented an increased state 3 compared to STZ Sed group when mitochondria were energized with succinate (**Figure 5.6 E**). No significant differences were observed between groups for state 4, RCR and ADP/O ratio in succinate-driven respiration of synaptosomal mitochondria (**Figure 5.6 F-H**). Nevertheless, STZ effect was observed for state 4 and exercise effect was observed for state 4, RCR and ADP/O respiratory endpoints.

Results from freshly isolated non-synaptosomal mitochondria revealed a decrease in the state 3 respiratory rate in the STZ Sed vs. Sal Sed, an impairment that was reverted with VPA, VPA+ET, and VPA+ET-post. Moreover, in STZ-AD condition, VPA+ET and VPA+ET-post increased state 3 respiratory rates compared with Sed group, and VPA+ET also increased state 3 respiratory rates, compared with the VPA group (**Figure 5.7 A**). No significant alterations were observed between groups in state 4 respiration using complex I energizing substrates G/M (**Figure 5.7 B**). Despite no alterations in Sed and in all exercised

groups induced by STZ were observed, the RCR in VPA+ET-post (both Sal and STZ) was higher than their Sed and VPA counterparts (**Figure 5.7 C**). Moreover, decreased ADP/O ratio was observed in STZ Sed *vs.* Sal Sed, which was reverted with STZ VPA+ET and STZ VPA+ET-post groups (**Figure 5.7 D**).

Despite no differences between groups and treatment conditions were observed in state 3, state 4 and ADP/O ratio of non-synaptosomal mitochondria energized with succinate (**Figure 5.7 E,F,H**), both Sal and STZ VPA+ET groups presented higher RCR compared with their Sed and VPA counterparts, while STZ VPA+ET-post group had an increased RCR compared to STZ Sed (**Figure 5.7 G**). Nevertheless, the exercise and AD effects were observed in state 3 and in state 4 (when using succinate), respectively.

5.4. Histomorphological analysis

Semi-quantitative and qualitative ultrastructural analyses of electron micrographs obtained from brain cortex and hippocampus were performed.

Hippocampus micrographs showed that STZ Sed animals presented general signs of damage, including the presence of lipofuscin-like granules and an increased number of swollen mitochondria (**Figure 5.8 A, B**). The VPA, VPA+ET and VPA+ET-post animals showed an increase in the number of good-shaped mitochondria (score 1) and a decrease in extensively damaged mitochondria (score 3) in icv STZ treated rats when compared with STZ Sed group (**Figure 5.8 B**). An increase in mitochondrial number was observed in both STZ exercised models compared to STZ Sed (**Figure 5.8 C**).

Photomicrographs also demonstrated that STZ Sed hippocampus presented a higher area of lipofuscin deposits compared to Sal Sed, which was reverted in VPA, VPA+ET, and VPA+ET-post. Moreover, in these physically active/exercised groups (VPA, VPA+ET, and VPA+ET-post), marked diminished lipofuscin accumulation was noted in both Sal and STZ-AD conditions (**Figure 5.8 D**). The STZ Sed animals showed fewer synapses number when compared to their Sal Sed counterparts. Importantly, in both Sal and STZ-AD conditions, increased

synapses number were noted for all exercised groups (VPA, VPA+ET, and VPA+ET-post) compared to their Sed counterparts (**Figure 5.8 E**).

The STZ administration along with a Sed status prompted deep mitochondria vacuolization and a reduction in the synapses number observed on the prefrontal cortex (**Figure 5.9 A**, **B**, **D**). A slight improvement in mitochondrial quality was observed in VPA STZ group when compared to Sed, while further improvements were observed in both ET-exercise regimens in STZ-treated animals (**Figure 5.9 A**, **B**). The number of mitochondria found *per* photomicrograph, as well as the number of synapses, increased in both STZ ET-exercised groups when compared to Sed counterparts (**Figure 5.9 C**, **D**).

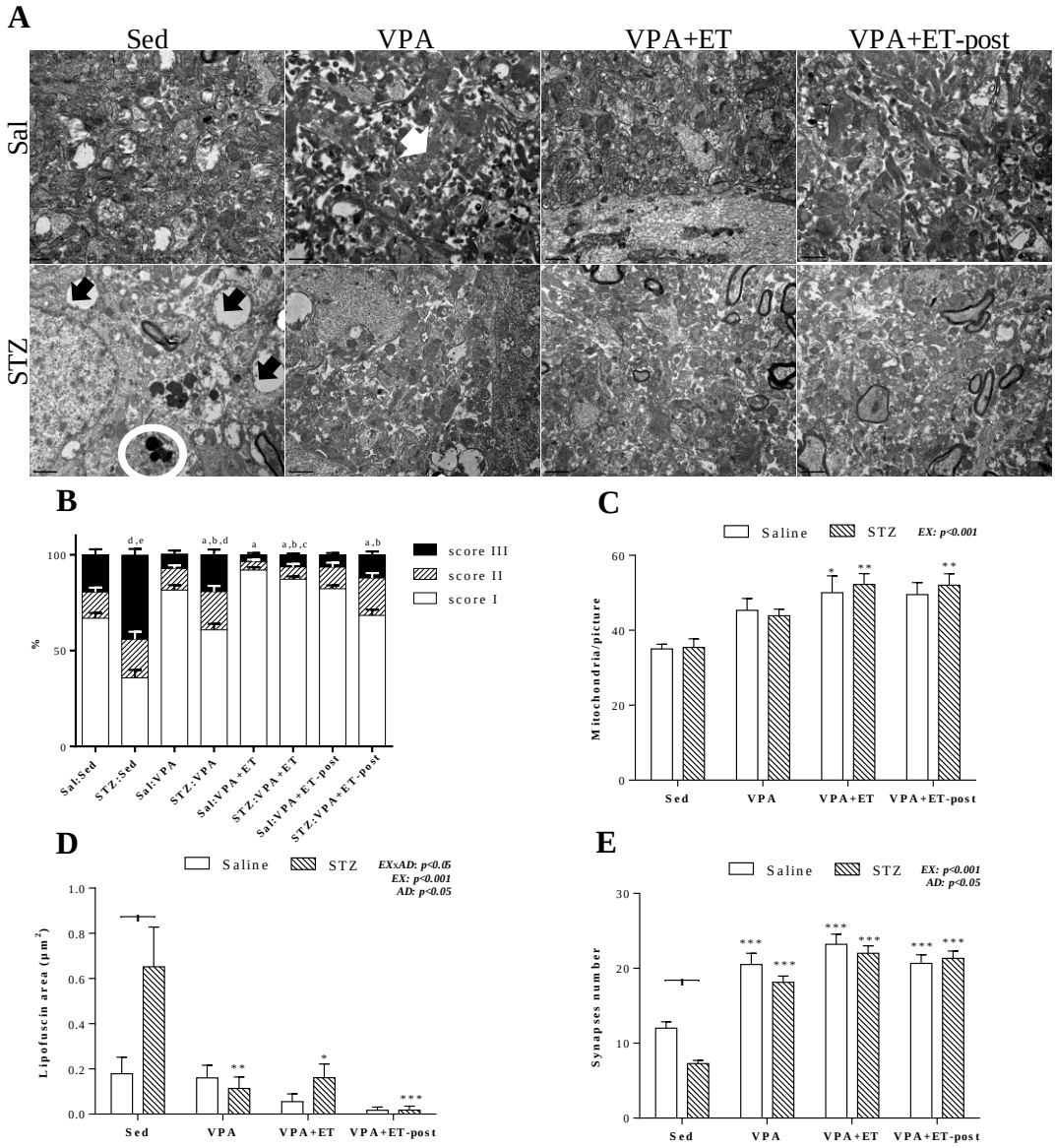


Figure 5.8: Effect of exercise and icv STZ administration at hippocampus ultrastructural level. (A) Representative electron photomicrographs of the hippocampus; (B) percentage of hippocampus mitochondrial alterations; (C) hippocampus mitochondrial density (number); (D) lipofuscin granules area (µm²) at the hippocampus and (E) synapses density (number) at the hippocampus. STZ, icv STZ administration; Sed, sedentary; VPA, voluntary physical activity; VPA+ET, VPA+endurance treadmill training; VPA+ET-post, VPA+ET only after the injection. At photomicrographs, scale bar represents 1µm, the white filled arrow indicates good shaped mitochondria and the black filled arrow indicates mitochondria vacuolization, the white circle indicates an extensive accumulation of lipofuscin granules (residual bodies). The percentage of abnormal hippocampal mitochondria were analyzed according to the score-based criteria previously established: score 1) no alterations; score 2) mitochondria were considered as abnormal only if presenting mild focal loss of cristae density; score 3) mitochondria evidencing extensive degeneration or even loss of cristae, intramitochondrial vacuoles, and mitochondria swelling. Values are expressed as means ± SEM); n=6-8 per group. a) *vs*. Sed counterpart for score I; b) *vs*. Sed counterpart for score III; c) *vs*. VPA counterpart for score I, d) *vs*. Sal for score 1; e) *vs*. Sal group for score 3; $p<0.001$ for all mitochondrial alterations (%). *) *vs*. Sed counterpart. One, two, and three repeated symbols correspond to $p<0.05$, $p<0.01$, and $p<0.001$, respectively. Significant exercise (EX), disease (AD) and/or interaction (EX x AD) effects are shown when applicable, along with their respective *p* values.

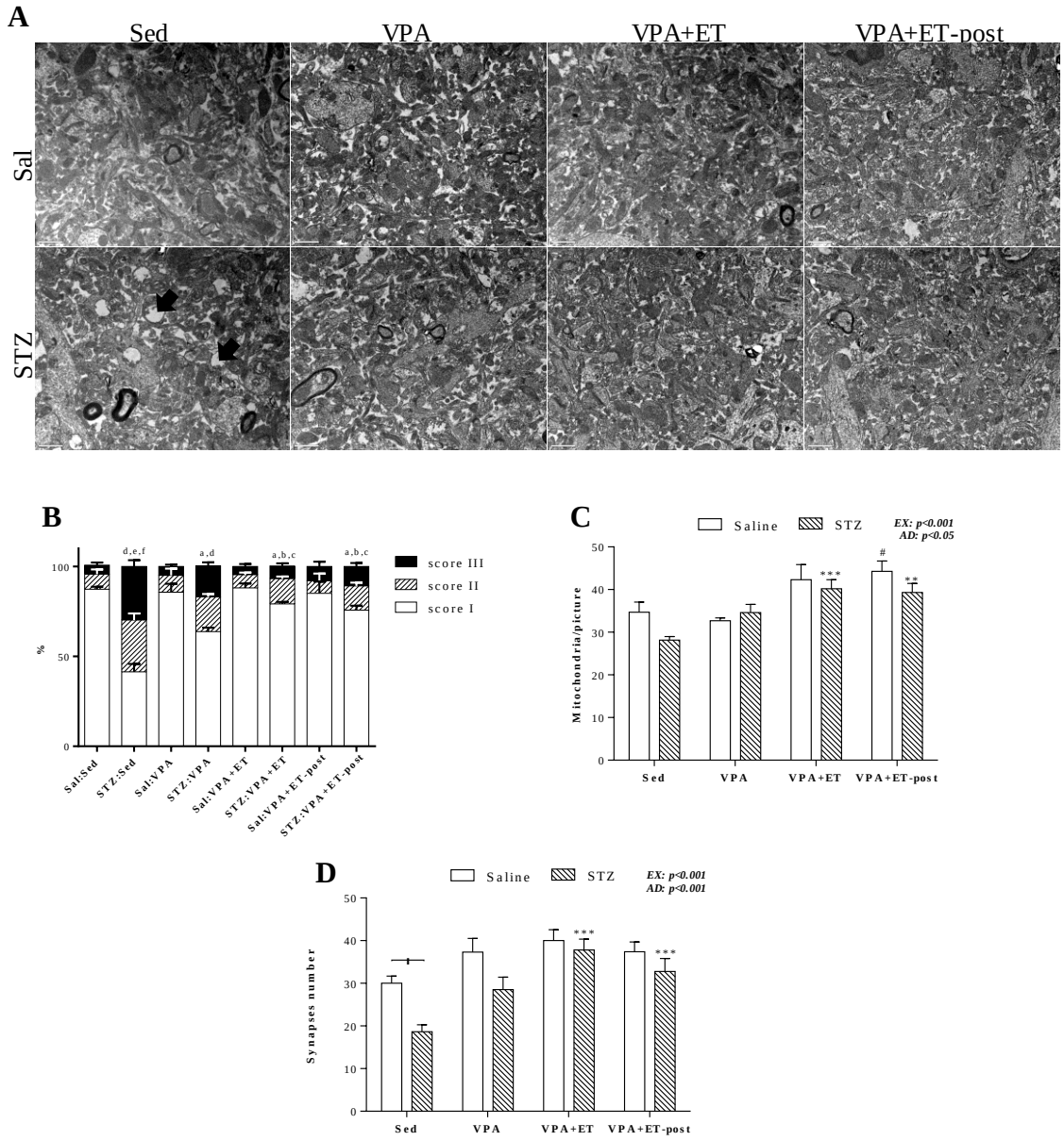


Figure 5.9: Effect of exercise and icv STZ administration at brain cortex ultrastructural level. (A) representative electron photomicrographs of the frontal cerebral cortex; (B) percentage of brain mitochondrial alterations; (C) brain mitochondrial density (number); (D) synapses density (number) at the prefrontal cortex. STZ, icv STZ administration; Sed, sedentary; VPA, voluntary physical activity; VPA+ET, VPA+endurance treadmill training; VPA+ET-post, VPA+ET only after the injection. At photomicrographs, scale bar represents 1µm and black filled arrow represents mitochondria vacuolization. The percentage of abnormal brain mitochondria were analyzed according to the score-based criteria previously established: score 1) no alterations; score 2) mitochondria were considered as abnormal only if presenting mild focal loss of cristae density; score 3) mitochondria evidencing extensive degeneration or even loss of cristae, intramitochondrial vacuoles, and mitochondria swelling. Values are expressed as means ± SEM); n=6-8 per group. a) *vs*. Sed counterpart for score I; b) *vs*. Sed counterpart for score II; c) *vs*. Sed counterpart for score III; d) *vs*. Sal for score 1; e) *vs*. Sal for score 2; f) *vs*. Sal for score 3; $p<0.05$ for all mitochondrial alterations (%). *) *vs*. Sed counterpart; # *vs*. VPA counterpart. One, two, and three repeated symbols correspond to $p<0.05$, $p<0.01$, and $p<0.001$, respectively. Significant exercise (EX) and disease (AD) effects are shown when applicable, along with their respective *p* values.

CHAPTER 6. [Discussion]

Discussion

As earlier mentioned in this thesis, the prevalence of AD and dementia is increasing, and no pharmacological treatment is still available (Song et al., 2018; Zucchella et al., 2018). It is known that physical exercise prevents and/or ameliorate the effects of a variety of diseases, including AD although the precise mechanism that leads to these positive adaptations remains elusive. Thus, rodent models have been extensively used to explore those unveiled mechanistic signaling pathways potentially involved in the effects of the distinct exercise-based strategies to mitigate the disease.

The icv injection of STZ is among the non-transgenic models to study the development of AD with a sporadic origin – which accounts for ≅ 95 % of all cases (Martin-Maestro et al., 2017; Ravelli, Rosario, Camarini, et al., 2017). The injection of STZ in both lateral cerebral rat ventricles allows a relevant model of chronic brain dysfunction that is characterized by long-term and progressive deficits in learning, memory, and cognitive behavior (Ravelli, Rosario, Camarini, et al., 2017; Salkovic-Petrisic et al., 2011). Indeed, the pathophysiological similarity of the icvSTZ-injection effects in animals and the pathological features observed in AD patients led to the extensive use of this model of sporadic AD in several studies (Lu et al., 2017; Zhao, Yang, Jin, Ma, & Feng, 2015).

While animal studies using endurance training as a therapeutic tool against the progression of AD consistently report positive outcomes for both transgenic and non-transgenic models, the effects of voluntary physical activity performed in running wheels does not seem to be so straightforward (Rao et al., 2015; Tapia-Rojas, Aranguiz, Varela-Nallar, & Inestrosa, 2016; Z. Q. Xu et al., 2013). Adlard and colleagues (Adlard et al., 2005) reported a reduction in amyloid plaque load and improved water maze learning in TgCRND8 mice promoted by the voluntary exercise, while Wolf and collaborators did not find any improvement neither regarding Aβ load nor in spatial learning in APP23 mice when running-wheel was used as a model to voluntarily exercise (Wolf et al., 2006). Richter and co-workers hypothesized that these conflicting findings might be caused by differences in either the onset of the access to the running wheel or in the period with free

access to the running wheel (Richter et al., 2008). In fact, recently Herring and affiliates demonstrated that in TgCRND8 mice, the earlier the running-wheel is acessible, the more pronounced is the therapeutic potential prompt by the voluntary physical activity (Herring et al., 2016). Moreover, very recent data from our group showed that self-paced free-running wheel mimics high-intensity interval training impact on rats' functional, physiological, and biochemical and morphological features of the skeletal muscle (Beleza J., 2019), which is in line with previous data also published by our group suggesting that endurance training, but also voluntary physical activity *per se* were able to mitigate doxorubicin-related impairments in brain cortex and cerebellum mitochondrial activity and apoptotic signaling (I. Marques-Aleixo et al., 2016).

To our knowledge, few studies have explored the impact of physical exercise in non-transgenic AD-like models, and even fewer explored its potential preventive role. Herein, we observed lower brain weights in sedentary icv-STZ-treated animals than in saline-treated rats (**Figure 5.2**). Yet, this condition was alleviated by chronic endurance training, although with a statistical difference presented just for VPA+ET-post group. Moreover, the brain-to-body weight ratios increased in the ET animals compared to those maintained under Sed conditions. These results are in agreement with some reports in human subjects over 60 years-old, in which an aerobic program session of 1h, 3 times/wk for 6 months increased the volume of gray and white matters in the cortical regions (Abd El-Kader & Al-Jiffri, 2016; Abe, 2012).

Regarding the impact of exercise, it was interesting to observe that the animals that were submitted to the treadmill endurance training were those that ran higher distances in the running wheel in the first 7 wks of protocol (**Figure 5.1**). In this sense, it seems that the treadmill endurance training stimulus prompt the animals to be more active even when they are not forced to run. Furthermore, it is in accordance with the idea that people that are seriously commited with training regimens impose a more active and healthy way of life for themselves and for their descendency.

In what concerns to the behavioral tests, no differences were observed between exercise models for Sal animals in each acquisition session day (**Figure 5.5 A, B**). Moreover, on the first water maze trial day, all the Sal animals reach the platform in less than 100 s, while all the STZ animals took on average much more than 100 s to achieve the same goal. However, after 4 days of trials, STZ ET groups reached the platform in the same time as Sal groups, whereas both STZ Sed and STZ VPA needed longer times to accomplish the same task. These data suggest that, on one hand the disease model was successfully implemented, as differences were noted in the first trials day between STZ and Sal, and on the other hand that ET was able to reverse the deleterious effects triggered by the STZ, considering the time to reach the platform in the last acquisition trial day.

Despite some studies on the topic, controversial results exist regarding the effectiveness of the different exercise types, protocols and durations. Actually, twelve days of voluntary running delayed the cognitive and non-cognitive impairments triggered by the pathology induced by icv-administrations of exogenous amyloid peptide (Q. Wang et al., 2013). Others showed that 4 weeks of treadmill exercise (30min/day; 5days/wks) also improved short-term memory by enhancing neurogenesis in amyloid beta-induced AD rats (Kim et al., 2014). Moreover, treadmill endurance training, with an exercise program as short as 20 min/day during 4 weeks, implemented after an STZ-icv-treatment mitigated the spatial learning ability impairments (Sim, 2014). On the other hand, 4 weeks of voluntary wheel running exercise after an icvSTZ administration was not enough to revert memory, learning and cognitive impairments in this AD model (Muller et al., 2012).

Our findings suggest that even life-long unlimited voluntary access to the running wheel it's not enough to revert the cognitive and phenotypic deficits associated with AD pathology triggered by the icvSTZ administration, whereas chronic treadmill endurance training (either lifelong or in later-life) seems to be able to prevent and reverse these impairments. Taken together, our results show that STZ triggers an acute pathological AD-like stimulus that is only reversed by ET, but not by the voluntary physical activity (*per se*) carried out in a running wheel. Considering data from other studies, including from our group (Beleza J., 2019),

regarding the impact of voluntary physical activity performed in free running wheels against several deleterious constrains, it is possible that tissue specificity, sensitivity of the techniques for data collection, susceptibility of the analyzed signaling pathways to distinct stimuli, and eventually different exercise regimens (time point and duration) can explain, at least in part, the discrepancies found in several studies (I. Marques-Aleixo et al., 2016). In line, as Muller and collaborators also hypothesized, the difference between these results could be partially explained by the influence of different exercise regimens on brain responses (Muller et al., 2012). However, considering the above-mentioned studies, it seems that the benefits promoted by the different regimens of physical exercise are dependent on the specific model of AD used in the studies. Therefore, special care is needed in the future, not only regarding results' interpretations but also concerning the use of experimental designs. In fact, the selected disease model could influence the adaptations promoted by exercise programs and consequently different inferences can be made.

A significant decline in bioenergetic function was already demonstrated in brains of icv STZ rats (Correia et al., 2013). Yet, treadmill exercise attenuates STZ-induced mitochondrial dysfunction seen both by an increase in cytochrome *c* oxidase activity and ATP synthesis (Lu et al., 2017). In agreement, we found positive alterations in *in vitro* mitochondrial oxygen consumption endpoints including ADP-driven state 3 respiration, state 4 and respiratory control ratio when complex I (glutamate/malate) and complex II (succinate)-related substrates were used in brain synaptosomal and non-synaptosomal mitochondria isolated from ET animals (**Figure 5.6** and **5.7**). These results extend mitochondrial-related adaptations resulting from physical exercise previously reported in transgenic animals (Bo et al., 2014; Koo, Kang, Oh, Yang, & Cho, 2017). Although we do not have conclusive data to assess whether the state 4 drop resulted in an improvement in the overall cell and mitochondrial metabolism, it appears to have contributed to a better coupling between the consumed oxygen and the phosphorylated ADP. Actually, there are results suggesting that mild-uncoupling may have a regulatory effect on mitochondrial function in non-ADP stimulating conditions through decreased ROS production (Caldeira da Silva, Cerqueira,

Barbosa, Medeiros, & Kowaltowski, 2008; Skulachev, 1996). Further studies are needed to clarify whether, in these cross-tolerance conditions involving sporadic AD and physical exercise combinations, mild increased coupling through decreased state 4 respiration might influence cellular and mitochondrial metabolism and redox state.

Deep mitochondrial morphological and morphometric changes have been reported in different brain regions, including in neurons from the hippocampus and neocortex from AD-patients (Baloyannis, 2011; Cadonic et al., 2015; Zhu, Perry, Smith, & Wang, 2013). Swollen and round mitochondria with occasional total loss of the inner structure are common features in neurons from AD biopsied brain and in M17 cells expressing Swedish AβPP mutant where excessive mitochondrial fission occurs (Baloyannis, 2011; X. Wang et al., 2008). Furthermore, mitochondrial mass is also significantly reduced in AD-patients' neurons (Baloyannis, 2011). Here, we assessed frontal-cortex and hippocampal morphology and found that although mitochondrial density (number) was similar between Sal and STZ-Sed groups, mitochondrial quality was dramatically altered in those groups (**Figure 5.8** and **5.9**). Also, the deep damaged (swollen) mitochondria and the synapses number reduction found in Sed STZ-infused rats were not observed in ET groups, which suggest a mitigating effect of exercise against the deleterious features associated with sporadic AD (**Figure 5.8** and **5.9**). Since synaptic dysfunction and synaptic loss are early events in the pathogenesis of AD (H. Du, Guo, & Yan, 2012) and the hippocampal synaptic function is crucial for learning ability and memory function (Eichenbaum, 2004; Kim et al., 2014), these results could explain, at least in part, the cognitive and behavioral improvements observed in the exercised animals of this study (Figure **5.3** to **5.5**). Some authors also support the hypothesis that lipofuscin aggregates may have an active rather than a passive role in the neurodegeneration process, particularly in AD pathology (Giaccone, Orsi, Cupidi, & Tagliavini, 2011; Moreno-Garcia, Kun, Calero, Medina, & Calero, 2018). In agreement, we found that Sed STZ-infused animals presented increased levels of lipofuscin deposits compared to Sal animals and that these larger agglomerates of lipofuscin granules were prevented by ET and VPA (**Figure 5.8**).

Studies from AD transgenic mice models and *post-mortem* human AD brains revealed several defects in cellular bioenergetics, alterations in antioxidant activity and increased ROS production (Garcia-Mesa et al., 2016), which are biological phenomena closely associated with mitochondrial physiology. Under physiological conditions, ROS are involved as important signaling molecules in distinct processes, such as immune response, inflammation, as well as synaptic plasticity, learning and memory (Schieber & Chandel, 2014). However, when produced in excess, those molecules can induce enhanced oxidative stress, and consequent proteins and DNA damage, as well as lipid peroxidation, being the mitochondrial structures first targets of toxicity (K. Leuner et al., 2012). In contrast, cumulative evidence support that physical exercise induces positive modulation of ROS production, antioxidant systems and related redox balance, which play a pivot role in the improvement of cognitive function and increased neurogenesis (Garcia-Mesa et al., 2016). Being the central nervous system particularly sensitive to abnormal oxidative stress conditions, studies confirmed that markers of lipid peroxidation are elevated in AD (Castellani et al., 2002). Conversely, the activity of antioxidant enzymes, such as superoxide dismutase (SOD) and glutathione peroxidase (GPX) was increased in stem and corpus striatum after an exercise program (Schrag et al., 2013). Accordingly, García-Mesa and co-workers showed that 3 months of physical exercise restored 3xTg-AD brain antioxidant capacity back to the levels of those non-transgenic mice (Garcia-Mesa et al., 2016). Despite the mentioned differences in the used AD models and chronic physical exercise regimens between these and our studies, it is possible that a positive redox modulation may be among the important mechanisms that justifies the obtained favorable outcomes regarding behavioral, morphological and mitochondrial function features in the studies presented in this thesis. Accordingly, other studies also correlate oxidative stress with AD-like pathology and the AD-related behaviors of apathy, anxiety, and cognitive loss (Garcia-Mesa et al., 2016). In fact, based in our results supporting that chronic ET (either lifelong or later-life) was able to prevent and reverse cognitive and phenotypic impairments associated with AD (**Figure 5.3** to **5.5**), we can speculate

that, at least in part, this amelioration can derive from improvements at antioxidant capacity level, corroborating the above referred hypothesis.

As mentioned earlier, in addition to the implications of mitochondrial physiology in cellular energy supply and redox status, mitochondrial function is involved in other important processes, such as cellular death, regulation of fusion, fission and biogenesis mechanisms, and associated to quality control through autophagy (DuBoff, Feany, & Gotz, 2013; Nixon, 2013). In AD subjects, apart from increased oxidative stress, an increase in neuronal death by apoptosis is also observed, which further supports the idea that mitochondria may trigger the abnormal onset of neuronal degeneration and death in AD (P. I. Moreira, Cardoso, Santos, & Oliveira, 2006). Besides, *in vitro* and *in vivo* models of AD have demonstrated excessive fission of mitochondria along with accumulation of dysfunctional mitochondria (Martin-Maestro et al., 2017; Nixon, 2013). Moreover, increased levels of Drp1, and reduced expression levels of genes related to mitochondrial biogenesis, including PGC-1α, TFAM, and NRF2 were observed in the brains of AD patients and mice (D. H. Cho et al., 2009; Godoy, Rios, Zolezzi, Braidy, & Inestrosa, 2014; Rice et al., 2014). Likewise, studies in which mitochondrial biogenesis was inhibited by RNA interference-mediated knockdown of PGC-1α suggest important roles for mitochondria in the formation of synapses in developing neuronal circuits, and for the maintenance of synapses in the adult hippocampus (Cheng et al., 2012). In line with the role of impaired PGC-1α in AD pathology, upregulation of PGC-1α can inhibit AD progression. In APP23 transgenic mice, the induction of PGC-1α gene with lentiviral vector-hPGC-1α injected in the hippocampus and cortex areas, decreased Aβ plaques and improved spatial and recognition memory (Katsouri et al., 2016).

Additionally, autophagy dysfunction in the brain of AD patients is widely documented (Martin-Maestro, Gargini, Perry, Avila, & Garcia-Escudero, 2016). This has been mainly related to the reduced degradative function, insufficient lysosomal pH acidification, and low hydrolase activity, impairing the recycling of damaged mitochondria and generating a mitophagy failure. However, the downregulation of genes involved in auto/mitophagy in AD was also already reported (D. S. Yang et al., 2011). Results from biological models of AD and from

subjects with sporadic late-onset AD suggest that impaired mitophagy contributes to synaptic dysfunction and cognitive deficits by triggering both Tau and Aβ accumulation through increases in oxidative damage and cellular energy deficits, establishing a vicious cycle. Again, despite not measured in the present study, signaling protein markers and/or other measures of these processes would be welcome in further studies using the present setup conditions regarding the cross-tolerance of the used exercise and AD models.

Interestingly, it seems that autophagy activation is also triggered during exercise. He and collaborators showed that treadmill exercise induces autophagy in the cerebral cortex of adult mice (He, Sumpter, & Levine, 2012). Further studies showed that treadmill exercise positively regulates autophagy by upregulating SIRT1 levels and AMPK activation in rat brain (Jeon, 2016). Furthermore, physical exercise seems to increase the density of organelles and to improve the efficiency/function of the mitochondrial network. The resulting functional improvement in the mitochondrial pool most likely results from mitochondrial biogenesis increased rates and efficient removal of impaired/damaged mitochondria (Steiner et al., 2011). Indeed, chronic treadmill running over 12 weeks increased PGC-1α expression in various areas of the mouse brain (Steiner et al., 2011). Exercise may trigger mitochondrial biogenesis in neurons through a mechanism involving BDNF signaling and upregulation of PGC-1α, a pathway that plays critical roles in synapse formation, maintenance and plasticity (Cheng et al., 2012). Thus, exercise may increase the number of well-functioning mitochondria in neurons by triggering pathways that stimulate both, mitochondrial biogenesis and mitophagy. As alterations in mitochondrial dynamics and biogenesis reflect mitochondria architecture and mass changes, we anticipate that the mitochondrial morphological improvements (**Figure 5.8** and **5.9**) promoted by exercise in the studies comprised in the present thesis had an impact on mitochondrial dynamics and biogenesis.

Evidence from AD *post-mortem* brains also showed a decrease in ATP levels and reduced respiratory chain complexes I, III, IV, and V activity and content (Bosetti et al., 2002), and in most neurons intact mitochondria are numerically reduced (Swerdlow, 2012). Positron emission tomography (PET) brain scans in living AD

patients also revealed diminished radiolabeled glucose uptake into neurons and biochemical analyses demonstrated reduction of the mitochondrial enzymes' activity involved in the TCA cycle and OXPHOS (Kapogiannis & Mattson, 2011). Eventually through auto(mito)phagy process impairment, dysfunctional mitochondria accumulate in neurons resulting in reduced cellular ATP levels and excessive ROS production, which can exacerbate mitochondrial damage, leading to the aberrant amyloidogenic processing of APP and pTau and subsequent formation of Aβ plaques and neurofibrillary tangles (Mattson, 2004; M. P. Mattson, M. Gleichmann, & A. Cheng, 2008). The Aβ plaques accumulation increases mitochondrial network impairment and leads to synaptic dysfunction, where the demand for ATP is critical for synaptic transmission (Norambuena et al., 2018).

Exercise is also an important modulator of neurotrophins, including IGF-1 and VEGF (Rendeiro & Rhodes, 2018; So et al., 2017), which are critical for nerve growth and brain nutrition supply (Echeverria, Barreto, Avila-Rodriguezc, Tarasov, & Aliev, 2017; Westwood et al., 2014). This is of importance as lower IGF-1 levels are associated with an increased risk of developing AD (Westwood et al., 2014) and lower VEGF levels are associated with the progressive loss of cognitive function in AD patients (Echeverria et al., 2017; H. Tang, Mao, Xie, Greenberg, & Jin, 2013). Additionally, it is important to note that exercise induces the reduction of inflammatory markers (CRP, TNF-α, and IL-6) (Sardi et al., 2011). Since increased markers of inflammation have been observed in AD post-mortem brains, the anti-inflammatory effects promoted by regular physical exercise may also contribute to explain the positive effects of exercise in the treatment of dementia, including AD (Pedersen, 2011). Likewise, following three weeks of voluntary wheel running, aged Tg2576 mice presented decreased levels of IL-1beta and TNF-α. Concurrently, critical cytokines for immune responses towards inflammation (IFNγ and MIP-1α) increased in the same AD-like model engaged in the exercise group (Nichol et al., 2008).

Mitochondrial dysfunction and neuroinflammation are observed in AD (Bosetti et al., 2002; Silva et al., 2013) and it is increasingly recognized that inflammation and mitochondrial dysfunction are interdependent phenomena (Wilkins, Carl,

Greenlief, Festoff, & Swerdlow, 2014). For this reason, we can speculate that the mitochondrial dysfunction observed in STZ Sed animals (**Figure 5.6** to .**5.9**) could be triggered by inflammatory mechanisms, and that these mechanisms might had been prevented/reversed by chronic endurance training programs.

So far, this was the longest study comparing different physical exercise models in non-transgenic rodent AD-like models. In addition to evidence that life-long wheel-voluntary running, *per se*, does not seem to be a sufficiently "strong" stimulus to counteract the pathology deleterious effects caused by this AD model, this study also showed that the beneficial cognitive and behavioral effects seen after 5 weeks of a treadmill training program (Rodrigues et al., 2010) remained at least for 12 weeks.

In addition to brain-local benefits, the profits prompt by exercise in the brain may also derive from systemically produced effects. During exercise, part of the muscle lactate production is shuttled to the brain (Dienel, 2012), acting in the brain as an energy source but also as a signaling molecule. However, the role of lactate in the brain has been also associated with long-term memory (Suzuki et al., 2011; J. Yang et al., 2014). In addition, Choi and collaborators found that ablation of neurogenesis prevented the beneficial effects of exercise in a mouse model of AD. However, induction of neurogenesis in the absence of exercise failed to improve memory. Only the combination of overexpression of BDNF with neurogenesis induction mimicked the memory improvements elicited by exercise, suggesting that boosting neurogenesis might protect against AD, but only when the health of the local brain environment is also improved (Choi et al., 2018). The effects of physical exercise seem to be multiple and acting at different levels of the central nervous system complexity. Perhaps because of this, despite increasing evidence that provide strong support for the implication of ROS in the AD etiology, clinical trials with antioxidant therapies either failed or delivered inconclusive results (Zhou, Li, Shi, & Ma, 2016).

Therefore, although depending on type and timing throughout lifespan, physical exercise represents an interesting tool that will ultimately modify the course of AD and perhaps other neurodegenerative disorders that feature mitochondrial

dysfunction. A further deeper understanding of the brain mitochondrial machinery adaptations induced by physical exercise will allow the development of new and more effective preventive and therapeutic strategies against this pathology based on exercise, particularly in the set of the used ecological and more representative AD and exercise models used in the studies comprised in the present thesis.

CHAPTER 7. [Conclusion]

Conclusion

In summary, our data suggest that:

- Endurance training improves spatial learning and long-term memory in a sporadic AD-like model, even in a later stage of life
- Endurance training counteracts brain cortex and hippocampus ultrastructural impairments, and cortex mitochondrial respiratory deficits caused by sporadic AD-like pathology in rats
- Life-long wheel-running is not enough to revert behavioral, cognitive, and mitochondrial respiratory deficits in icv-STZ rats.

These findings will contribute to better understand the effects of different exercise regimens in the prevention and treatment of the disease in this specific model of sporadic AD.

CHAPTER 8. [References]

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MINI-SYMPOSIUM: ENERGY DEMAND AND ENERGY SUPPLY IN ALZHEIMER'S DISEASE

Physical Exercise and Brain Mitochondrial Fitness: The Possible Role Against Alzheimer's Disease

T.C. Bernardo[1]; I. Marques-Aleixo[1]; J. Beleza[1]; P.J. Oliveira[2]; A. Ascensão[1]; J. Magalhães[1]

[1] CIAFEL—Research Centre in Physical Activity, , Health and Leisure, Faculty of Sport, University of Porto, Porto, Portugal.
[2] CNC—Centre for Neuroscience and Cell Biology, UC-Biotech, Biocant Park, University of Coimbra, Coimbra, Portugal.

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Corresponding author:
Telma C. Bernardo, Research Centre in Physical Activity Heath and Leisure, Faculty of Sport, University of Porto, Rua Dr. Plácido Costa 91, 4200-450 Porto, Portugal (E-mail: *telmasbernardo@gmail.com*)

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Abstract

Exercise is one of the most effective strategies to maintain a healthy body and mind, with particular beneficial effects of exercise on promoting brain plasticity, increasing cognition and reducing the risk of cognitive decline and dementia in later life. Moreover, the beneficial effects resulting from increased physical activity occur at different levels of cellular organization, mitochondria being preferential target organelles. The relevance of this review article relies on the need to integrate the current knowledge of proposed mechanisms, focus mitochondria, to explain the protective effects of exercise that might underlie neuroplasticity and seeks to synthesize these data in the context of exploring exercise as a feasible intervention to delay cognitive impairment associated with neurodegenerative conditions, particularly Alzheimer disease.

INTRODUCTION

Alzheimer disease (AD), the most common neurodegenerative disorder in which the nervous system progressively and irreversibly deteriorates, affects millions of people worldwide. The etiology of AD has a genetic background in 1%–5% of the population (familial early-onset <60 years), while the majority, accounting for more than 95%, represent sporadic cases (late-onset >60 years) (183). As AD is mainly a late-onset age-dependent disorder, it is estimated that its prevalence will increase along with the increase in life expectancy, exacerbating the societal and economic impact in the coming years. AD clearly is associated with systemic manifestations that extend beyond the central nervous system. In fact, triggered by environmental and endogenous factors, the risk for brain dysfunction and AD is augmented by obesity, diabetes, hypertension, hypercholesterolemia and chronic inflammation (151).

The symptoms of AD appear several years after the disease initiation and are characterized by a progressive cognitive decline, mostly related with memory and thinking language impairment, confusion and disorientation (88). Additionally, AD is related with neurobehavioral disarrangements, including apathy, depression, agitation and anxiety (88). The AD brain is further characterized by decreased neuronal cell proliferation (95, 96), survival (115, 201) and differentiation (15, 56), and a progressive loss of neurons and synapses number in specific brain regions, particularly in the hippocampus, followed by changes in the cortical and subcortical structures and complexity (21, 50, 115).

From a histopathological point of view, extracellular deposition of senile plaques (SP) mainly composed of amyloid β peptide (Aβ) and intracellular deposition of neurofibrillary tangles (NFT), comprised of hyperphosphorylated tau are common features of AD clinical diagnosis stage. In addition, an early intracellular accumulation of Aβ, preceding the formation of extracellular Aβ deposits and NFT formation in the brains of AD patients (89, 90, 117, 149) and of AD mouse models, may be a key factor in the induction of neuronal stress characterizing the progression of AD (167, 180, 215, 221, 222, 235).

Mitochondria have a central role in cellular energy metabolism; however, these organelles are also involved in several other important cellular tasks, such as intracellular calcium regulation and redox and apoptotic signaling. The progressively accumulation of Aβ within mitochondria has also been associated with the onset and progression of AD neuronal homeostasis perturbation. Accordingly, Swerdlow and Khan (211) proposed the "mitochondrial cascade hypothesis" to explain sporadic late-onset AD. Briefly, this hypothesis suggests that mitochondrial deregulation is the primary event in AD sporadic pathology leading to SN and NFT deposition. The precise mechanism behind this is still elusive, although some studies indicate that gradual accumulation of Aβ within mitochondria may be the link for mitochondria-mediated toxicity (35, 97). Moreover, mitochondrial dysfunction comprises an increased production of reactive oxygen species (ROS) namely superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (•OH), which further enhances Aβ production closing a vicious circle (124).