The interplay between vascular and mitochondrial abnormalities in type 2 diabetes and Alzheimer's disease

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I- Abbreviations

- ***(AP)-1 –*** Activator protein
- ***3xTg-AD –*** Triple transgenic mice for Alzheimer's Disease
- ***Acetyl-CoA***- Acetyl coenzyme A
- ***AD*** - Alzheimer's Disease
- ***ADMA –*** Asymmetric dimethylarginine
- ***ADP -*** adenosine diphosphate
- ***AGEs –*** advanced glycation end products
- ***AMPK –*** 5' AMP-activated protein kinase
- ***ANG II***- Angiotensin II
- ***ANP –*** Atrial natriuretic peptide
- ***APAF-1 –*** Apoptotic protease activating factor 1
- ***APH-1A*** – Anterior pharynx defective 1 homolog A
- ***APOEε4 -*** Apolipoprotein ε4 allele
- ***APP*** – Amyloid precursor protein
- ***Atg4*** – Autophagy-related protein 4
- ***ATP -*** adenosine triphosphate
- ***Aβ -*** Beta amyloid protein
- ***BACE*** – β-site APP cleavage enzyme
- ***Bad –*** Bcl-2-associated death promoter
- ***Bax –*** Bcl-2–associated X protein
- ***BBB***- Blood brain barrier
- ***BNIP3***- Bcl2/adenovirus E1B 19kD-interacting protein-3
- ***BOLD*** – blood oxygenation level dependent
- ***BSA -*** bovine serum albumin
- ***C99*** – 12 kDa C-terminal fragment of APP
- Ca^{2+} - calcium cation
- ***CAA*** – Cerebral amyloid angiopathy
- ***CBC –*** Complete blood count
- ***CBF***- Cerebral blood flow
- ***CCCP*** - carbonylcyanide m-chlorophenylhydrazone
- ***Chol***- Cholestrol

- ***CK*** – Creatinine kinase
- ***CNS***- Central nervous system
- ***CRP*** - C-reactive protein
- ***CsA*** - cyclosporin A
- ***CSF***- Cerebrospinal fluid
- ***CuZnSOD*** – Cuper/Zinc superoxide dismutase
- ***DAB*** – 3,30-diaminobenzidine
- ***DAG*** - diacylglycerol
- ***db/db*** – Homozygous $Lepr^{db}$ mice
- ***DEC-1*** – defective chorion-1
- ***DMEM*** - Dulbecco's modified Eagle medium
- ***DMSO*** – dimethyl sulfoxide
- ***DNA*** - desoxyribonucleic acid
- ***DOC*** – Sodium deoxycholate
- ***DTNB*** - 5,5'-ditiobis(2-nitrobenzoic acid)
- ***DTT*** – Dithiothreitol
- ***ECF***- Enhanced chemifluorescence
- ***ELISA*** – Enzyme-Linked Immunosorbent Assay
- ***eNOS*** - endothelial nitric oxide synthase
- ***EPO***- Erythropoietin
- ***ER*** – Endoplasmatic reticulum
- ***ERK*** – Extracellular signal-regulated kinases
- ***ET-1*** - vasoconstrictor endothelin-1
- Fe^{2+} - Iron cation
- ***GD*** – Gestational diabetes
- ***GFAT*** - glutamine:fructose-6-phosphate amidotransferase
- ***GK*** - Goto-Kakizaki
- ***Glut-1***- Glucose transporter 1
- ***GPx*** - glutathione peroxidase
- ***GR*** - glutathione reductase
- ***GSH*** - glutathione
- ***GSK-3***- glycogen synthase kinase-3
- ***GSSG*** – glutathione dissulfide

- ***H_2O_2*** **-** hydrogen peroxide
- ***Hb1Ac*** – Glycated hemoglobin
- ***HCT*** **–** Hematocrit
- ***HDL*** – High- density lipoprotein
- ***Het***- Heterozygous DOCK7^{m}/Lepr^{db}
- ***HG***- High glucose
- ***HGB*** **–** Hemoglobin
- ***HIF-1α*** **–** Hypoxia-inducible factor 1 alpha
- ***HO•*** **-** hydroxyl radical
- ***HPLC*** **–** High-performance liquid chromatography
- ***HRP*** – Horseradish peroxidase
- ***ICAM*** **–** Intercellular Adhesion Molecule
- ***icv***- intracerebroventricular
- ***IDE***- Insulin degrading enzyme
- ***IGF-1*** – Insulin growth factor 1
- ***IIS***- Insulin/insulin-like growth factor signaling pathway
- ***IL*** **-** interleukin
- ***iNOS*** **–** Inducible nitric oxide synthase
- ***IR***- Insulin Receptor
- ***IRS-1***- Insulin Receptor substrate 1
- ***IU*** **–** International Units
- ***Keap1*** – Kelch-like ECH-associated protein 1
- ***LG*** **–** Low glucose
- ***MAPK*** **–** Mitogen-activated protein kinases
- ***MBMEC*** – Mice brain microvascular endothelial cells
- ***MCI*** – Mild cognitive impairment
- ***MCP-1*** **-** monocyte chemoattractant protein-1
- ***MDA*** **-** malondialdehyde
- ***MnSOD*** **-** manganese superoxide dismutase
- ***Mo*** **–** Monocytes
- ***MRI*** **–** magnetic resonance imaging
- ***mtDNA***- Mitochondrial DNA
- ***MTOR***- mechanistic target of rapamycin

- ***N_2*** – Nitrogen
- ***NADH*** **-** reduced nicotinamide adenine dinucleotide
- ***NADPH*** **-** reduced nicotinamide adenine dinucleotide phosphate
- ***NF-kB*** **–** nuclear factor kB
- ***NFTs*** - Neurofibrillary tangles
- ***NIX*** **–** Nip3-like protein X
- ***NMDA*** **–** N-Methyl-D-aspartate
- ***NO*** **-** nitric oxide
- ***NR2B*** – NMDA receptor subtype 2B
- ***Nrf2*** – Nuclear factor (erythroid-derived 2)-like 2
- ***O_2*** **–** Molecular oxygen
- ***$O_2^{-\bullet}$*** **-** superoxide anion radical
- ***$ONOO^-$*** **- P**eroxynitrite
- ***OPT*** **-** ophthalaldehyde
- ***OsO_4*** **–** Tetroxide osmium
- ***OXPHOS*** **–** Oxidative phosphorylation system
- ***PBS*** **-** phosphate buffered saline
- ***Peg***- Polyethylene Glycol
- ***PGI_2*** **–** prostacyclin
- ***PHD*** – Prolyl hydroxylase domain
- ***Pi***- Inorganic phosphate
- ***PI3K***- phosphatidylinositol 3-kinase
- ***PKB***- Protein kinase B
- ***PKC*** **-** protein kinase C
- ***PMSF*** – Phenylmethanesulfonyl Fluoride
- ***$PPAR_\gamma$*** **-** peroxisome proliferator-activated receptor gamma
- ***PrPc*** – Cellular prion protein
- ***PS*** **–** Presenilins
- ***PTP*** **-** permeability transition pore
- ***PVDF*** – Polyvinylidene difluoride
- ***RAAS***- Renin-angiotensin-aldosterone system
- ***RAGE*** **–** AGEs receptor
- ***RBC*** **–** Red Blood Cells

- ***RBMEC*** – Rat brain microvascular endothelial cells
- ***RCR –*** respiratory control ratio
- ***RDW –*** Red Cell Distribution Width
- ***RHD123***- Rhodamine 123
- ***RNS –*** Reactive nitrogen species
- ***ROS -*** reactive oxygen species
- ***SD –*** Sprague Dawley
- ***SOD -*** superoxide dismutase
- ***SPECT*** – single photon excitation computed tomography
- *S**TZ***- Streptozotocin
- ***T1D***- Type 1 diabetes
- ***T2D***- Type 2 diabetes
- ***TBA -*** thiobarbituric acid
- ***TBS*** – Tris-buffered saline
- ***TBS-T*** – Tris-buffered saline 0.1% tween
- ***TCA -*** tricarboxylic acid
- ***TG –*** triglycerides
- ***TGF-α*** – Transforming growth factor-α
- ***TMB -*** 3,3', 5,5'-tetrametilbenzidina
- ***TMPD*** – N, N, N′, N′-tetrametyl-*p*-phenylenodiamine
- ***TNF-α*** - tumor necrosis factor-α
- TPP^+ **-** tetraphenylphosphonium ion
- ***UDP -*** uridine diphosphate
- ***v*** **-** mitochondrial volume
- ***V -*** volume of incubation medium
- ***VCAM –*** Vascular cell adhesion protein
- ***VEGF***- Vascular endothelial growth factor
- ***W -*** Wistar
- ***WHO -*** World Health Organization
- ***WT***- Wild- Type mice
- ***ΔE –*** Deflection of the electrode potential from the base line
- $\Delta\psi_m$ –Mitochondrial transmembrane potential

II- Abstract

Alzheimer´s disease (AD) is the most common form of dementia worldwide and its prevalence is increasing due to population aging, especially in developed countries. Evidence indicates that type 2 diabetes (T2D), normally associated with obesity, is becoming an epidemic, mainly due to unhealthy lifestyles characterized by the lack of physical exercise and high-calorie intake. Together with aging, T2D is a main risk factor for several diseases including vascular dementia and AD. In fact, several ongoing research studies aim to decipher the mechanistic links between T2D and AD. The work presented in this Thesis clarifies the involvement of vascular and mitochondrial anomalies in the neurodegenerative events predisposing to AD. In chapters 4 and 5, the impact of hypoxia, T2D and aging in the oxidative status of brain vessels and synaptosomes was studied. In chapter 4, chronic hypoxia was induced by exposing 3- and 12-month-old Wistar rats to 10% O_2 for 7 days using a hypoxic chamber. 3- and 12-month-old Wistar animals under normoxia were used as control animals. In chapter 5, 3- and 12-month-old GK rats, a spontaneous non-obese animal model for T2D, and respective Wistar control animals were used. Hypoxia occurs in aging and chronic diseases of the brain contributing to cells degeneration and, eventually, death. Chronic hypoxia and T2D promoted an oxidative imbalance in brain vessels and synaptosomes, this effect being potentiated by aging. Nevertheless, those alterations presented a tissue-specific pattern suggesting that different brain cells or structures behave differently when exposed to the same deleterious events. Another interesting aspect was that chronic hypoxia produced alterations similar to those induced by T2D supporting the idea that T2D is also associated with hypoxic events mainly due to vascular anomalies.

In chapters 6 and 7 we evaluated and compared the effects of AD and T2D in mice behavior, cognitive function, cerebral vasculature and mitochondrial function, oxidative status and ultrastructure. For this purpose, we used T2D mice, whose diabetes was induced by exposing the animals to a 20% sucrose solution *ad libitum* during 7 months, and 3xTg-AD mice. In short, diabetic and 3xTg-AD mice presented similar behavioral and cognitive alterations, an increased permeability of the cerebral vasculature, particularly in the cortex and hippocampus, as well as a similar profile of mitochondrial anomalies. Interestingly, diabetic mice presented a significant increase in amyloid β (Aβ) protein levels in both cortex and hippocampus, the two main areas affected in AD. These results support the idea that T2D predispose to AD development.

Because vascular anomalies associated to AD and T2D are deeply associated to endothelial dysfunction, the effects of high glucose and $A\beta_{1-40}$ in brain microvascular endothelial cells from normal and db/db diabetic rodents were also studied (chapter 8). High glucose increased the susceptibility of endothelial cells to $A\beta_{1-40}$, mitochondrial superoxide playing a key role in this process.

Altogether, these findings show that cerebral vasculature and mitochondria are key targets in T2D and AD, and associated events such as chronic hypoxia, which may contribute to synaptic degeneration and neuronal death. Furthermore, our results suggest that mitochondrial abnormalities underlie vascular alterations and vice-versa. Finally, the work presented herein reinforces the idea that, besides aging, T2D is a main risk factor for AD development.

III- Resumo

A doença de Alzheimer (AD) é a forma mais comum de demência a nível mundial e a sua incidência continua a aumentar, de um modo proporcional ao aumento no envelhecimento da população, especialmente em países desenvolvidos. Evidências incontestáveis mostram que um excessivo consumo de substâncias ricas em açúcar está na base de doenças como obesidade e diabetes tipo 2 (T2D). Do mesmo modo, resultados de estudos epidemiológicos revelam que existe uma correlação entre T2D e AD. Assim, é seguro afirmar que o envelhecimento e a T2D são dois dos maiores fatores de risco para o desenvolvimento de doenças neurodegenerativas, nomeadamente AD. No entanto, o exato mecanismo pelo qual estas duas patologias levam ao estabelecimento de disfunção cognitiva e morte das células neuronais continua a ser assunto de controvérsia, sendo que as alterações cardiovasculares ganham destaque como possíveis responsáveis por este processo. De facto, tanto o envelhecimento como a T2D são caracterizados por modificações vasculares e episódios de hipoxia contribuindo desse modo para a deterioração e morte das células neuronais. Com base nestes resultados, o principal objetivo desta dissertação foi avaliar e comparar o efeito da idade e da prevalência de T2D em vasos cerebrais e sinaptossomas, verificando se existe similaridade entre essas alterações e as presentes num modelo animal de AD (ratinho 3xTg-AD). Encontra-se ainda descrito que a disfunção mitocondrial e o stresse oxidativo são dois dos intervenientes mais importantes na fisiopatologia de AD, como tal, propusemos ainda avaliar se a disfunção endotelial a nível do cérebro pode interferir com as mitocôndrias cerebrais, alterando o seu normal funcionamento e induzindo morte neuronal.

Através de estudos *in vivo* fomos capazes de demonstrar que o envelhecimento e a T2D induzem um aumento dos níveis de stresse oxidativo dependente da idade, bem como, uma diminuição dos sistemas de defesa antioxidante nos vasos cerebrais. Apresentamos ainda nesta dissertação, resultados que mostram que essas alterações são de algum modo semelhantes às observadas em casos de hipoxia crónica demonstrando que o comprometimento vascular devido a aumento dos níveis de stresse oxidativo no envelhecimento e T2D podem induzir diminuição na distribuição de oxigénio ao cérebro comprometendo a sua homeostasia, aumentando a predisposição para neurodegeneração.

Além do anteriormente descrito, fomos ainda capazes de demonstrar num modelo de ratinho para T2D que as alterações observadas nos vasos cerebrais são semelhantes às observadas nos ratinhos 3xTg-AD. Comprovamos ainda, que essas alterações parecem interferir com o normal funcionamento das mitocôndrias cerebrais induzindo a libertação de fatores pro-apoptóticos através do poro mitocondrial de permeabilidade transitória promovendo a morte celular. Observámos ainda que algumas destas alterações são específicas de acordo com o tipo celular e que os vasos cerebrais estão na linha da frente de defesa do cérebro sofrendo alterações diferentes das observadas nos sinaptossomas. Numa tentativa de compreender melhor o mecanismo pelo qual estas alterações ocorriam, através de estudos *in vitro*, fomos capazes de demonstrar que as células endoteliais são os intervenientes principais neste aumento de suscetibilidade para neurodegeneração, e que o aumento na produção do ião superóxido pela mitocôndria parece ser o evento chave responsável por um aumento na suscetibilidade destas células a insultos tóxicos, nomeadamente a normal deposição de abeta que ocorre com o envelhecimento.

Deste modo, ao longo desta dissertação, os nossos resultados mostram que as alterações cerebrovasculares podem ser a ligação que falta para o melhor conhecimento da estreita associação entre T2D e AD, através da disrupção da barreira hematoencefálica com indução de disfunção mitocondrial, culminado num aumento da morte neuronal.

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Chapter 1- Introduction

1.1- An overview about Alzheimer's disease

Diseases resulting from degenerative changes in the nervous system markedly impact the lives of millions and cause growing public health challenges. Sporadic Alzheimer's disease (AD) is one of the most common forms of dementia and with the increase in life span the number of cases is raising substantially. Indeed, AD accounts for 50–60% of all dementia cases and, in 2010, the number of known AD cases was ≈ 35 million people worldwide, and it is expect to reach 140 million people by 2050 (Querfurth and LaFerla, 2010). AD is the most aggressive form of dementia and is easily distinguished from other forms of dementia (Humpel, 2011). However, the exact mechanism causing AD establishment is still unknown hampering the development of an effective therapeutic strategy.

AD is a progressive, chronic, neurodegenerative disease that slowly destroys neurons and causes severe cognitive disability, with the sporadic cases covering, at least, 95% of all individuals affected by the disease. In fact, the differential susceptibility to develop AD and the course of the illness, as well as the late age of onset in major part of the cases, suggest that epigenetic and environmental components, (Zawia et al., 2009), as well as alterations in physiological systems such as cholesterol (Koudinov and Koudinova, 2005, Lane and Farlow, 2005, Poirier, 2005), mitochondria and apoptosis (Eckert et al., 2003), insulin signaling and energy metabolism (Hoyer, 2002, 2004), are involved in the pathogenesis of AD, with aging constituting the most obvious risk factor for developing sporadic AD. Furthermore, the apolipoprotein E (ApoE) ε4 allele is associated with increased risk of AD while other alleles such as ε2 seems to protect against AD (Rao et al., 2013). Concerning familial AD, which it is estimated to affect less than 5% of all AD cases and affecting subjects at younger ages,

follow a more aggressive clinical course than sporadic AD (Robakis, 2013), and is associated with various genetic mutations in the genes coding for amyloid precursor protein (APP) and the presenilins (PS) 1 and 2 (Zekanowski et al., 2004, Hooli et al., 2012, Hunter et al., 2013).

Neuropsychological evaluation is the first step in the diagnosis of AD, which documents the pattern and degree of deficits in different domains of cognitive function. Different cognitive behaviors have strict relationship to specific brain structures and can, therefore, be used to describe the pattern and distribution of the disease (Pena-Casanova et al., 2012). Depression and apathy are the most common symptoms observed in AD individuals, although the incidence of verbal and physical agitation is also high across all stages of the disease (Lyketsos et al., 2011). Concerning cognitive changes in AD, individuals usually start with memory impairment, that seems to be very specific and focused on encoding and storage of new information (Pena-Casanova et al., 2012). Symptoms initially manifest through the reduction in recall recent events and, as the disease progresses, delusions, hallucinations, and aggression become more common as well as severe problems affecting language, visuospatial skills, judgment, problem solving, sleep disturbances, and disorientation to time and place (Young and Bennett, 2010, Lyketsos et al., 2011, Accardi et al., 2012). These alterations result in loss of control over bodily functions and complete dependence on others for the individual's activities of daily living (**Figure 1.1**) (Wasling et al., 2009).

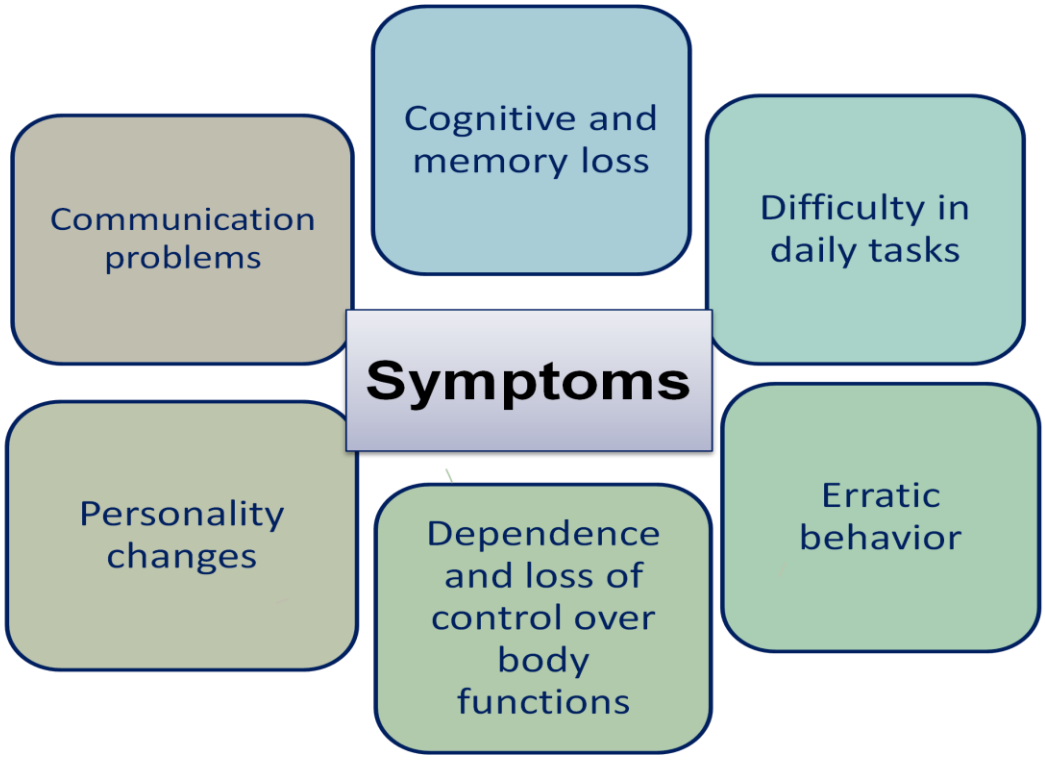


Figure 1.1- ***Neuropsychiatric symptoms in Alzheimer's disease***

Regarding pathological markers, the major features of this disease are severe beta-amyloid (Aβ) deposition in both, brain parenchyma and blood vessels, tau pathology, with formation of neurofibrillary tangles (NFTs), death of cholinergic neurons, microglial activation and inflammation.

Several hypotheses have been proposed in an attempt to explain AD pathogenesis including the amyloid cascade, tau, vascular and mitochondrial hypotheses. The amyloid cascade hypothesis, first proposed by Hardy and Higgins (1992) and the most prevalent theory until recently, postulates that an imbalance between the production and clearance of Aβ activates a deleterious cascade guided by the formation of neurotoxic Aβ oligomers, and larger Aβ assemblies (Hardy and Higgins, 1992, Armstrong, 2011). The deposition of Aβ may occur in the brain parenchyma and in the walls of small brain arteries, leading to cerebral amyloid angiopathy (CAA). At the molecular level, the generation of Aβ peptide involves the

site-specific cleavage of the precursor protein by β-site APP cleavage enzyme (BACE) and presenilin (Ng et al., 2010). Cleavage of APP by BACE generates an approximately 100 kDa soluble N-terminal fragment and a 12 kDa C-terminal fragment (C99), which can be further cleaved by γ-secretase generating 40 and 42 amino acid long Aβ peptides ($A\beta_{1-40}$ and $A\beta_{1-42}$), the latter of which is the most prone to self-aggregate into oligomers and plaques (the amyloidogenic pathway) (Lorenzo and Yankner, 1994). The extracellular accumulation of Aβ consequently stimulates the initiation of neurotoxic cascades that eventually lead to cytoskeletal changes, neuronal dysfunction and cellular death (De-Paula et al., 2012). More recently, it was showed that soluble Aβ, but not amyloid plaques, is the trigger of the pathological cascades that characterize AD (Dong et al., 2012). Klyubin and coworkers (2008) showed that Aβ dimers obtained from human cerebrospinal fluid (CSF) suppress hippocampal synaptic plasticity in vivo confirming previous studies showing a reduction in postsynaptic receptors and disruptions of synaptic morphology in cultured hippocampal neurons (Walsh and Selkoe, 2004, Lacor et al., 2007). Furthermore, exposure to Aβ oligomers have been observed to disrupt calcium signaling (Nimmrich et al., 2008), affect the function of N-Methyl-D-aspartate (NMDA) receptors (De Felice et al., 2007, Shankar et al., 2007), induce oxidative stress (Resende et al., 2008), mitochondrial dysfunction (Eckert et al., 2008) and cause tau-dependent microtubule disassembly (King et al., 2006). More recently, it was demonstrated that oligomeric Aβ activates Fyn kinase through a connection with cellular prion protein (PrPc) culminating in the phosphorylation of subtype 2B subunit of NMDA receptors (NR2B), with consequent dendritic spine loss and altered synaptic function (Um et al., 2012).

The tau hypothesis postulates that tau protein hyperphosphorylation is the mechanism that drives AD pathogenesis, upon which a crowd of signaling mechanisms

converge, and tau lesions are present in all stages of the disease (Maccioni et al., 2009, Braak et al., 2011). The followers of this hypothesis support that, at the beginning of the disease, only abnormally high levels of tau protein, which lie free in the cytosol, form nonbiodegradable aggregates. Due to unknown causes, susceptible brain cells start to present unusual high levels of phosphorylated tau protein that does not connect to microtubules, staying free in the cytosol (Braak and Del Tredici, 2012). Those tau protein modifications lead to its oligomerization and long-term production of NFTs that culminate in neuronal damage. As a result of neuronal damage, tau oligomeric forms and filaments are released to the extracellular environment, instigating microglial cells activation and stimulating a injurious cycle leading to progressive neuronal degeneration and death (Maccioni et al., 2010).

The mitochondrial cascade hypothesis posits that heritage determines mitochondrial function and durability, and, this mitochondrial durability, influences how mitochondria change with age and when mitochondrial alterations reach a threshold, AD pathology may be triggered. In fact, mitochondrial DNA (mtDNA) or protein damage may be responsible for declining mitochondrial function and increasing toxic side-products (Swerdlow and Khan, 2004, Young and Bennett, 2010). A positive correlation seems to exist between basal mitochondrial reactive oxygen species (ROS) production rates and accumulation of mtDNA damage (Swerdlow and Khan, 2004). With aging those mutations can lead to a general decline in mtDNA copy number, which would result in a reduction in oxidative phosphorylation, decreased membrane potential, and subsequent Ca^{2+} deregulation with subsequent opening of the mitochondrial permeability transition pore and increased neuronal death (Readnower et al., 2011). Furthermore, ROS can also react with Aβ causing the formation of dityrosine cross-linking between Aβ peptides, which leads to enhanced oligomerization and

aggregation (Massaad, 2011). Also the activity of β-secretase seems to be upregulated by ROS, increasing the amyloidogenic pathway and, consequently, Aβ production (Guglielmotto et al., 2009). Additionally, it was reported an imbalance in mitochondrial dynamics in AD patients, favoring mitochondrial fission that decrease the number of mitochondria and increase their size (Hirai et al., 2001, Readnower et al., 2011).

The vascular hypothesis, first proposed by de la Torre and Mussivand (1993) emphasize that cerebral perfusion declines during aging and worsens in the presence of vascular risk factors (de la Torre, 2000). The development of chronic brain hypoperfusion leads to a decrease in delivery of nutrients such as glucose and oxygen, highly necessary to maintain a healthy pool of brain cells. Since neuronal cells present a high-metabolic demand, a decrease in cerebral blood flow (CBF) deeply impacts neuronal function and survival that may reach a critical threshold where brain function is permanently compromised (de la Torre, 2010a).

However, each hypothesis by itself does not explain all the known histopathological and biochemical lesions seen in AD. Indeed, Aβ and mitochondrial abnormalities appear to have a cause-and-effect relationship, where the production of Aβ potentiates mitochondrial alterations and vice-versa. In addition, rare cases of "pure" AD have been observed, since the majority of patients with AD present cerebrovascular damage (Roy and Rauk, 2005, Young and Bennett, 2010). Therefore, due to its complexity, the prevention and treatment of AD represents one critical goal of biomedical research.

1.2- Aging and the brain

For several years, scientists believed that aging was a stochastic, uncontrolled weakening process, with modest or no genetic influence, that increased the susceptibility for disease development (Cohen and Dillin, 2008).

Nowadays it is well accepted that there are at least three autonomous metabolic pathways controlling the aging process with vital roles in lifespan. Those pathways include dietary restriction, the insulin/insulin-like growth factor 1 (IGF-1) signaling (IIS) pathway and, most recently, it was found that also the rate of mitochondrial respiration can determine lifespan (Cohen and Dillin, 2008, Hou and Taubert, 2012). In fact, the physiological process of aging is considered the main risk factor for all major human diseases, and is thought that intervening in the aging process could be the solution for preventing the onset of these age-related diseases (Baker et al., 2010).

Concerning the brain, the physiological process of aging leads to biochemical, structural and functional alterations (Seidler et al., 2010). In fact, grey matter atrophy and a small degree of white matter changes were showed by neuroimaging studies in the human cortex, involving mainly prefrontal regions, parietal, and temporal association cortices, and the insula and cingulum (Resnick et al., 2003, Sowell et al., 2003, Raz et al., 2005, Du et al., 2006, Swerdlow, 2011, Terribilli et al., 2011). Also a reorganization of cortical connectivity patterns was observed during normal aging with a consistent loss of axonal integrity (Madden et al., 2009, Koch et al., 2010, Wu et al., 2012, Zhu et al., 2012, Li et al., 2013) and a shift in regional efficiency from parietal and occipital to frontal and temporal neocortex (Gong et al., 2009). However, Rapp and Gallagher (1996) reported that old rats, showed a reduced performance in the Morris maze spatial task without presenting hippocampal neurodegeneration (the hippocampus is the brain

area mainly implicated in spatial orientation) supporting the theory that the cognitive decline that occurs with normal aging should be due to functional alterations, which could be responsible for a distorted interneuronal communication leading to cognitive decline (Pascale et al., 2007). This progressive and continuing gathering of harmful changes in structure and function over time should also have consequences in the electrical activity of the brain since its alteration may contribute to age-associated physiological and behavioral changes with accentuated decreases in neurological function (Baker et al., 2010).

In 1956, Harman proposed the free radicals hypothesis, that postulates that high levels of free radicals have an essential role in the aging process. In fact, cell aging result from increased oxidative stress, perturbed energy homeostasis, accumulation of damaged lipid proteins, and nucleic acids. It is well known that the brain is particularly defenseless to oxidative damage as a result of its high oxygen consumption rate, abundant lipid content, and relative lack of antioxidant enzymes as compared to other tissues. Additionally, aging is associated with declines in expression and activity of key endoplasmic reticulum (ER) molecular chaperones that may contribute to protein misfolding, accumulation and aggregation leading to a decline in cellular function (Brown and Naidoo, 2012).

1.3- Diabetes Mellitus, a world pandemic

Diabetes is a chronic metabolic disorder that results from a complex interplay between genetic predisposition and environmental factors (Ross et al., 2004). Currently, it is considered one of the main threats to human health, and its burden continues to rise due to changes in lifestyle (Zimmet et al., 2005). Indeed, almost 6% of world's adult

population, an estimated 366 million people worldwide, suffer from diabetes and the number of cases are expected to double until 2030 (Wild et al., 2004, Benhalima et al., 2012). As Robert Beaglehole and colleagues at the World Health Organization (WHO) pointed out *"The world is facing a growing diabetes epidemic of potential devastating proportions. Its impact will be felt most severely in developing countries"* (Amanda and Nigel, 2004). The most common symptoms associated to diabetes include polyuria (excessive urine production), polydipsia (increased fluid intake), polyphasia (excessive hunger), blurred vision, weight loss (most common on type 1 diabetes) and fatigue. The WHO recognizes three main types of diabetes: type 1 diabetes (T1D), type 2 diabetes (T2D) and gestational diabetes (GD).

T1D, previously known as insulin-dependent diabetes mellitus or juvenile diabetes, is a chronic disease, more common among children and young adults, that results from an autoimmune idiopathic destruction of the pancreatic β-cells leading to the absolute deficiency of insulin production (Azar et al., 1999). It has been suggested that the main cause(s) underlying the autoimmune process is a multifactorial combination of genetic, immunologic and non-genetic factors. In this regard, the inability of pancreas to synthesize enough insulin to maintain euglycemia makes insulin therapy indispensable for T1D patients. Indeed, the lack of insulin causes an increase of fasting blood glucose that begins to appear in the urine above the renal threshold (about 190-200 mg/dl in most people), thus connecting to the symptom by which the disease was identified in antiquity, sweet urine (glycosuria) (Dhatariya, 2008). Glycosuria causes the patients to urinate more frequently, and drink more than normal (polydipsia) (Takada et al., 2007).

GD is normally acquired by pregnant women in late pregnancy, after the baby's body has been formed, but while the baby is growing. It is a condition in which women

without previously diagnosed diabetes exhibit high levels of blood glucose during pregnancy (Ovadia and Dixit, 2012). However, the precise level of glucose intolerance characterizing gestational diabetes has been controversial over the last three decades (Wendland et al., 2012). Gestational diabetes affects about 4% of all pregnant women and this incidence is increasing with higher obesity in the pregnant population, lifestyle changes and migration (Ovadia and Dixit, 2012) and is associated with an increased risk of complications for both the mother and the baby during pregnancy and birth. In addition, women with gestational diabetes are at higher risk of developing T2D later in life (Benhalima et al., 2012).

T2D makes up about 80-95% of cases of diabetes and is more common in middle- or late-age people. The number of T2D cases is increasing uncontrollably due to population aging, overweight and obesity, resulting from unhealthy diets and sedentary lifestyles. In fact, T2D prevalence is increasing in young people (Sicree et al., 2003, Hviid et al., 2004, Zimmet et al., 2005, Zimmet, 2011, Song, 2012). In 2004, the United Nations General Assembly recognized T2D as a chronic, debilitating, costly disease, representing the fifth leading cause of death in developed countries (Wild et al., 2004, Shaw et al., 2010, Sanghera and Blackett, 2012). T2D is characterized by insulin resistance in peripheral tissues, since type 2 diabetic patients are able to produce insulin however, the rate of glucose uptake is reduced resulting in a state of hyperglycemia, which in turn stimulates more insulin secretion in order to compensate the high levels of blood glucose ensuing in a secondary hyperinsulinemia (Libby and Plutzky, 2002, Eckel et al., 2005, Jellinger, 2007). When T2D is diagnosed, patients are initially treated with dietary restrictions and exercise to facilitate glucose uptake. However, many patients are unable to change their lifestyle patterns, lose weight or increase physical exercise sufficiently to achieve a better glucose control. Subsequently, the only solution is the

prescription of oral hypoglycemic drugs to further control hyperglycemia. These drugs either increase insulin secretion or reduce glucose output from the liver. A final treatment for T2D consists of daily insulin injections when hypoglycemic drugs are no longer effective in maintaining normal blood glucose (Awad et al., 2004, Tibaldi and Rakel, 2007).

Over time, high glucose levels in the bloodstream can lead to severe complications such as vision loss, cardiovascular diseases, kidney disorders, nerve damage and brain degeneration (MacKinnon, 1999, Hviid et al., 2004, Cade, 2008).

1.4- Type 2 diabetes as a risk factor for Alzheimer's disease

Evidence shows that people suffering from T2D are more vulnerable to AD compared with healthy individuals (Biessels and Kappelle, 2005, Brands et al., 2005, Lu et al., 2009, Profenno et al., 2010, Sims-Robinson et al., 2010, Baker et al., 2011). Indeed, an approximately twofold increased risk of AD (risk ratios vary between 1.5 and 4.0) exist in T2D patients (Peila et al., 2002, Luchsinger et al., 2004). It has also been shown that 80% of AD patients exhibit impaired glucose tolerance or T2D, which suggests that these diseases may share a recognizable pathogenetic mechanism accountable for the loss of brain cells and β-cells (Janson et al., 2004, Correia et al., 2012). Evidence from the literature also demonstrates a pronounced cortical, subcortical, and hippocampal atrophy (10–15% of volume decrease), leukoariosis and white matter abnormalities, usually referred as diabetic encephalopathy, as well as an increase in cognitive impairment (Arvanitakis et al., 2004, Roriz-Filho et al., 2009, Milone, 2012). It was also proposed that diabetes may accelerate the onset of AD, instead of increasing the long-term risk (Pasquier et al., 2006, Correia et al., 2012).

Furthermore, cross-sectional studies demonstrate that verbal memory, processing speed, and brief cognitive screening measures are of inferior quality in diabetic patients that among controls (Roriz-Filho et al., 2009). However, this theory is controversial because clinical diagnosis of AD does not always match with the underlying neuropathology (i.e. AD-type or vascular) (Echavarri et al., 2012). Additionally, neuritic plaques and NFTs, the pathological hallmarks of AD, accumulate more abundantly in the brains of diabetic patients relative to control subjects (Milone, 2012). These observations were further confirmed by studies in rodents indicating that T2D increases anxiety-like behavior and "behavioral despair" in rodents such as ob/ob mice and db/db mice (Collin et al., 2000, Miyata et al., 2007, Sharma et al., 2010, Reagan, 2012).

Among all the metabolic alterations present in T2D, insulin deregulation and hyperglycemia seem to be the main (or at least more studied) alterations involved in the increased incidence of AD in T2D patients.

In 2005, Steen and coworkers hypothesized that AD could be the "type 3 diabetes" after a postmortem analysis of 45 AD patients brains that demonstrated a consistent decrease in brain insulin levels, in particular in the frontal cortex (Steen et al., 2005). In fact, insulin has been shown to be essential for memory, learning, neuronal survival, and longevity processes (Wozniak et al., 1993, Plum et al., 2005). Moreover, recent data showed that later stages of AD are associated with a pronounced decrease (up to 80%) in insulin concentration, IGF-1, and insulin receptor (IR) compared to age-matched control brains (Accardi et al., 2012). It was also demonstrated that the direct administration of insulin into the brain via the intranasal route [avoiding the blood-brain barrier (BBB) resistance] improved hippocampus-dependent memory in subjects with early AD or mild cognitive impairment (MCI) (Reger et al., 2006, Reger et al., 2008),

supporting the idea that central insulin deficiency could contribute to cognitive impairment (Hildreth et al., 2012).

Insulin is best known for its involvement in the regulation of glucose metabolism in peripheral tissues. However, insulin also achieves the central nervous system (CNS), through a saturable transport system (Banks, 2004), where it is involved in the control of energy balance. Additionally, insulin stimulates and enhances neuroplasticity (Wozniak et al., 1993, Reger and Craft, 2006, Reagan, 2007) affecting numerous brain functions including cognition, memory and synaptic plasticity through the signaling pathways activated by the complex insulin/ IR (Zhao and Alkon, 2001).

The peripheral hyperinsulinemia that characterizes T2D causes a reduction in the transport of insulin across the BBB creating a state of central insulin deficiency (Craft, 2007), the so called "insulin-resistant brain state", which contributes to cognitive impairment and neurodegeneration (Accardi et al., 2012, Reagan, 2012). Furthermore, a reduction in insulin levels and IR expression was previously reported in AD brains (Frolich et al., 1998, Steen et al., 2005). A role of insulin in the overexpression of glycogen synthase kinase-3β (GSK-3β) seems to be a major player in AD establishment. Indeed, it is known that insulin inhibits GSK-3β via the activation of phosphatidylinositol 3-kinase (PI3K) pathway. So, an impairment of the insulin signaling pathway decreases PI3K activation and, consequently, increases GSK-3β activation, potentiating tau protein phosphorylation and intraneuronal Aβ accumulation (Accardi et al., 2012). When tau protein is hyperphosphorylated it loses the capacity to stabilize the microtubules, and accumulates and aggregates into NFTs contributing to neurodegenerative events (Sima and Li, 2006). Interestingly, the loss of IGF-1 signaling was demonstrated to increase tau protein hyperphosphorylation and NFTs accumulation

in the brains of Igf1-/- (Cheng et al., 2005) and insulin receptor substrate-2 (IRS-2)-disrupted mice, a model of T2D (Schubert et al., 2003).

Recurrently, AD patients also present high fasting plasma insulin levels, decreased CSF insulin levels, and/or decreased CSF/plasma insulin ratio, besides increased Aβ levels in plasma (Watson and Craft, 2004). This evidence suggests that a decrease in insulin clearance may provoke an elevation of plasma Aβ levels (Accardi et al., 2012). *In vitro* studies also found that defects in insulin signal transduction affect the autophagic flux, a controlled degradation process involved in the elimination route of dysfunctional organelles or aggregated proteins in cells. Autophagy decline occurs through the inhibition of the mechanistic target of rapamycin (mTOR) pathway resulting in an alteration of APP processing, through the enrichment of secretase proteins in the autophagosomes. Furthermore, insulin signal transduction defects are also involved in the modulation of γ-secretase activity (Phiel et al., 2003) increasing Aβ concentration (Milone, 2012, Son et al., 2012). A role for insulin in Aβ transport from the ER and trans-Golgi network, the main site for Aβ generation, to the plasma membrane was also demonstrated (Gasparini et al., 2001). So, alterations in the insulin pathway may significantly reduce the intracellular concentration of Aβ derivatives, with an increase in extracellular Aβ levels (Gasparini et al., 2001). On the other hand, the insulin-degrading enzyme (IDE), a major Aβ-degrading enzyme, might be competitively inhibited by the elevated levels of insulin that occur under diabetic conditions, resulting in decreased Aβ degradation (Gasparini and Xu, 2003). Supporting these data, *in vivo* studies performed in animals subjected to the disruption of brain insulin function by the intracerebroventricular (icv) injection of streptozotocin (STZ) showed the presence of numerous behavioral, neurochemical and structural features that resemble those found in human sporadic AD (Grunblatt et al., 2007, Salkovic-Petrisic

and Hoyer, 2007). icvSTZ treated animals also displayed several classical features of AD including elevated APP, Aβ, and phosphorylated tau protein levels, neuronal cell loss, diminished acetylcholine levels and cerebral atrophy (Correia et al., 2011).

Compelling evidence demonstrates that this abnormal glucose metabolism can also be associated with T2D (Zhao and Townsend, 2009) through alterations in glucose supply, transport and utilization. Indeed, poor glycemic control, that can be evaluated by glycated hemoglobin (HbA1c) levels, appears to increase the risk of dementia (Xu et al., 2009). Actually, studies involving T2D patients demonstrated an inverse relationship between serum HbAlc and working memory (Perlmuter et al., 1984, Munshi et al., 2006), executive functioning (Munshi et al., 2006), learning (Reaven et al., 1990), and complex psychomotor performance (Reaven et al., 1990, Sommerfield et al., 2003). Those results support the hypothesis that a poor glucose control is associated with worsening cognitive function. Glucose is one of the major fuels used by the brain however, the breakdown of glucose is also essential for neurotransmitters synthesis such as acetylcholine and glutamate (Schulingkamp et al., 2000). In opposition, high levels of glucose exert toxic effects causing progressive functional and structural abnormalities in the brain (Arvanitakis et al., 2004). The harmful effects of hyperglycemia are mediated by an increased flux of glucose through the polyol and hexosamine pathways, activation of protein kinase C, and increased production of advanced glycation end products (AGEs), which lead to several cellular alterations like disturbances in intracellular second messenger pathways, increased levels of ROS and lipid peroxidation products, and decreased antioxidant defenses (Gispen and Biessels, 2000, Brownlee, 2001). Those pathogenic processes were observed in the CNS of experimental models of T2D and are proposed to contribute to the development of AD (Reagan, 2012). The formation and accumulation of AGEs in various tissues occur

during normal aging, but in the presence of T2D this process is enhanced and appears to be related with NFTs formation (Goh and Cooper, 2008). Furthermore, a clinical study showed that AGE's immunostaining was increased in post-mortem brain slices from diabetic AD patients, compared with non-diabetic AD subjects (Girones et al., 2004). This evidence suggests that the increased formation of AGEs, as a result of cerebral glucose metabolism impairment, may potentiate Aβ accumulation, tau protein hyperphosphorylation and oxidative stress.

Moreover, insulin resistance leads to an impairment of intraneuronal glucose metabolism. Indeed, data suggest that reduced cerebral glucose metabolism and insulin resistance accompany memory deficits in pre-diabetic individuals and T2D patients (Baker et al., 2011). Besides, it was also found a reduction in brain glucose metabolism in transgenic animal models of AD (Valla et al., 2008). The altered brain glucose metabolism is closely associated with mitochondrial dysfunction and a decrease in adenosine triphosphate (ATP) production, by around 50% at the beginning of AD, putting at risk the ATP-dependent processes involved in normal cell functioning (Mattson et al., 2001, Moreira et al., 2007a). Furthermore, glucose transporter-1 (GLUT-1) is significantly reduced in aged humans and in AD transgenic mice, which coincides with hippocampal atrophy and can justify the impairment in glucose distribution and utilization in AD brains (Correia et al., 2012).

Additionally, both insulin deregulation and hyperglycemia seem to be involved in brain endothelial dysfunction contributing to AD establishment (**this topic will be expanded in chapters 1.6.3.1 and 1.6.3.2**).

Interestingly, there is also evidence demonstrating that the association between diabetes and AD pathology is reinforced in APOEε4 allele carriers that, by itself, is

considered a risk factor for AD development (Milone, 2012). Actually, individuals with T2D who possess the APOEε4 allele have a two times higher risk of developing AD, as compared with non-diabetic subjects carrying the APOEε4 allele (Roriz-Filho et al., 2009). Furthermore, Aβ deposition is markedly increased in individuals with both diabetes and the APOEε4 genotype (Messier, 2003, Watson and Craft, 2004). The mechanism(s) underlying this synergistic connection remain(s) controversial but it is known that the APOEε4 allele decreases the capacity of neuronal repair, which associated with a diminished antioxidant activity, seems to contribute to Aβ deposition (Beffert et al., 1998). It was also demonstrated that AD patients with an APOEε4 allele exhibit lower levels of IDE in the hippocampus than those without an APOEε4 allele (Roriz-Filho et al., 2009). These results suggest that increased Aβ deposition may result from both the decreased expression of IDE in individuals with an APOEε4 allele or by the increased use of IDE for insulin levels regulation in individuals with diabetes, which decreases the clearance of Aβ by IDE (Peila et al., 2002, Dore et al., 2009).

1.5- Mitochondria and its role in cell homeostasis

Mitochondria are organelles that evolved from aerobic bacteria that in primitive times invaded proto-eukaryotic cells as parasites and developed a symbiotic relationship becoming intracellular organelles (Dromparis and Michelakis, 2012). Mitochondria are mainly devoted to energy production, but their functions can go far beyond that function. Undeniably these organelles are involved in several vital processes such as cell growth and differentiation, cell cycle control and cell death. Moreover, mitochondria are described as dynamic organelles whose shape and distribution in cells is strongly synchronized by processes of fission and fusion, biogenesis and autophagy,

resulting in a relatively steady mitochondrial population (Osellame et al., 2012). Mitochondrial oxidative phosphorylation system (OXPHOS) is the major source of high-energy compounds in the cell (Beal, 2005, Niizuma et al., 2009), where the oxidation of sugars, fats and proteins can be catabolised to acetyl-coenzyme A (Acetyl-CoA), which afterwards, goes through the tricarboxylic acid (TCA), which take place in the mitochondrial matrix (Luzikov, 2009). The TCA cycle generates the electron donors nicotinamide adenine dinucleotide (NADH) and succinate that donate electrons to mitochondrial complexes I and II, respectively. Electrons from these complexes are transferred to coenzyme Q, complex III, cytochrome c, complex IV and, finally, to molecular oxygen (O_2) that is reduced to water. According to data accumulated during the last decades, these mitochondrial complexes aggregate and form supercomplexes that can include up to 50 subunits, and the complete respiratory chain of higher organisms can contain about 90 subunits (Luzikov, 2009). The electron transport system is organized in this way in order to regulate ATP production **(Figure 1.2)**. Indeed, part of the energy of those electrons is used to pump protons from the matrix to the intermembrane space, generating a potential difference across the inner mitochondrial membrane. This voltage gradient is eventually used by ATP synthase to generate ATP from adenosine diphosphate (ADP) and inorganic phosphate (Pi) in the final stage of OXPHOS (Osellame et al., 2012) (**Figure 1.2)**.

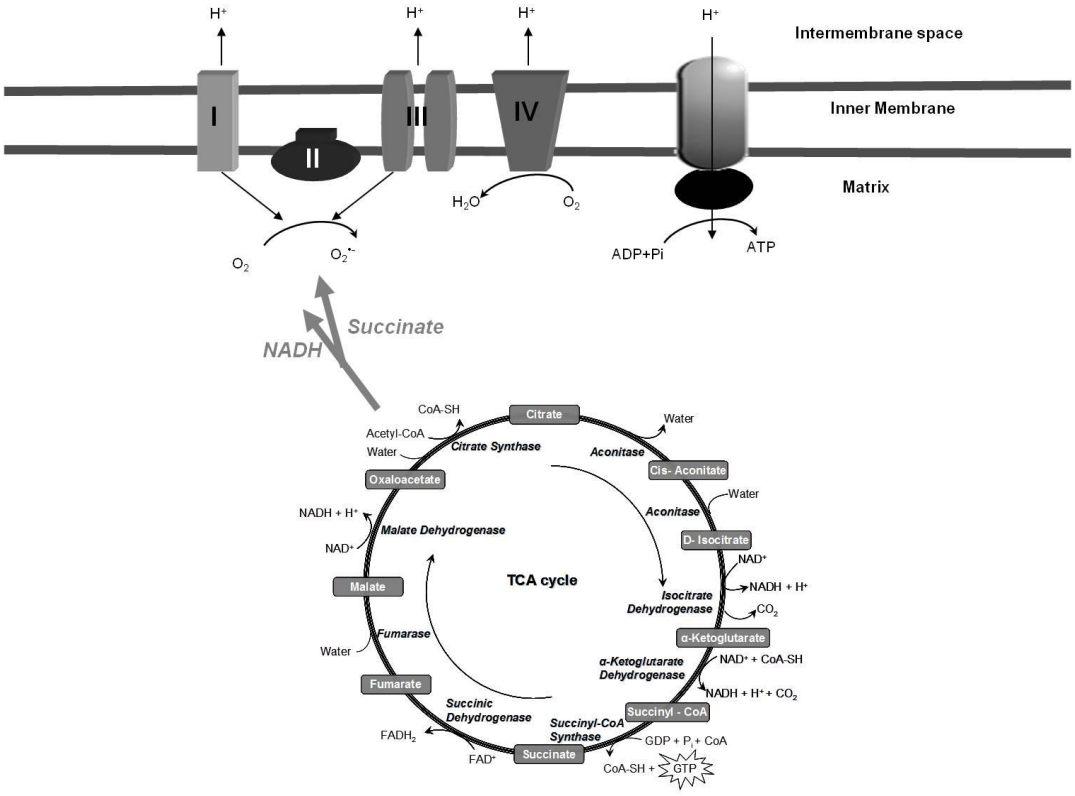


Figure 1.2- ***Mitochondrial respiratory chain and tricarboxylic cycle.*** The primary physiological function of mitochondria is to generate adenosine triphosphate (ATP), through oxidative phosphorylation via the electron transport chain. Glucose enters in the tricarboxylic acid (TCA) cycle where nicotinamide adenine dinucleotide (NADH) and succinate are produced in order to provide electrons to mitochondrial complexes I and II, respectively, of the respiratory chain. Electrons from these complexes are transferred through the respiratory chain, with concomitant basal production of reactive oxygen species namely superoxide ion ($O_2^{\bullet-}$) and, finally transferred to molecular oxygen that is reduced to water. The electron gradient produced by the respiratory chain is used by ATP synthase to generate ATP from ADP and Pi. *Adapted from (Carvalho et al., 2011).*

The ATP generated through OXPHOS is utilized by cells to maintain their normal functions. During the phosphorylation of ADP to ATP, the oxidation of NADH and succinate by O_2, leads to a continuous production of ROS (Hoye et al., 2008), such as superoxide anion ($O_2^{\bullet-}$), nitric oxide ($NO^{\bullet}$), hydroxyl radical (HO^{-}), peroxynitrite ($ONOO^{-}$), and hydrogen peroxide (H_2O_2) (Goetz and Luch, 2008). Indeed, the process of OXPHOS is not 100% efficient and around 20% of protons suffer synchronized

proton leak. The continuous electron leak from the respiratory chain leads to the generation of ROS and possibly thermogenic processes (Richter and Kass, 1991, Beal, 1996, Cai and Jones, 1999, Ricquier and Bouillaud, 2000). These reactive species may play a dual role, deleterious or beneficial depending on their levels (**Figure 1.3**) (Valko et al., 2006).

Low/moderate levels of ROS and reactive nitrogen species (RNS) are necessary for the activation of several physiological cellular responses, including cell defense against infectious agents and survival signaling pathways (**Figure 1.3**) (Sheu et al., 2006). Most cell types have been shown to elicit a small oxidative burst generating low concentrations of ROS when they are stimulated by cytokines, growth factors and hormones, e.g. interleukin-1α (IL- 1α), interleukin 6 (IL-6), interleukin 3 (IL-3), tumor necrosis factor-α (TNF-α) and angiotensin II (ANGII), among others (Thannickal and Fanburg, 2000). These observations led to the assumption that the initiation and/or proper functioning of several signal transduction pathways rely on the action of ROS as signaling molecules, which may act on different levels in the signal transduction cascade including inflammation, cell cycle progression, apoptosis, aging, and cancer (Park et al., 2011b, Miki and Funato, 2012). ROS can thus play a very important physiological role as secondary messengers (Lowenstein et al., 1994, Storz, 2005, McBride et al., 2006). Moreover, under normal conditions, ROS production is balanced by an efficient system of antioxidants, molecules that are capable to neutralize reactive species above a certain threshold preventing oxidant damage (Lenaz et al., 2002, Shen, 2010). Through the regulation of redox homeostasis, low levels of reactive species can, in turn, induce the endogenous antioxidant phase 2 enzymes, the Kelch-like ECH-associated protein 1(KEAP1)/ nuclear factor (erythroid-derived 2)-like 2 (Nrf2) cascade, and increase the expression of several antioxidant defenses including manganese

superoxide dismutase (MnSOD), catalase and glutathione reductase (GR) **(Figure 1.3)** (Droge, 2002, Akhtar et al., 2012). ROS play also a central role in the regulation of autophagy through autophagy-related protein 4 (Atg4) activity, which increase cell survival after removal of pathogens (Scherz-Shouval et al., 2007).

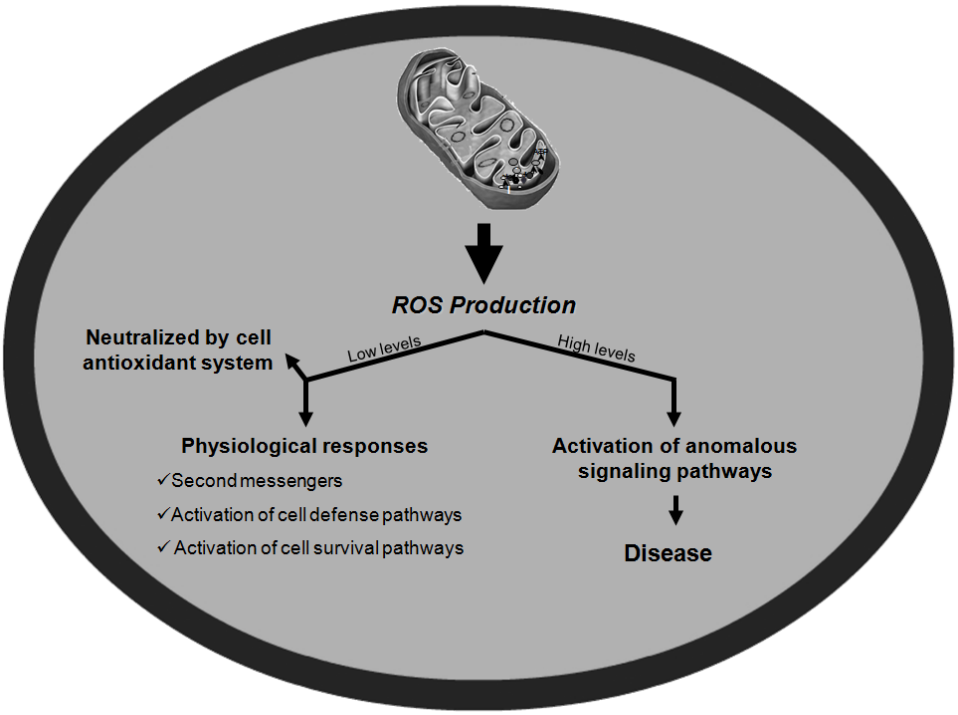


Figure 1.3- ***Mitochondrial reactive oxygen species production***. Mitochondria are intracellular organelles mainly devoted to energy production. During this process an increase in reactive oxygen species (ROS) production occurs. Under normal conditions, ROS production is balanced by an efficient system of antioxidants that will neutralize them, thereby, preventing oxidative damage by maintaining low/moderate levels of ROS that act as second messengers, which are also necessary for the activation of several physiological cellular responses, including cell defense and survival. However, high levels of ROS promotes mitochondrial oxidative damage and activates anomalous signaling mechanisms related to various disease states including diabetes, atherosclerosis, and some neurodegenerative diseases such as Alzheimer's disease, among others. *Adapted from (Carvalho et al., 2010a).*

However, high levels of ROS promote oxidative stress and activate anomalous signaling mechanisms related to various disease states (Brown and Borutaite, 2001, Sheu et al., 2006). Actually, the term ‘‘oxidative stress” describes adverse interactions of O_2, or its reactive derivatives, with biomolecules causing a disequilibrium between the generation of cellular damaging molecules and the cellular capacity for detoxification (Valko et al., 2006). In the physiological process of aging or in pathological processes, the production of ROS exceeds the scavenging capacity of endogenous systems, resulting in the damage of cellular components such as proteins, lipids, and nucleic acids (Trendeleva et al., 2012). Besides being one major source of ROS, mitochondria are also the preferential targets of these reactive species. The mtDNA is particularly susceptible to oxidative damage and all the polypeptides that are encoded by mtDNA are components of the OXPHOS (Wallace, 1992). This increased susceptibility of mtDNA to oxidative damage is probably due to its lack of protective histones, limited repair capabilities, and proximity to the electron transport chain (Linnane et al., 1989, Miquel, 1991, Wallace, 1992, Moreira et al., 2008). The oxidative damage of mitochondrial biomolecules impairs mitochondrial function and potentiates the release of cytochrome C and activation of the intrinsic death pathway **(Figure 1.4)** (Hoye et al., 2008). Indeed, cytochrome C binds to apoptotic protease activating factor-1 (APAF-1) and this complex is able to recruit procaspase-9, that can cleave and activate the downstream executioner caspase-3 leading to cell death (Osellame et al., 2012).

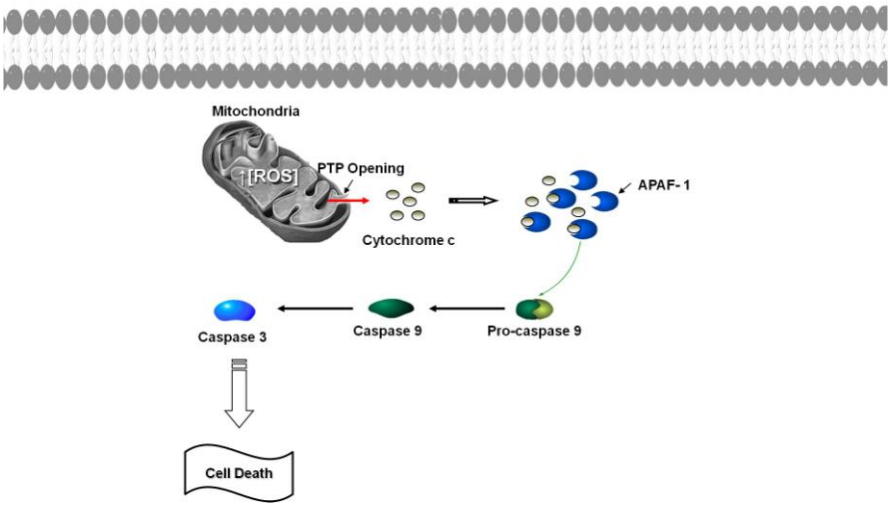


Figure 1.4 – Mitochondrial-mediated cell death. The impairment of mitochondria is intimately associated with an increase in reactive oxygen species (ROS) levels, a decrease in ATP levels and calcium (Ca^{2+}) dyshomeostasis. One important phenomenon associated with mitochondrial dysfunction and oxidative stress is the induction of the permeability transition pore (PTP). The sudden increase in the permeability of the inner mitochondrial membrane plays a key role in apoptotic cell death by facilitating the release of apoptogenic factors such as cytochrome c. Once released to the cytosol, cytochrome c interacts with apoptotic protease activating factor-1 (APAF-1), which cleaves pro-caspase 9 into an active form, caspase-9, that in turn activates caspase-3, resulting in the activation of apoptotic cell death pathway. *Adapted from (Carvalho et al., 2011)*.

1.5.1- Brain mitochondrial dysfunction: a common story between two giants

As discussed above, an exaggerated production of ROS by the respiratory chain affects, in the first place, mitochondrial components (Richter and Kass, 1991, Beal, 1996, Cai and Jones, 1999) exacerbating mitochondrial dysfunction and activating deleterious signaling mechanisms related to various diseases including diabetes and neurodegenerative diseases (**Figure 1.4**) (Brown and Borutaite, 2001, Moreira et al., 2007b, Moreira et al., 2009a). Indeed, it has been hotly debated if diabetes-associated oxidative stress actually triggers and/or propagates neurodegeneration. Previous studies from our laboratory showed that oxidative stress and mitochondrial dysfunction are

typical phenomena that occur in the brains of diabetic Goto- Kakizaki (GK) rats, an animal model of T2D, leading to neurodegeneration (Santos et al., 2001, Moreira et al., 2003, Moreira et al., 2005b). Also Silva and co-workers (2009) showed that diabetes exacerbates mitochondrial dysfunction, oxidative stress and neurodegeneration in retinal cells. It is also known that in diabetic cells more glucose is oxidized in the TCA cycle generating high levels of electron donors, NADH and succinate. These electron donors accelerate the respiratory chain activity and, as a result of this, the voltage gradient across the mitochondrial membrane raises until a critical threshold is reached. The increase in the mitochondrial membrane potential ($\Delta\Psi m$) exacerbates $O_2^{\bullet-}$ production activating the stress-related metabolic pathways: the polyol and hexosamine pathways, protein kinase C (PKC), and potentiating the formation of AGE's (Brownlee, 2005).

Concerning AD, in the last years a growing body of evidence demonstrated that mitochondrial abnormalities and ROS generation are involved in the pathogenesis of the disease (Nunomura et al., 2001, Zhu et al., 2006, Moreira et al., 2008), with oxidative stress occurring prior to cytopathology (Hirai et al., 2001, Nunomura et al., 2009). Indeed, several *in vitro* and *in vivo* studies corroborate the idea that mitochondrial impairment and oxidative stress are key players in AD development. Studies from the Bogdanovic's group (2001) showed an increase in oxidative stress in brains form patients with Swedish familiar AD bearing the APP670/671 mutation. In the same year, Pratico's group (2001) reported similar findings in Tg2576 APP transgenic mice. Increased oxidative stress levels were also described in mitochondria of platelets and fibroblasts from AD patients (Beal, 2005, Reddy, 2007). Quantitative morphometric, molecular and cellular studies focusing on mitochondria demonstrated the existence of increased levels of abnormal organelles in AD (Eckert et al., 2011, Silva et al., 2011), which may explain the increased oxidative stress in this pathology. It was also proposed

that age-related oxidative stress and damage potentiates mtDNA damage altering the normal mitochondrial functioning (Santos et al., 2010b). Valla and colleagues (2006) demonstrated a reduction in the activity of respiratory chain complexes, specifically in complexes III and IV, in mitochondria isolated from platelets of patients with AD. In fact, irregularities in mitochondrial cristae were observed in neurons of subjects with AD (Baloyannis et al., 2004). Additionally, the mitochondrial α-ketoglutarate dehydrogenase activity, a crucial mitochondrial enzyme complex that arbitrates oxidative metabolism, was found to be markedly reduced in either injured or relatively undamaged areas in human AD brains (Mohsenzadegan and Mirshafiey, 2012). All the mitochondrial alterations described above may explain in part the changes in electron transfer and the subsequent increase in ROS production that could underlie AD development (Feldhaus et al., 2011). Data from the literature also support the idea that APP and Aβ can be contributing factors to mitochondrial dysfunction in AD through their interaction with mitochondrial proteins resulting in the blockage of mitochondrial import channels, impaired mitochondrial transport, disruption of the electron transfer chain and increased ROS levels that, consequently, increase mitochondrial damage activating apoptotic pathways (Spuch et al., 2012)

Altogether, these studies indicate that mitochondrial dysfunction, oxidative stress and apoptotic cell death are intimately involved in AD and T2D.

In the next chapters we will discuss the involvement of T2D-induced mitochondrial dysfunction in BBB integrity and permeability that might compromise brain homeostasis potentiating AD development.

1.6- Brain endothelium: the defender of the brain

The BBB is the major, and highly regulated, exchange surface between the blood and brain parenchyma. This barrier consists of a monolayer of brain capillary microvessels, endothelial cells on the blood side and perivascular cells on the brain side, whose main function relates to neurovascular homeostasis (Li et al., 2012). Initially, it was believed that the endothelium was just a physical barrier surrounding the vessels, isolating the circulating blood from the tissues that selectively receive the nutrients and hormones carried by the blood. However, further research revealed that the endothelium is also a key regulator of vascular homeostasis (Fleming et al., 1996). The highlight of endothelial biology occurred in 1998, when the Nobel Prize in Physiology or Medicine was awarded jointly to Robert F. Furchgott, Louis J. Ignarro and Ferid Murad, for their discoveries regarding the role of $NO^{\bullet}$ as a signaling molecule controlling vasodilatation (Natali and Ferrannini, 2012). Endothelial cells, characterized by a high simplicity and particular morphology, are important modulators of the vascular function (Vane et al., 1990) since in CNS the normal signaling requires a high controlled environment.

Under physiological conditions, only a simple monolayer of healthy endothelium is able to react to physical and chemical signals regulating the endothelial release of mediators with vasodilator, vasoconstrictor, procoagulant, fibrinolytic, adhesion, growth and differentiation properties (Deanfield et al., 2005, Deanfield et al., 2007, Potenza et al., 2009b). The endothelium is also involved in the disposal of metabolic waste products, inhibition of platelets and leukocyte adhesion to the vascular surface, $NO^{\bullet}$ production, and control of volume and electrolyte content of the intra- and extravascular spaces (Libby and Plutzky, 2002, Mann et al., 2003, Kolluru et al., 2012). Furthermore, the endothelium has a primary role in maintaining blood fluidity and in

the restoration of vessel wall integrity avoiding extensive bleedings (Hadi and Suwaidi, 2007).

In response to hormones, neurotransmitters, and physical and chemical stimuli, endothelial cells release mediators with opposing vascular effects to coordinate changes in regional blood flow, transport and distribution of nutrients, and disposal of metabolic waste products. Under physiological conditions, molecules including endothelin-1 (ET-1), $NO^{\bullet}$, prostacyclin (PGI2), angiotensin (ANG) II, among others, act in concert to maintain hemodynamic balance, adequate O_2 supply, and nutrient delivery to tissues (Feletou and Vanhoutte, 1999, Potenza et al., 2009a). These molecules play a fundamental role in controlling the functional and structural integrity of the arterial wall and may be important in physiological processes and in pathological mechanisms underlying vascular diseases (Tirapelli et al., 2009).

1.6.1- When the brain endothelium breaks: Hypoxia and mitochondrial involvement

A disparity between the production of vasodilatation and vasoconstriction-species underlies the impairment of endothelial cell functions, including their barrier functions and angiogenic properties (Deanfield et al., 2005, Goligorsky, 2005, Kolluru et al., 2012).

The brain integrates diverse central and peripheral signals to maintain homeostasis (Ronnett et al., 2009). This organ has a high energy demand, and although it represents only 2% of the body weight, it receives 15% of cardiac output and accounts for 20% of total body O_2 consumption with an average consumption of approximately 3,5ml/100g/min (Moreira et al., 2009b, Nunomura et al., 2009, Ronnett et al., 2009).

Glucose is the major fuel in the brain and is transported across the cell membranes by facilitated diffusion mediated by glucose transporter proteins. More than any other organ, the brain is entirely dependent on a continuous supply of glucose from the circulation since glucose is almost the sole substrate for energy metabolism (Duelli and Kuschinsky, 2001). This extraordinary energy requirement is largely driven by energy needed to maintain ion gradients across the neuronal plasma membrane that is critical for the generation of action potentials. Despite its high energy demand to maintain "housekeeping" functions, the brain cannot store energy very well. Cerebral energy only sustains brain function for a few minutes before irreversible injury, which result from metabolic failure (Awad and Committee, 1993).

Moreover, cells utilize O_2 as the final electron acceptor in the aerobic metabolism of glucose to generate ATP, which fuels most active cellular processes. This clearly illustrates that brain function depends on a continuous and highly-regulated delivery of blood to meet energy requirements and to clear deleterious metabolic by-products. Physiological cellular demand for O_2 can also vary depending on tissue requirements at a given moment (Taylor, 2008). As such, complex cellular O_2 sensing systems have evolved for tight regulation of O_2 homeostasis and avoid or, at least, minimize brain damage (Acker and Acker, 2004). In fact, even short periods of cerebrovascular impairment can lead to neuronal damage by compromising the steady supply of essential metabolites such as O_2, amino acids and glucose (Bell and Zlokovic, 2009, Kalaria, 2009).

A drop in tissue O_2 levels to the point where O_2 demand exceeds supply (termed hypoxia) leads rapidly to a metabolic crisis and represents a severe threat to ongoing physiological function and, ultimately, viability (Taylor, 2008). Hypoxia has been implicated in CNS pathology in a number of disorders including stroke, head trauma,

neoplasia and neurodegenerative diseases (Bharke, 1993, Acker and Acker, 2004, Hainsworth et al., 2007).

Cellular and molecular pathways underlying hypoxic neurotoxicity and cell death are multifaceted and complex and involve several cellular responses, including oxidative stress, altered ionic homeostasis, mitochondrial dysfunction, and activation of apoptotic cascades (Jayalakshmi et al., 2005, Maiti et al., 2006, Hota et al., 2007). Furthermore it has been reported that hypoxia triggers ROS generation by mitochondria at complex III **(Figure 1.5).** The $O_2^{\bullet-}$ is converted to H_2O_2 through SOD, and directly inhibit prolyl hydroxylase domain (PHD) enzymes by oxidizing the essential non heme-bound iron inducing hypoxia-inducible factor-1alpha (HIF-1α) stabilization avoiding its degradation by the proteasome (Chandel et al., 2000, Hirota and Semenza, 2001, Gorlach et al., 2003, Bell and Chandel, 2007, Hagen, 2012). HIF-1α is a transcriptional activator involved in the adaptation to hypoxic stress**,** and its subunits are O_2 sensitive since they are substrates of a family of proline and asparagine hydroxylases, which utilize dioxygen, ferrous iron (Fe^{2+}) and 2-oxoglutarate to catalyze the hydroxylation of specific residues on HIF-1α subunits. In hypoxia, this repression is removed and the HIF-1α pathway becomes rapidly activated (Taylor, 2008). HIF-1α is a master transcriptional regulator of hypoxia-dependent gene expression and its transcriptional activation is a stress response, developed through evolution, to allow cells to avoid a bioenergetic crisis in low oxygen levels environments. Whether and to what extent the HIF-system may participate in the disease process remains to be elucidated. Indeed, current data would support a dual role of the HIF-1α system, depending on whether its stabilization is the cause or the consequence of the disease (Acker and Acker, 2004). Although the mechanism is not fully understood, HIF-1α system activates a physiological pathway that covers a wide array of physiological responses to hypoxia,

ranging from mechanisms that increase cell survival to those inducing cell cycle arrest or even apoptosis (Acker and Acker, 2004). These mechanisms combine cooperatively to activate HIF-1α to maximal levels under decreasing O_2 concentrations (Acker and Acker, 2004).

Previous studies show that, in a early phase, HIF-1α activates a survival pathway that involve the expression of angiogenic and vasodilators genes such as vascular endothelial growth factor (VEGF), inducible nitric oxide synthase (iNOS) and erythropoietin (EPO) (Bernaudin et al., 1999, Brines et al., 2000, Aminova et al., 2005). However, sustained and prolonged activation of the HIF-1α pathway may lead to a transition from neuroprotective to cell death responses. The long-lasting activation includes responses with adverse effects on cell function due to the activation of specific proteins involved in cell-cycle arrest and apoptotic cell death such as defective chorion-1 (DEC-1), Bcl2/adenovirus E1B 19kD-interacting protein-3 (BNIP3), its orthologue Nip3-like protein X (NIX), PUMA and cyclin G2 expression (**Figure 1.5**). In addition, direct stabilization through the pro-apoptotic protein p53 has been suggested by studies demonstrating physical and functional interactions between HIF-1α and p53 (Acker and Plate, 2002). The protein p53 is a master regulator of cell death by inducing apoptosis through the control of apoptosis-related gene expression (Schmitt et al., 2002). In response to certain death stimuli, a fraction of stabilized p53 rapidly translocates to mitochondria launching a rapid pro-apoptotic response in a transcription-independent manner that jump-starts and amplifies the slower transcription-dependent response (Marchenko et al., 2000, Mihara et al., 2003, Erster et al., 2004).

Sydow and Münzel (2003) also showed that ROS were responsible for the increase in asymmetric dimethylarginine (ADMA), an endogenous NOS inhibitor, increasing the risk of endothelium dysfunction (Masuda et al., 1999, Fard et al., 2000,

Lu et al., 2010). However, more studies are needed to elucidate if mitochondrial ROS are responsible for ADMA activation and the subsequent decrease in $NO^{\bullet}$ bioavailability culminating in O_2 deprivation. In fact, it is known that $NO^{\bullet}$ mediates electron transport chain inhibition suppressing mitochondrial function and allowing O_2 to diffuse culminating in neuronal cell death (Dromparis and Michelakis, 2012).

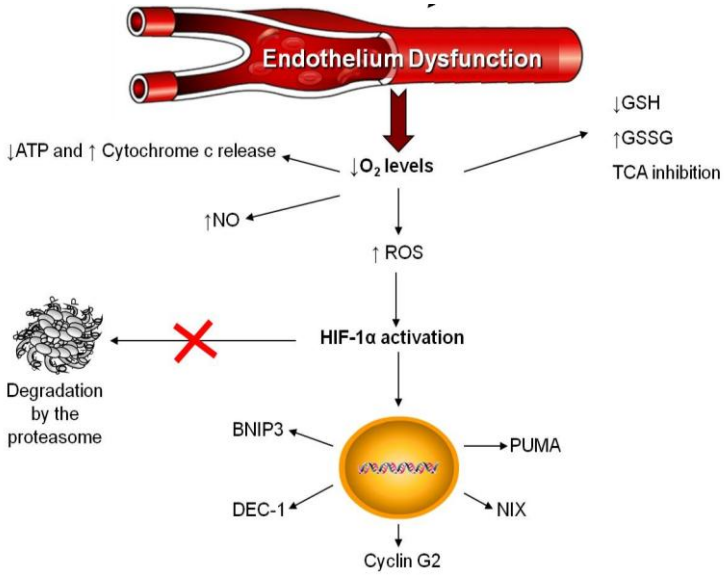


Figure 1.5- ***Hypoxia-mediated cell death in neurodegeneration.*** Hypoxia has been implicated in several pathologies of the central nervous system. In Alzheimer's disease the deposition of the amyloid β (Aβ) protein in brain vessels potentiate the occurrence of hypoxic phenomena. A drop in tissue oxygen levels to the point where oxygen demand exceeds supply rapidly leads to a metabolic crisis putting in danger the ongoing physiological functions. This metabolic crisis comprises a severe energy (ATP) drop that results from an impairment of mitochondria function, including the inhibition of the tricarboxylic acid (TCA) cycle. These alterations are intimately associated with an increase in the production of reactive oxygen species (ROS) as well as reactive nitrogen species, namely nitric oxide (NO) and a concomitant decrease in antioxidants namely glutathione (GSH), the first line of defense against oxidative stress. Hypoxia-inducible factor-1α (HIF-1α) is a transcription factor that is oxygen sensitive. In physiological conditions, HIF-1α is continuously degraded by the proteasome. However, in the presence of low levels of oxygen (O_2) and increased levels of ROS, HIF-1α is activated and translocated to the nucleus where it will bind to hypoxia response elements (HREs) increasing the expression of pro-apoptotic proteins, namely the defective chorion-1 (DEC-1), Bcl2/adenovirus E1B 19kD-interacting protein-3 (BNIP3), its orthologue Nip3-like protein X (NIX), PUMA and cyclin G2 leading to cell death. See text for more complete information. *Adapted from (Carvalho et al., 2011)*.

1.6.2- Brain endothelial dysfunction and AD

Recent evidence suggests a profound involvement of endothelial dysfunction in AD-related cerebral hypoperfusion and AD pathophysiology (Lange-Asschenfeldt and Kojda, 2008). Indeed, clinical imaging, epidemiological and pharmacotherapy studies showed that the prevalence of cerebrovascular disease in AD is higher when compared with non-demented age-matched controls. In fact, cerebrovascular abnormalities such as decreased microvascular density, basement membrane thickening, endothelial and pericyte damage and diminished glucose transport across the BBB were already described in AD brains (Humpel, 2011). Expression of inflammatory markers, perivascular fibrosis, capillaries with fewer branches, atrophic vessels, changes in vessel diameter, accumulation of collagen, atherosclerotic plaques, CAA, microglial activation in degenerating endothelial cells or thrombotic lesions have also been described in AD brains and were correlated with the severity of disease (Farkas and Luiten, 2001, Jellinger and Attems, 2010). However, until now, it was very difficult to establish if these changes are an initial cause for the development of AD or if they occur in late stages of the disease (Humpel, 2011).

Of notice is also the role of hypoxia in Aβ generation process. Several studies showed that hypoxia-associated AD establishment are due to defective Aβ clearance (Zlokovic, 2005, Deane et al., 2009), amplified influx of peripheral Aβ across the BBB (Deane et al., 2003, Eisele et al., 2010), and/or higher expression of APP (Atwood et al., 2002, Kumar-Singh et al., 2005, Cullen et al., 2006, Weller et al., 2008). All the described alterations have as a consequence an increase in Aβ accumulation in the brain (Zlokovic, 2011). Indeed, Wang and collaborators (2006) demonstrated that the expression of anterior pharynx defective 1 homolog A (APH-1A), a component of the γ-secretase complex, and the γ-secretase-mediated Aβ and Notch intracellular domain

generation are regulated by HIF-1α. In 2006, Sun and colleagues observed that hypoxia leads to an increase in APP processing enzymes. The authors reported a functional hypoxia-responsive element in the BACE1 gene promoter and an increase in β-secretase activity, with consequent increase in the generation and deposition of cytotoxic Aβ peptide and neuritic plaque formation (Sun et al., 2006). Additionally, it was also found that hypoxia potentiated the memory deficit in Swedish mutant APP transgenic mice (Sun et al., 2006). Subsequent studies confirmed the involvement of hypoxia in the up-regulation of β-secretase potentiating the production of Aβ (Guglielmotto et al., 2009, Ng et al., 2010). It was also reported that this effect is mediated by mitochondrial ROS (Guglielmotto et al., 2009). These results clearly demonstrate that hypoxia resulting from brain endothelial dysfunction can facilitate AD pathogenesis, and provide a molecular mechanism linking vascular factors to AD. Zhang and collaborators (2007) also showed that acute hypoxia increases the expression and the enzymatic activity of BACE1 by up-regulating the level of BACE1 mRNA, resulting in increased APP C99 fragment and Aβ levels. This study strengthens the hypothesis that oxidative stress is a basic common mechanism of Aβ accumulation. Additionally, also a decrease in the expression of neprilysin and endothelin-converting enzyme, both involved in Aβ degradation, was observed in mouse primary cortical and hippocampal neurons an in human neuroblastoma cells, respectively (Fisk et al., 2007, Wang et al., 2011).

Besides alterations in Aβ processing and clearance, the literature also provides evidence that chronic cerebral hypoperfusion increases the expression of APP in neurons and in dystrophic axons in rats (Stephenson et al., 1992; Kalaria et al., 1993; van Groen et al., 2005).

Koike and coworkers (2010, 2011) also reported that even transient changes in CBF, caused by the bilateral occlusion of the common carotid arteries of young triple-

transgenic AD (3xTg-AD) mice, could potentiate AD neuropathological alterations by increasing tau protein and Aβ levels, after 4 minutes of occlusion, and those prevailed for at least 3 weeks, amplifying process mediated by an increase in β-secretase protein expression (Koike et al., 2010, 2011). Increases in brain Aβ levels may in turn potentiate neurovascular (Deane et al., 2003, Bell and Zlokovic, 2009) and neuronal (Yan et al., 1996, Walsh et al., 2002, Takuma et al., 2009) dysfunction (Meyer-Luehmann et al., 2006, 2008, Eisele et al., 2010), which increase cerebral amyloidosis (Zlokovic, 2008).

In humans, AD was also correlated with extensive CBF reductions, mainly in areas of the parietal cortex, and many vascular alterations such as atherosclerosis, stroke and cardiac disease, could result in cerebrovascular dysfunction and trigger AD pathology (Johnson et al., 2005, Rocchi et al., 2009, Schuff et al., 2009). Interestingly, magnetic resonance imaging (MRI), transcranial doppler measurements, and single photon excitation computed tomography (SPECT) have established that areas with decreased CBF match to regions with significant neuropathological alterations in AD, suggesting that the reduction in CBF is an early event in AD pathogenesis (Hu et al., 2010, Pimentel-Coelho and Rivest, 2012). Moreover, brain hypoperfusion was also demonstrated in patients with amnestic MCI (Luckhaus et al., 2008). In fact, it was showed by functional MRI that monitorization of CBF changes in specific brain regions, via blood oxygenation level dependent (BOLD) contrast can be used to recognize MCI patients that will probably progress to AD in up to 3 years of follow-up (Habert et al., 2011, Pimentel-Coelho and Rivest, 2012). Indeed, it was showed that patients with MCI presented a delay in CBF response, during a task that evaluate episodic memory and this delay in fMRI-BOLD signal becomes more pronounced in AD patients (Rombouts et al., 2005). This suggests that vascular activation and cerebral

hypoperfusion seem to precede cognitive deficits and are present in the early stages of AD pathogenesis, since MCI is considered a potential transitional state between normal aging and dementia.

1.6.3- Diabetes-associated brain endothelium dysfunction

The idea that diabetic neuropathy is a vascular disease came in 1983, when Price observed areas of nerve degeneration supplied by severely atheromatous posterior tibial arteries with occlusion of small microscopic vessels (Pryce, 1893). Sixty six years later, Fagerberg (1959) published, for the first time, a study showing the link between neuropathy and microvessels damage. Later, Cameron and Cotter (1997) demonstrated that reduced nerve perfusion is a contributing factor in the etiology of diabetic neuropathy. Since then, several other studies were performed to elucidate the mechanisms underlying this association. Due to its importance in homeostasis regulation and involvement in several pathologies, the endothelium became a target of scientific interest. Presently, there is a consensual notion that increased oxidative stress compromises endothelial cells function preceding the morphological alterations that occur during the progression of diabetes and its complications such as neurologic disorders (Okouchi et al., 2006, Potenza et al., 2009b). As previously discussed, hyperglycemia and insulin signaling alterations are major players in oxidative stress, which, in turn, can induce endothelial dysfunction and, consequently, development of neurodegenerative diseases. The excessive $O_2^{\bullet -}$ production under hyperglycemia leads to direct damage of the endothelium since it rapidly reacts with $NO^{\bullet}$ to form the highly reactive intermediate $ONOO^-$ (Beckman, 1996). This reaction is about three to four times faster than the dismutation of $O_2^{\bullet -}$ by SOD. $ONOO^-$ in high concentrations is

cytotoxic and may cause oxidative damage to proteins, lipids and DNA (Beckman, 1996). $ONOO^-$ may react with the endothelial nitric oxide synthase (eNOS) co-factor BH4 converting it into pterins, mainly dihydropterin and dihydrobiopterin, exacerbating endothelial dysfunction (Milstien and Katusic, 1999). However, $ONOO^-$ may directly inhibit insulin-stimulated $NO^\bullet$ production by enhancing serine phosphorylation of IRS-1 (Potashnik et al., 2003), which results in impaired IRS-1-mediated activation of PI 3-K/Akt pathway that reduces $NO^\bullet$ bioavailability and accelerates its degradation. In fact, several studies reported an impairment of $NO^\bullet$-mediated dilation of cerebral arterioles in diabetic rats (Mayhan et al., 1991, Pelligrino et al., 1992) and a decrease in $NO^\bullet$ bioavailability in aortic tissue of GK rats when compared with control rats (Bitar et al., 2005). However, Chantemele and co-workers (2009) reported that diabetes increases the expression of eNOS. The same authors reported that a sharp dissociation between eNOS and its product $NO^\bullet$ in the vascular tissue of GK rats may occur (Belin de Chantemele et al., 2009).

Increased circulating levels of ET-1 has also been found in patients with diabetes (Kalani, 2008). Indeed, El Boghdady and Badr (2012) reported that ET-1 levels were significantly increased in diabetic patients with neuropathy, which is in accordance with previous reports showing that diabetic patients with retinopathy had increased levels of ET-1, in comparison with patients without retinopathy (Takahashi et al., 1990). It was also found that the improvement of blood glucose levels did not affect plasma ET-1 concentrations (Kakizawa et al., 2004). This increase in ET-1 levels in diabetic patients was correlated with the development of cardiovascular disease due to its pro-inflammatory and profibrotic effects (Kalani, 2008). Besides its vasoconstrictor properties, ET-1 can also inhibit $NO^\bullet$ production leading to several complications. According to Pawar and co-workers (2009), an increase in ET-1 levels could result from

the increased levels of ROS in diabetic brain. Indeed, the up-regulation of ET-1 by ROS was also observed in other vascular diseases such as pediatric pulmonary hypertension (Black and Fineman, 2006). Inversely, *in vitro* studies have shown that ROS can be produced in response to ET-1 increases via a small G protein p21ras (Ras)-dependent mechanism (Piechota et al., 2010).

Another consequence of hyperglycemia and increased ROS generation is the activation of the transcription factor nuclear factor kB (NF-κB) (Hattori et al., 2000, Yorek and Dunlap, 2002). In patients with T1D, activation of NF-κB in peripheral blood mononuclear cells was positively correlated to HbA1c levels (Hofmann et al., 1998). Schiefkofer et al. (2003) demonstrated in non-diabetic volunteers that exposure to hyperglycemia (180 mg/dl, 10 mmol/l) for 2 hours caused NF-κB activation in peripheral blood mononuclear cells. Upon activation, NF-κB translocates from the cytosol to the nucleus where it influences the expression of many genes. In endothelial cells, NF-κB activation leads to increased expression of adhesion molecules leading to increased attachment of monocytes to the endothelial cell monolayer (**Figure 1.6**) (Takami et al., 1998, Yorek and Dunlap, 2002).

Finally, the renin-angiotensin-aldosterone system (RAAS) has also been widely implicated in the development and progression of both micro- and macrovascular complications of diabetes. Indeed, hyperglycemia could alter the normal function of the endothelium with subsequent increase of ANGII levels, which might promote the formation of ROS that induce endothelial dysfunction and, consequently, cell death (Hayashi et al., 2010).

In the next sections we will further discuss the role of hyperglycemia and insulin resistance in diabetes-induced endothelial dysfunction.

1.6.3.1- Hyperglycemia

Since the brain is a high energy consumer, the percentage of glucose that is transported to the brain is very high. For this reason, it is clear that brain endothelium function is highly compromised in T2D patients. Animal studies showed that not only prolonged but also acute hyperglycemic episodes cause endothelial dysfunction (Taylor and Poston, 1994, Lash et al., 1999). Although several mechanisms could be involved in this phenomenon, oxidative stress seems to be the prompt event. Indeed, in the presence of high levels of glucose several deleterious pathways are activated culminating in several metabolic and cellular abnormalities (Correia et al., 2008b, Correia et al., 2010a).

As previously discussed, hyperglycemia can activate the polyol pathway through the increase in the enzymatic conversion of glucose to the polyalcohol sorbitol, with concomitant decreases in reduced nicotinamide adenine dinucleotide phosphate (NADPH) and glutathione (GSH) (Brownlee, 2001). NADPH is the cells' principal reductant and provides reducing equivalents to the glutathione peroxidase (GPx)–GR system (Zhang et al., 2000, Wu et al., 2001). The decrease in antioxidants like GSH increases the sensitivity of cells to oxidative stress.

Furthermore, high levels of glucose inside the cells also increase the synthesis of diacylglycerol (DAG), which is a critical activating cofactor of the classic isoform of PKC (Derubertis and Craven, 1994, Xia et al., 1994, Koya et al., 1997, Koya and King, 1998). When PKC is activated, it exerts a variety of effects on gene expression decreasing eNOS and increasing the vasoconstrictor ET-1, and the transforming growth factor-α (TGF-α). These alterations have several consequences including blood flow anomalies, capillary occlusion and increased vascular permeability (Studer et al., 1993, Feener et al., 1996, Kuboki et al., 2000).

The formation of AGEs resulting from increased glucose levels has also been implicated in the pathogenesis of the major microvascular complications of diabetes mellitus (Basta et al., 2004, Cooper, 2004, Stitt et al., 2004). Indeed, AGEs and their precursors appear to damage cells through the modification of intracellular proteins including those involved in the regulation of gene transcription (Yan et al., 1994), diffusion to extracellular environment and modification of extracellular matrix molecules nearby (McLellan et al., 1994) and alteration of the signaling between the matrix and the cell. The impairment of the signaling causes cellular dysfunction and alterations in blood circulating proteins (Charonis et al., 1990) that can bind and activate AGE receptors, thereby causing an increase in $O_2^{\bullet-}$ production (Schmidt et al., 1999, Wautier et al., 2001) and promoting macrophages-mediated inflammation in the vessel walls (Chavakis et al., 2004). AGEs decrease $NO^{\bullet}$ bioavailability and eNOS expression by accelerating eNOS mRNA degradation (Chakravarthy et al., 1998, Xu et al., 2003). By the activation of NF-κB pathways, AGEs also enhance expression of ET-1 in endothelial cells (Quehenberger et al., 2000), therefore altering the balance between NO and ET-1 favouring vasoconstriction and endothelial dysfunction.

The hexosamine pathway is an additional pathway of glucose metabolism and can mediate some of the toxic effects of this monosaccharide (Du et al., 2000, Brownlee, 2001). This pathway starts with the conversion of fructose 6-phosphate to glucosamine 6-phosphate by the rate-limiting enzyme glutamine: fructose-6-phosphate amidotransferase (GFAT) (James et al., 2002), an effect that is exacerbated under hyperglycemic conditions. Glucosamine-6 phosphate is converted into uridine diphosphate (UDP) *N*-acetyl glucosamine, a substrate for the glycosylation of important intracellular factors (McClain and Crook, 1996). UDP *N*-acetyl glucosamine can interfere with the integrity and functionality of cells by increasing the expression of

several gene products that could cause cellular alterations involved in diabetes-related complications (Newsholme et al., 2007). So, it can be concluded that hyperglycemia could compromise brain homeostasis increasing neurodegenerative events (**Figure 1.6**).

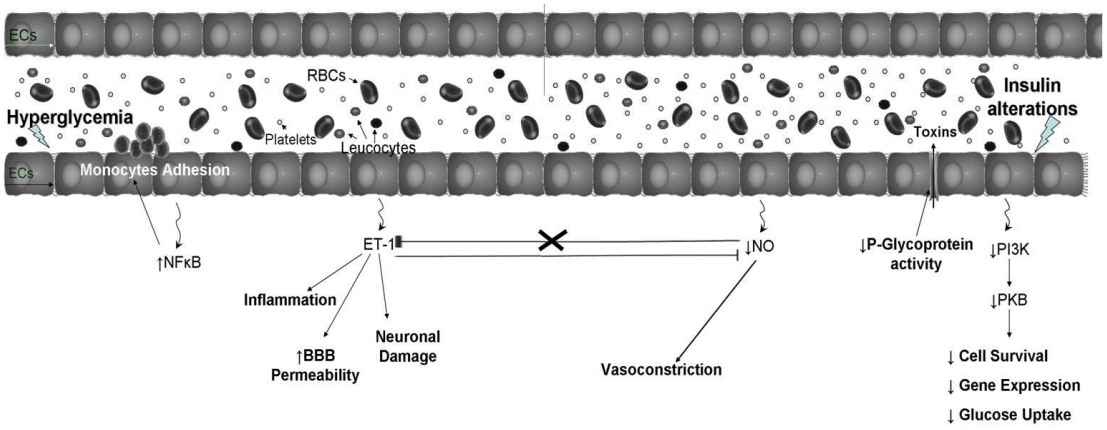


Figure 1.6- ***Hyperglycemia and insulin signaling alterations are intimately associated with endothelial dysfunction***. Under a state of hyperglycemia and altered insulin signaling, high levels of reactive oxygen species are produced activating several deleterious pathways. Under these conditions, there is an increase in transcription factor nuclear factor kB (NF-κB) activation leading to an increase in monocytes adhesion to the vessel walls, which initiates an atherosclerotic state. Furthermore, an increase in endothelin-1 (ET-1) and a decrease in nitric oxide ($NO^{\bullet}$) levels that contribute to an increase in blood-brain barrier (BBB) permeability, inflammation, vasoconstriction and, consequently, neuronal damage. Insulin alterations also lead to a decrease in P-glycoprotein activity, decreasing the efflux of toxins produced by cells to the circulating blood. Furthermore, insulin decreases phosphoinositide 3-kinase (PI3K) activity and, subsequently, protein kinase B (PKB) activity leading to a decrease in cell survival, gene expression and glucose uptake. RBCs- red blood cells; ECs- endothelial cells; CNS- central nervous system. *Adapted from (Carvalho et al., 2010a)*

1.6.3.2- Insulin resistance

The binding of insulin to its receptor, activates the insulin β-subunit, and stimulates autophosphorylation of certain tyrosine residues (Baserga, 1999), which leads to a cascade of events culminating in the activation of several signaling pathways (Saltiel and Kahn, 2001, Cardoso et al., 2009). Besides its essential effects on glucose and lipid metabolism, insulin has also a number of biological vascular actions including stimulation of sympathetic activity, and production of both ET-1 and $NO^{\bullet}$ from endothelium contributing to overall metabolic homeostasis (Montagnani and Quon, 2000). Consequently, abnormal insulin concentrations and/or impaired insulin action will interfere and provoke a profound impact on vascular homeostasis (Potenza et al., 2009b). Indeed, activation of eNOS by insulin increases production of $NO^{\bullet}$ that reduces the expression of adhesion molecules in endothelium, promotes vasorelaxation, and inhibits proliferation in vascular smooth muscle. On the other hand, insulin stimulates the release of ET-1 that increases expression of adhesion molecules, favors platelet aggregation, and promotes vascular smooth muscle cells contraction, migration, and proliferation (**Figure 1.6**).

Due to its differing effects, insulin signaling disturbances have also a pivotal role in brain endothelium dysfunction. Liu and collaborators (Liu et al., 2006, 2007, 2009) showed that the function and expression of P-glycoprotein was significantly down-regulated in the BBB of STZ-induced diabetic rats and mice and this event was reverted by insulin treatment, suggesting that insulin is responsible for the regulation of this protein. P-glycoprotein is an important efflux transporter in the BBB, and has an important role in the integrity of the barrier protecting the brain against many exogenous toxins (**Figure 1.6**) (Schinkel, 1999, Sun et al., 2003).

Also vasodilation stimulated by $NO^{\bullet}$ is highly regulated by insulin levels (Vincent et al., 2002, 2003). Recent *in vivo* and *in vitro* studies suggest that insulin crosses the vascular endothelium via a transcellular, receptor-mediated pathway (Wang et al., 2008). Emerging data indicate that insulin may act on the endothelium to facilitate its own transendothelial transport. In diabetes, the presence of altered insulin levels and function in endothelium leads to insufficient PI3K-mediated activation. Subsequently, an impairment in the activation of PKB occurs, which interferes with cell survival, regulation of growth, gene expression, glucose uptake, and glycogen synthesis (**Figure 1.6**) (Avogaro et al., 2010). It has been shown in muscle and blood vessels of insulin-resistant subjects (Bjornholm et al., 1997, Jiang et al., 1999, Cusi et al., 2000) that PKB activation leads to inadequate activation of eNOS with subsequent decrease of $NO^{\bullet}$ bioavailability and enhanced ET-1 release contributing to endothelial dysfunction (Potenza et al., 2009a). Again, mitochondria and high glucose levels are causative events since mitochondrial ROS production in brain exacerbates insulin resistance and progression from pre-diabetes to early diabetes or from mild to more severe T2D (Herlein et al., 2010). Although PI3K/Akt signaling is impaired in insulin-resistant individuals, activation of the mitogen-activated protein kinases (MAPK) pathway by insulin tends to be preserved (Bjornholm et al., 1997, Hsueh and Law, 1999, Jiang et al., 1999, Cusi et al., 2000, Eringa et al., 2004). In insulin-resistant subjects, hyperinsulinemia may lead to overstated pro-atherosclerotic responses through the MAPK pathway, promoting vascular smooth muscle cell proliferation (Hsueh and Law, 1999) and ET-1 production (Eringa et al., 2004).

IGF-1, a potent vessel growth promoter in the brain, is also involved in T2D-induced brain endothelium dysfunction. In physiological conditions, IGF-1 is critical for brain growth (Russo et al., 2005) and after development, IGF-1 becomes a pleiotropic

peptide involved in numerous processes aimed to maintain brain homeostasis, intervening in basic energy regulatory loops, cell protein sorting and cell-to-cell communication (Trejo et al., 2004). In general, IGF-1 actions in vasculature are similar to those of insulin. For example, physiological up- and down-regulation of brain vessel density is regulated by IGF-1 that triggers the production of the angiogenic factor VEGF and, consequently, leads to enhanced endothelial cell proliferation. The literature shows that diabetic patients present an impairment of the IGF-1 physiological actions that may contribute to vasoconstriction (Sowers, 1996). This fact may be due to a reduction in IGF-1-induced eNOS phosphorylation and concomitant increase in eNOS activity leading to $NO^{\bullet}$-dependent relaxation of vessels (Imrie et al., 2009). Furthermore, a straight interaction seems to exist between IGF-1 and AMP-activated protein kinase (AMPK), a cell energy sensor that intervenes in angiogenic signaling and interacts with IGF-1. Several studies revealed an impairment in IGF-1 receptor and AMPK signaling in mouse models of AD, preceding the onset of disease, which may ultimately lead to disturbed vessel homeostasis and therefore to compromised nutrient supply to the brain (Fernandez et al., 2007). Moreover, IGF-1 binding to its receptor also seems to activate an intracellular signal transduction pathway that may modulate the mitochondrial pathway (Li et al., 2003, Delafontaine et al., 2004). Li and co-workers (Li et al., 2009) demonstrated that enhanced IGF-1 signaling inhibits glucose-induced apoptosis via protection of mitochondrial function, specifically through the preservation of the $\Delta\Psi m$ and avoiding the release of cytochrome C (Li et al., 2009). Deregulation of this pathway contributes to cell death and neurodegeneration.

Although the triggers of neurodegenerative events remain a topic of intensive debate, the possible contribution of cerebrovascular deficiencies has been vigorously investigated in recent years (Farkas and Luiten, 2001). A decreased CBF, lower

metabolic rates of glucose and O_2 and a compromised structural integrity of the cerebral vasculature, particularly of the microvessels, are representative degenerative features of the brain vascular system associated to aging and T2D. The brain vascular system is regulated independently of the systemic circulation (van Beek et al., 2008, Grinberg et al., 2012) and a decrease in blood supply to the brain or disturbed microvascular integrity in cortical regions may occupy an initial or intermediary position in the chain of events finishing with cognitive dysfunction (Farkas and Luiten, 2001). Several studies have been conducted to establish the role of T2D-induced endothelial dysfunction and consequent reduction in CBF and hypoxia in neurodegeneration, particularly in AD. Subsequently, revealing the role of vascular alterations and preventing T2D-induced endothelial dysfunction could be an important step in the discovery of new therapies for the prevention of AD (Pimentel-Coelho and Rivest, 2012).

Chapter 2- Objectives

2.1- Objectives

T2D and aging are two major risk factors for AD development. Although T2D and AD share several common features, the mechanisms linking both pathologies remain under discussion. Here, we aimed to clarify the role of mitochondrial and cerebrovascular anomalies in the T2D-AD connection. The work presented in this Thesis helps to answer the following questions:

- ✓ How do chronic hypoxia and aging affect the oxidative status of brain vessels and synaptosomes?
- ✓ What is the impact of T2D in the oxidative status of brain vessels and synaptosomes? Does aging play a role? Can Aβ potentiate endothelial dysfunction caused by high glucose levels?
- ✓ What is the effect of T2D and AD on mice cerebral vasculature? Do mitochondrial abnormalities play a role? Are those alterations similar in T2D and AD? If so, can they cause similar behavioral, cognitive and neuropathological alterations?

Understand how T2D interferes with brain vessels, particularly the endothelium, which may jeopardize BBB integrity and, consequently, neuronal health is of utmost importance. In fact, the alterations promoted by T2D may predispose to the development of neurodegenerative events, including AD. The clarification of these issues, including the mechanisms underlying those effects may help identify new therapeutic targets for T2D-associated neurodegeneration and AD.

Chapter 3- Material and Methods

3.1- Animals and experimental design

Chapters 4 and 5: Male Wistar and GK rats (3- and 12-month-old) were housed in our animal colony (Animal Facility, Faculty of Medicine/Center for Neuroscience and Cell Biology, University of Coimbra). In **chapter 5**, Wistar rats were randomly divided into two groups: 1) control group that was maintained under normoxia and 2) hypoxic group that was maintained for 1 week in an O_2 controlled normobaric hypoxic chamber containing mix gas of 10% O_2:90% N_2, where the introduction of N_2 gas leaded to a decrease in O_2 levels. O_2 concentration inside the chamber was confirmed by an O_2 monitor (Proox Model 110, Biospherix, Redfield, New York) (**Figure 3.1**). Hypoxia was interrupted once for roughly ten minutes for animal care. Rats were maintained under controlled light (12 hours day/night cycle) and humidity with free access (except in the fasting period) to water and powdered rodent chow (URF1; Charles River).

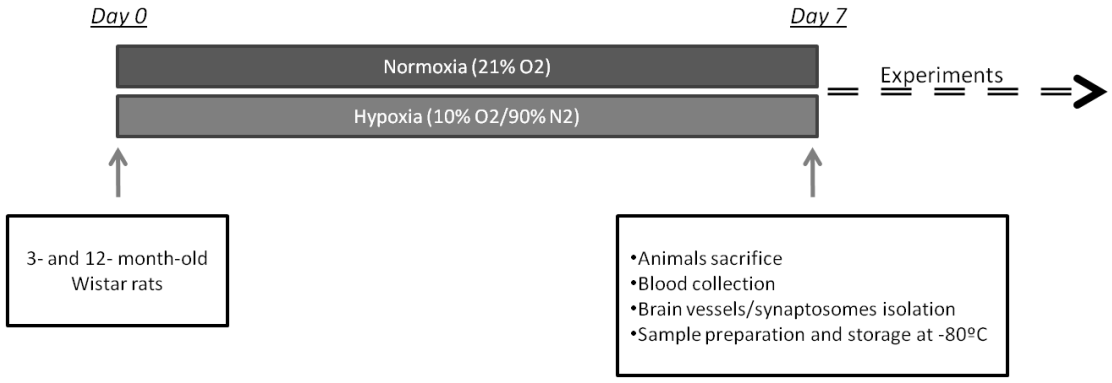


Figure 3.1- ***Experimental design*****.** Male Wistar rats (3- and 12-month-old) were divided in two groups. The hypoxic group was maintained for 1 week in an O_2 controlled normobaric hypoxic chamber containing mix gas of 10% O_2:90% N_2, where the introduction of N_2 gas leaded to a decrease in O_2 levels. The normoxic group was maintained for 1 week at atmospheric O_2 levels. Adhering to procedures approved by the Federation of Laboratory Animal Science Associations (FELASA), the animals were sacrificed by cervical displacement and decapitation at the end of hypoxic or normoxic period. Blood was collected, brain vessels and synaptosomes were isolated and samples were prepared and stored at -80ºC until assayed. Adapted from (Carvalho et al., 2010b)

Chapters 6 and 7: Male wild type mice (WT) and triple transgenic mice for AD (3xTg-AD) (4-months-old) (**Figure 3.2**) were housed in our animal colony (Animal Facility, Faculty of Medicine/Center for Neuroscience and Cell Biology, University of Coimbra). WT mice were randomly divided into two groups: 1) control group and 2) sucrose-treated animals with free access to 20% sucrose solution during 7 months. Mice were maintained under controlled light (12 hours day/night cycle) and humidity with free access (except in the fasting period) to water (WT and 3xTg-AD mice at basal conditions) or 20% sucrose solution (T2D) and powdered rodent chow (URF1; Charles River).

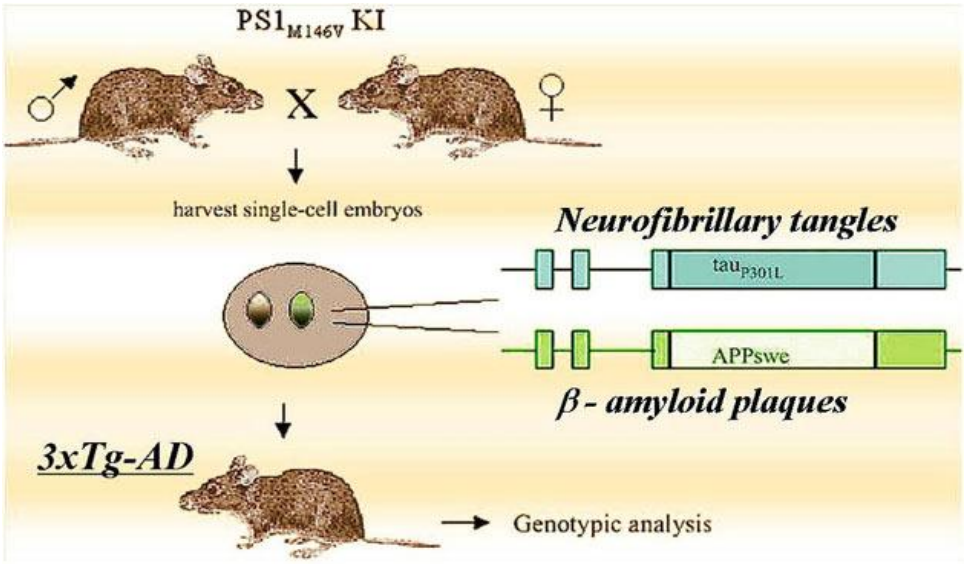


Figure 3.2- ***Triple transgenic mouse model for Alzheimer's disease.*** The 3xTg-AD mice develop an age-related and progressive neuropathological phenotype that includes both plaque and tangle pathology. 3xTg-AD mice were obtained through a pronuclear microinjection technique, where two independent transgene constructs encoding human APPSwe and tauP301L (4R/0N) were inserted into single-cell embryos produced from mutant homozygous PS1M146V knockin mice. The injected embryos were reimplanted into foster mothers and the resultant progeny genotyped. The neuropathology initiates with a regional pattern closely mimicking that observed in AD. Synaptic dysfunction is an early change that precedes the accumulation of the hallmark pathological lesions. *Adapted from Oddo et al., 2003*

Adhering to procedures approved by the Federation of Laboratory Animal Science Associations (FELASA), the animals were sacrificed by cervical displacement and decapitation at the end of treatment periods.

Chapter 8: The animal protocol was approved by the Institutional Animal Care and Use Committee of Tulane University School of Medicine. All experiments complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. For rat brain microvascular endothelial cells culture, male Sprague–Dawley rats at 10 weeks of age were obtained from Harlan laboratory and for mice brain microvascular endothelial cells culture, 11 weeks old db/db mice, a homozygous mice model for the diabetes spontaneous mutation ($Lepr^{db}$) and the heterozygous mice ($Dock7^{m}/Lepr^{db}$) (**Figure 3.3**) were obtained from Jackson's Laboratory. Rodents were housed in the animal care facility and received standard rat or mice chow and tap water ad libitum.

Figure 3.3- ***Homozygous mice model for the diabetes spontaneous mutation ($Lepr^{db}$) and the heterozygous mice ($Dock7^{m}/Lepr^{db}$).*** Mice homozygous for the diabetes spontaneous mutation ($Lepr^{db}$) become obese at approximately three to four weeks of age. Elevations of plasma insulin begin at 10 to 14 days and elevations of blood sugar at 4 to 8 weeks. Homozygous mutant mice are polyphagic, polydipsic, and polyuric. The severity of disease on this genetic background leads to an uncontrolled rise in blood sugar, severe depletion of the insulin-producing β-cells of the pancreatic islets, and death by 10 months of age. Exogenous insulin fails to control blood glucose levels and gluconeogenic enzyme activity increases. Peripheral neuropathy and myocardial disease are seen in C57BLKS-$Lepr^{db}$ homozygotes. Wound healing is delayed, and metabolic efficiency is increased. *Adapted from http://jaxmice.jax.org/strain/000642.html*

3.2- In vivo / ex vivo studies

Behavioral tests

1) *Open field tests* - Two distinct open field tests were used to measure different parameters. The locomotor activity was measured in an open field arena (30 x 30 cm, divided into 9 squares) and the exploratory behavior of the animals was evaluated by counting the total number of line crossings over a period of 8 minutes. This test was also used for habituation of the animal to the open field arena before the object recognition test. To analyze parameters such as grooming, rearing, time moving and resting and the time spent before to start the exploration of the field (freezing behavior), we used a different open field connected to a water maze tracking system (Water 2020, HVS Image, Buckingham, UK) that monitored each mouse for 30 minutes. For both tests, each mouse was placed in the centre of the open field and the experimenter stepped out of the view of the animal.

2) Object recognition test - Memory performance was also evaluated by the object recognition test consisting of two 5 minutes sessions (120 minutes after habituation): the first with two identical objects (sample session) and the second (test session, 120 minutes after) with two dissimilar objects (a familiar and a novel one). Exploration of objects was considered when animals were facing the objects with the nose up to 2 cm away from the object. The time exploring each object was recorded and recognition object index was calculated by the ratio of the time spent exploring the novel object minus the time exploring the familiar object over the total exploration time of both objects, as previously described (Costa et al., 2008).

3) Y maze test - Spatial memory performance was evaluated using the modified Y-maze test, in a Plexiglas apparatus consisted of 3 arms (w: 5 cm, l: 35 cm, h: 10 cm) in a Y shape, separated by equal angles. The test consists in subjecting animals to two 8

minutes sessions (trial and test) separated by a 120 minutes inter-trial interval (Dellu et al., 1992, Dellu et al., 1997, Akwa et al., 2001). During the trial session 1 arm was separated by a guillotine wall that made it inaccessible to the animals so, they were allowed to explore only 2 arms, and during the test session the guillotine was removed allowing the exploration behavior in the 3 arms. Memory performance was evaluated by measuring the percentage of time spent exploring the novel arm over the time spent exploring all arms.

4) Elevated plus maze test - This test evaluates the anxiety of the animals. The elevated plus maze consisted of 4 arms (30×5 cm), connected by a common central area (5×5 cm). Two opposite facing arms were open, while the other two opposite facing arms were enclosed by 15 cm high walls. The entire plus maze was elevated to the height of 40 cm. Each mouse was placed in the centre of the maze facing a closed arm. Each mouse was monitored for 5 minutes and the number of entries into the open and closed arms was recorded, as well as the time spent in the open arms. Decreased time spent in the open arms indicates an increased level of anxiety.

Determination of biochemical parameters - Blood glucose levels were determined by a glucose oxidase reaction, using a commercial glucometer (Glucometer-Elite Bayer, Portugal) and compatible reactive tests (Ascencia Elite Bayer, Portugal).

Chapters 4 and 5: Complete blood count (CBC) analyses were performed after animal decapitation and collection of total blood in 1 ml tubes containing EDTA (Aquisel®) and analyzed in a Coulter MAXM analyzer.

Triglycerides (TG) levels were measure in plasma collected from total blood that was centrifuged at 2500 rpm x 5 minutes (Eppendorf Centrifuge 5415C) (Daniele et al.,

2010), at 4ºC, and analyzed in Cx4 Beckman auto-analyzer. TG levels were evaluated by the timed-endpoint method (Hongzong et al., 2007). TG in the sample were hydrolyzed to glycerol and free fatty acids by the action of lipase. A sequence of three coupled enzymatic steps using glycerol kinase, glycerophosphate oxidase and horseradish peroxidase caused the oxidative coupling of 3,5-dichloro-2-hydroxybenzenesul-fonic acid with 4-aminoantipyrine to form a red quinoneimine dye. The TG levels in the sample were obtained through the Beer's law against the absorbance of a standard.

Total CK activity of the plasma was measured, at 30º C, using a Cx4 Beckman auto–analyzer (Oliver and Rawlinson, 1955). Briefly, samples were assayed with and without creatine phosphate as a substrate in order to eliminate activity not due to creatine phosphokinase. The absorbance changes were directly proportional to CK activity and expressed in international units (IU) per litre, in which 1 U of enzyme catalyses the conversion of 1 µmol substrate per minute, at 30 ºC.

Plasma C-reactive protein (CRP) levels were measured in Cx4 Beckman auto-analyzer by a turbidimetric method (Boyden et al., 1947). In the reaction solution, CRP combined with a specific antibody to form an insoluble antigen-antibody complex. The Cx4 Beckman auto-analyzer system monitored the change in absorbance at 340 nm. The change in absorbance was proportional to the concentration of CRP in the sample and was used to calculate and express CRP concentration based on a multi-point, nonlinear calibration curve.

Chapters 6 and 7: Blood insulin levels were determined using a commercial mouse insulin kit (Mercodia, Arium Barbosa Portugal).

Glucose tolerance was evaluated using the intraperitoneal glucose tolerance test. In brief, an intraperitoneal injection of glucose (1.8 g/kg body weight) was administered to fasted mice and the levels of blood glucose were monitored immediately before and 30, 60, 90 and 120 minutes after glucose administration.

Blood HbA1C levels were determined using a commercial DCA Vantage™ analyzer (Siemens HealthCare Diagnostics, Portugal) and compatible HbA1C reactive tests (Siemens HealthCare Diagnostics, Portugal).

Blood triglycerides and cholesterol levels were determined using a commercial Accutrend Plus Monitor (Accutrend - Roche, Portugal) and compatible reactive tests for triglycerides and cholesterol, respectively.

The plasma levels of CRP (BioVendor Laboratorni Medicina a. s.), E-selectin (R&D Systems, Inc.), intercellular adhesion molecule-1 (ICAM-1) (R&D Systems, Inc.), vascular cell adhesion molecule-1 (VCAM-1) (R&D Systems, Inc.) and atrial natiuretic peptide (ANP) (Phoenix Europe GmbH) were measured with commercially available kits.

Brain vessels isolation – Brain vessels were isolated from rodents by following a previously described method (McNeill et al., 1999). Briefly, mice were decapitated, and the whole brain minus the cerebellum was rapidly removed, washed, minced, and homogenized, at 4°C, in phosphate buffer [PBS; 0.01M (8.5g/L NaCl and 1.42g/L Na_2HPO_4; pH=7.4)] and then centrifuged 3x 2000 rpm x 5 minutes (Sigma 3-16K Refrigerated Centrifuge Swing-out rotor, Sigma 11133). The supernatants were collected for synaptosomes isolation. The pellet was then resuspended in PBS and layered over dextran 16% and then centrifuged at 5000 rpm x 20 minutes. The

supernatant and the middle layer were collected and the above steps repeated. Finally, the two resulting pellets were resuspended in PBS and stored at -80ºC. Protein concentration was determined by the biuret method calibrated with bovine serum albumin (BSA) (Gornall et al., 1949).

Synaptosomes isolation- Synaptosomes were isolated following a method described by Hajós (1975), with slight modifications. Briefly, the supernatants were collected during brain vessels isolation (as previously described) and centrifuged at 11000 rpm x 10 minutes (Sorvall RC-5B Refrigerated Superspeed Centrifuge). The resulting pellet was resuspended in 10 ml of homogenization medium (0.32 M sucrose, 10 mM HEPES, and 0.5 mM EGTA-K^+, pH 7.4) and centrifuged at 2500 rpm x 10 minutes. Then, the supernatant was centrifuged at 10000 rpm x 10 minutes. The supernatant was discharged and the pellet was resuspended in 10 ml of washing medium (0.32 M sucrose, 10 mM HEPES, pH 7.4) and centrifuged at 10000 rpm x 10 minutes. The final pellet containing synaptosomes was resuspended in 200 µl of washing medium. Protein concentration was determined by the biuret method calibrated with BSA (Gornall et al., 1949).

Isolation of brain mitochondria – Brain mitochondria were isolated from mice by the method of Rosenthal et al. (1987), with slight modifications, adding 0.02% digitonin to free mitochondria from the synaptosomal fraction. In brief, a mouse was decapitated, and the whole brain minus the cerebellum was rapidly removed, washed, minced, and homogenized, at 4°C, in 10 ml of isolation medium (225 mM mannitol, 75 mM sucrose, 5 mM HEPES, 1 mM EGTA, 1 mg/ml BSA, pH 7.4) containing 1.5 mg of

bacterial protease type VIII (Subtilisin). Single brain homogenates were brought to 20 ml and then centrifuged at 2500 rpm x 5 minutes (Sorvall RC-5B Refrigerated Superspeed Centrifuge). The pellet, including the fluffy synaptosomal layer, was resuspended in 10 ml of the isolation medium containing 0.02% digitonin and centrifuged at 10000 rpm x 10 minutes. The brown mitochondrial pellet without the synaptosomal layer was then resuspended in 10 ml of isolation medium and centrifuged at 10000 rpm x 5 minutes. The pellet was resuspended in 10 ml of washing medium (225 mM mannitol, 75 mM sucrose, 5 mM HEPES, pH 7.4) and centrifuged at 10000 rpm x 5 minutes. The final mitochondrial pellet was resuspended in 100 µl of the washing medium. Mitochondrial protein was determined by the biuret method calibrated with BSA (Gornall et al., 1949).

Measurement of mitochondrial respiration - Oxygen consumption of the brain mitochondria was registered polarographically with a Clark oxygen electrode (Estabrook, 1967) connected to a suitable recorder in a thermostated water-jacketed closed chamber with magnetic stirring. The reactions were carried out at 30 °C in 1 ml of the standard medium (100 mM sucrose, 100 mM KCl, 2 mM KH_2PO_4, 5 mM Hepes and 10 µM EGTA, pH 7.4) with 0.5 mg of protein. The respiratory state 2 of mitochondrial respiration was initiated with 5 mM succinate (mitochondrial energization through complex II) in the presence of 2 µM rotenone (inhibitor of complex I). RCR is the ratio between respiratory states 3 (consumption of oxygen in the presence of succinate and 155 nmol ADP/mg protein) and 4 (consumption of oxygen after ADP has been phosphorylated). The ADP/O index is expressed by the ratio between the amount of ADP added to the reaction medium and the oxygen consumed during respiratory state 3.

Measurement of mitochondrial transmembrane potential ($\Delta\Psi_m$) - $\Delta\Psi$m was monitored by evaluating the transmembrane distribution of the lipophilic cation tetraphenylphosphonium (TPP^+) with a TPP^+-selective electrode prepared according to Kamo et al. (1979) using an Ag/AgCl-saturated electrode (Tacussel, model MI 402) as reference. TPP^+ uptake has been measured from the decreased TPP^+ concentration in the medium sensed by the electrode. The potential difference between the selective electrode and the reference electrode was measured with an electrometer and recorded continuously in a Kipps and Zonen recorder. The voltage response of the TPP^+ electrode to log [TPP^+] was linear with a slope of 59 ± 1, in a good agreement with the Nernst equation. Reactions were carried out in a chamber with magnetic stirring in 1 ml of the standard medium containing 3 µM TPP^+. This TPP^+ concentration was chosen in order to achieve high sensitivity in measurements and to avoid possible toxic effects on mitochondria (Jensen and Gunther, 1984). The $\Delta\Psi$m was estimated by the equation: $\Delta\Psi m\ (mV) = 59 \log(v/V) - 59 \log(10\Delta E/59-1)$, as indicated by Kamo et al. (1979) and Muratsugu et al. (1977). v, V, and ΔE stand for mitochondrial volume, volume of the incubation medium and deflection of the electrode potential from the baseline, respectively. This equation was derived assuming that TPP^+ distribution between the mitochondria and the medium follows the Nernst equation, and that the law of mass conservation is applicable. A matrix volume of 1.1 µl/mg protein was assumed. No correction was made for the "passive'' binding contribution of TPP^+ to the mitochondrial membranes, because the purpose of the experiments was to show relative changes in potentials rather than absolute values. As a consequence, we can anticipate a slight overestimation on $\Delta\Psi$m values. However, the overestimation is only significant at $\Delta\Psi$m values below 90 mV, therefore, far from our measurements. Mitochondria (0.5 mg/ml) were energized with 5 mM succinate in the presence of 2 µM rotenone. After a

steady-state distribution of TPP^+ had been reached (ca. 1 minute of recording), $\Delta\Psi m$ fluctuations were recorded.

Measurement of mitochondrial enzymatic activities: NADH-cytochrome C reductase (complex I-III) and succinate-cytochrome C reductase (complex II-III) activities were assayed spectrophotometrically in 100 mM phosphate buffer (pH 7.4), at 30 °C. For the determination of NADH-cytochrome C reductase and succinate-cytochrome C reductase, samples (100 µg/ml) were added to 0.2 mM NADH or 20 mM succinate, respectively, and 1 mM KCN. The reaction was started with 0.1 mM cytochrome C and followed in a Jasco V5060 UV/VIS spectrophotometer at 550 nm, as previously described (Hatefi and Rieske, 1967, King, 1967). The activities were determined using the molar extinction coefficient 19.6 mM-1 cm-1 and expressed as nmol/minute/ml.

Cytochrome oxidase activity was measured polarographically by monitoring O_2 consumption with a Clark-type oxygen electrode (YSI Model 5331, Yelloe Spring Inst) connected to a suitable recorder in a thermostated water-jacketed closed chamber under magnetic stirring at 30 °C in 1 ml of the reaction medium (130 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 5 mM KH_2PO_4, 5 mM Hepes-Tris, pH 7.4) supplemented with 2 µM rotenone, 10 µM cytochrome c and 100 µg of protein. The reaction was initiated with 5 mM ascorbate plus 0,25 mM N, N, N′, N′-tetrametyl-*p*-phenylenodiamine (TMPD) and finished with 1 mM KCN. The activity was determined by evaluating oxygen consumption and expressed as nAtgO/ minute/ mg protein.

The above-mentioned mitochondrial enzymatic activities were corrected with the citrate synthase activity (Estabrook, 1967). Mitochondrial citrate synthase activity

was determined in 50 mM Tris-HCl (pH 7.8) containing 0.1% Triton X-100, 0.5 mM 5-5′-dithio-bis (2-nitrobenzoic acid) DTNB, 2 mM acetyl-CoA. The reaction was started with 5 mM oxaloacetate and followed spectrophotometrically at 412 nm, according to Coore and coworkers (1971). The activity was determined using the molar extinction coefficient 13.6 mM-1 cm-1 and expressed as nmol/minute/ml.

Aconitase activity was determined according to Krebs and Holzach (1952). Briefly, samples (200 µg) were diluted in 0.6 ml buffer containing 50 mM Tris-HCl and 0.6 mM $MnCl_2$ (pH=7.4), and sonicated for 10 seconds. Aconitase activity was immediately measured spectrophotometrically by monitoring at 240 nm the *cis*-aconitase after the addition of 20 mM isocitrate at 25º C. The activity of aconitase was calculated using a molar coefficient of 3.6 mM^{-1} cm^{-1} and expressed as U/mg protein/minute. One unit was defined as the amount of enzyme necessary to produce 1 µM *cis*-aconitate per minute.

Determination of adenine nucleotide levels - At the end of each $\Delta\Psi m$ measurement, 250 µl of each sample was promptly centrifuged at 14000 rpm x 2 minutes (Eppendorf Centrifuge 5415C) with 250 µl of 0.3 M perchloric acid ($HClO_4$). The supernatants were neutralized with 10 M KOH in 5 M Tris and again centrifuged at 14000 rpm x 2 minutes. The resulting supernatants were assayed for adenine nucleotide by separation in a reverse-phase high performance liquid chromatography (HPLC). The HPLC apparatus was a Beckman-System Gold, consisting of a 126 Binary Pump Model and 166 Variable UV detector controlled by a computer. The detection wavelength was 254 nm, and the column was a Lichrospher 100 RP-18 (5µm) from Merck. An isocratic elution with 100 mM phosphate buffer (KH_2PO_4; pH 6.5) and 1.2% methanol was performed with a flow rate of 1 ml/minute. The required time for each analysis was 5

minutes. Adenine nucleotides were identified by their chromatographic behaviour (retention time, absorption spectra and correlation with standards).

Measurement of Ca^{2+} fluxes – Mitochondrial Ca^{2+} fluxes were measured by monitoring the changes in Ca^{2+} concentration in the reaction medium using a Ca^{2+}-selective electrode (Moreno and Madeira, 1991). The reactions were conducted in an open vessel with magnetic stirring in 1 ml of the standard reaction medium containing 0.5 mg protein and 80 nmol $CaCl_2$/mg protein. The reaction started with the addition of 5 mM succinate. The inhibitors of the mitochondrial permeability transition pore (PTP) 2 mg/ml oligomycin plus 1 mM ADP were added 2 minutes before mitochondrial energization.

Electron Microscopy – At the end of each Ca^{2+} flux measurements, 1 ml of each sample was promptly centrifuged at 14000 rpm x 2 minutes (Eppendorf Centrifuge 5415 R). The pellets, containing mitochondria, were fixed for electron microscopy by the addition of 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.3, and incubated for 2 hours, at 4°C. After centrifugation (13200 rpm x 3 minutes; Eppendorf Centrifuge 5415 R), the pellets were washed with 0.1 M PBS, pH 7.3. The fixed and washed pellet was resuspended in 1% osmium tetroxide (OsO_4) buffered with phosphate buffer 0.1 M, pH 7.3. After 2 hours of incubation, membranes were washed with phosphate buffer (pH 7.3; without OsO_4). After preincubation in 1% agar, the samples were dehydrated in grade ethanol and embedded in Spurr. The ultrathin sections were obtained in an LKB ultramycrotome Ultrotome III, stained with methanolic uranyl acetate followed by lead citrate, and examined with a Jeol Jem-100SV electron microscope operated at 80 kV.

Measurement of malondialdehyde (MDA) levels - MDA levels were determined by HPLC, as previously described (Wong et al., 1987, Knight et al., 1988, Draper and Hadley, 1990). In brief, 250 µl of protein were added to 250 µl H_2O, 500 µl TBA 42 mM and 250 µl H_3PO_4 0.44 M. The samples were boiled during 1 hour and then, 500 µl of each sample was added to 500 µl methanol/NaOH 1 M (10:1) and centrifuged at 3000 rpm x 10 minutes. MDA levels were determined by liquid chromatography, performed in a Gilson HPLC apparatus with a reverse phase column (RP18 Spherisorb, S5 OD2). The samples were eluted from the column at a flow rate of 1ml/minute and detection was performed at 532 nm. The MDA content was calculated from a standard curve prepared using the thiobarbituric acid-MDA complex and was expressed as nmol/mg protein.

Measurement of H_2O_2 levels - H_2O_2 levels were measured fluorimetrically using a modification of the method described by Barja (1999). Briefly, samples (0.2 mg) were incubated at 30ºC in 1.5 ml of phosphate buffer, pH 7.4, containing 0.1 mM EGTA, 5 mM KH_2PO_4, 3 mM $MgCl_2$, 145 mM KCl, 30 mM Hepes, 0.1 mM homovalinic acid and 6 U/ml horseradish peroxidase. After 15 minutes, the reaction was stopped with 0.5 ml cold stop solution (0.1 M glycine, 25 mM EDTA-NaOH, pH 12). Fluorescence of supernatants was determined at 312 nm excitation and 420 nm emission wavelengths. The H_2O_2 levels were calculated using a standard curve of H_2O_2.

Measurement of GSH and GSSG levels - GSH and GSSG levels were determined with fluorescence detection after reaction of the supernatant containing H_3PO_4/NaH_2PO_4-EDTA or H_3PO_4/NaOH, respectively, of the deproteinized

homogenates solution with o-phthalaldehyde (OPT), pH 8.0, according to Hissin and Hif (1976). In brief, samples (200 µg) were resuspended in 1.5 ml phosphate buffer (100 mM NaH_2PO_4, 5 mM EDTA, pH 8.0) and 500 µl H_3PO_4 4.5% and rapidly centrifuged at 50000 rpm x 30 minutes (Beckman, TL-100 Ultracentrifuge). For GSH determination, 100 µl of supernatant was added to 1.8 ml phosphate buffer and 100 µl OPT. After thorough mixing and incubation at room temperature for 15 minutes, the fluorescence of the solution was measured at 420 nm and 350 nm emission and excitation wavelength, respectively. For GSSG determination 250 µl of the supernatant were added to 100 µl of N-ethylmaleymide and incubated at room temperature for 30 minutes. After the incubation, 140 µl of the mixture were added to 1.76 ml NaOH (100 mM) buffer and 100 µl OPT. After mixing and incubation at room temperature for 15 minutes, the fluorescence of the solution was measured at 420 nm and 350 nm emission and excitation wavelength, respectively. The GSH and GSSG contents were determined from comparisons with a linear GSH and GSSG standard curve, respectively.

Measurement of vitamin E content - Extraction and separation of vitamin E (α-tocopherol) from samples were performed by following a method previously described by Vatassery and Younoszai (1978). Briefly, 1.5 ml sodium dodecyl sulfate (10 mM) was added to 0.2 mg of protein, followed by the addition of 2 ml ethanol. Then, 2 ml hexane and 50 µl of KCl 3M were added, and the mixture was vortexed for about 3 minutes. The extract was centrifuged at 2000 rpm x 10 minutes (Sorvall RT6000 Refrigerated Centrifuge) and 1 ml of the upper phase, containing n-hexane (n-hexane layer), was recovered and evaporated to dryness under a stream of N_2 and kept at -80ºC. The extract was dissolved in n-hexane, and vitamin E content was analyzed by reverse-phase HPLC. A Spherisorb S10w column (4.6 x 200 nm) was eluted with n-

hexane modified with 0.9% methanol, at a flow rate of 1.5 ml/minute. Detection was performed by an UV detector at 287 nm. The levels of vitamin E were calculated as nmol/mg protein.

Measurement of GPx activity - GPx activity was determined spectrophotometrically by following the method of Flohé and Gunzler (1984). Briefly, the activity of GPx was measured upon a 5 minutes incubation, in the dark, of 100 μg of each sample with 0.5 mM phosphate buffer (0.25 M KH_2PO_4, 0.25 M K_2HPO_4 and 0.5 mM EDTA, pH 7.0), 0.5 mM EDTA, 1 mM GSH and 2.4 U/ml of GR. The quantification occurred after the addition of 0.2 mM NADPH and 1.2 mM tertbutylhydroperoxide, at 340 nm, and continuous magnetic stirring, for 5 minutes, in a Jasco V560 UV/VIS Spectrophotometer. The measurements were made against blanks prepared in the absence of NADPH. GPx activity was determined using the molar extinction coefficient 6220 M-1 cm-1 and expressed as nmol/min/mg protein.

Measurement of GR activity - For the activity of GR, 100 μg of each sample were incubated for 1 minute with 1 ml phosphate buffer (containing 0.2 M K_2HPO_4 and 2 mM EDTA, pH 7.0) and 2 mM NADPH. The measurements were initiated with the addition of 20 mM GSSG, at 340 nm, at 30ºC, with continuous magnetic stirring for 4 minutes, against blanks prepared in the absence of GSSG, using a Jasco V560 UV/VIS Spectrophotometer (Carlberg and Mannervik, 1985). GR activity was determined using the molar extinction coefficient 6220 M-1 cm-1 and expressed as nmol/min/mg protein.

Measurement of SOD activity - SOD activity was determined spectrophotometrically at 550nm (Flohe and Otting, 1984). After the incubation of 100 µg of protein in 1.4 ml of phosphate buffer (50 mM K_2HPO_4 and 100 µM EDTA, pH 7.8), 0.025 mM hypoxanthine, 0.025% Triton X-100, 0.1 mM nitrobluetetrazolium (NBT) in the presence (MnSOD) or absence (total SOD) of 1.33 mM KCN and CuZnSOD was calculated by the subtraction of MnSOD activity to the total SOD activity. The reaction was started with the addition of 0.025 U/ml xanthine oxidase and the reaction was allowed to continue for 200 seconds, at 25 ºC, with continuous magnetic stirring. The measurements were performed in a Jasco V560 UV/VIS Spectrophotometer, against a blank, prepared in the absence of hypoxanthine. The activity of SOD was calculated using a standard curve, prepared with different concentrations of SOD commercially available and expressed as nmol/min/mg protein.

Measurement of catalase activity- Catalase activity was measured by oxygen production ratio after the addition of 1 µM of H_2O_2 to 25 µg of protein, and monitored polarographically with a Clark oxygen electrode (YSI Model 5331, Yellow Springs Inst) connected to a suitable recorder in a 1 ml thermostated, water-jacketed closed chamber with magnetic stirring, as previously described by Estabrook (1967). The reactions were carried out at 25º C in 1 ml of the reaction medium (130 mM sucrose, 50 mM KCl, 2.5 mM $MgCl_2$, 2.5 mM KH_2PO_4, 100 µM EGTA, 5 mM Hepes at pH 7.4). Catalase activity was determined by measuring oxygen produced as H_2O_2 is decomposed and expressed as nAtgO/ minute/ mg protein.

Evaluation of Aβ and p-tau proteins levels by western blot - Brains were homogenized in buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 1% Sodium deoxycholate (DOC) and 0.1 % Sodium dodecyl sulfate (SDS) (pH 7.4), protease inhibitors (commercial protease inhibitor cocktail from Roche), phosphatase inhibitors (commercial phosphatase inhibitor cocktail from Roche), 0.1 M Phenylmethanesulfonyl Fluoride (PMSF) (Sigma), 0.2 M Dithiothreitol (DTT) (Sigma), frozen three times in liquid nitrogen and centrifuged at 14.000 rpm for 10 minutes (Eppendorf Centrifuge 5415C). The supernatants represent the cytosolic fractions and the resulting pellets the membrane fractions. The amount of protein content in the samples was measured using the BCA protein assay kit (Pierce). The samples were resolved by electrophoresis in 10% SDS–polyacrylamide gels and transferred to Polyvinylidene difluoride (PVDF) membranes. Non-specific binding was blocked by gently agitating the membranes in 5% BSA and 0.1% Tween in Tris-buffered saline (TBS) for 1 hour at room temperature. The blots were subsequently incubated with specific primary antibodies (1:1.000 mouse monoclonal human beta amyloid clone 6E10 from Signet Laboratories; 1:1.000 mouse monoclonal PHF-tau MAb (clone AT8) from Thermo Fisher Scientific; or 1:10.000 monoclonal anti-alpha-tubulin antibody from SIGMA), overnight at 4 °C, with gentle agitation. Blots were washed three times (3x 15 minutes), with TBS containing 0.1% Tween (TBS-T) and then incubated with secondary antibodies for 2 hours at room temperature with gentle agitation. After three washes with TBS-T specific bands of immunoreactive proteins were visualized after membrane incubation with enhanced chemifluorescence (ECF) for 5 minutes in a VersaDoc Imaging System (Bio-Rad), and the density of protein bands was calculated using the Quantity One Program (Bio-Rad).

Evaluation of Aβ deposition by immunohistochemistry- Rodent brains were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The fixed brains were immersed for cryoprotection with 30% sucrose in 0.1 M PBS, pH 7.4. Fixed frozen sections were cut at 40 µm thickness, collected in PBS containing 0.1% sodium azide until use. To abolish endogenous peroxidase activity, brain sections were treated with 3% H_2O_2 for 15 minutes followed by rinsing with PBS. A subsequent blocking step with 3% goat serum in 0.5% BSA, 0.2% Triton X-100 in PBS was carried out for 30 minutes at room temperature to prevent non-specific antibody binding. Slices were incubated with primary antibody (1:1,000 mouse monoclonal human beta amyloid clone 6E10 from Signet Laboratories) overnight at 4ºC with gentle agitation. Antibodies were detected with biotinylated goat anti-mouse IgG (Sigma B0529, 1:200). Sections were further processed with extravidin–peroxidase conjugate (Sigma S5512, 1:500) and antibody localization was visualized with 0.04% DAB (3,30-diaminobenzidine, Sigma D4168)/0.0015% H_2O_2 as chromogen. Primary antibodies were omitted in control sections. Brain slices were than mounted in a slide and let to dry overnight at room temperature. Counterstaining was performed with hematoxilin staining.

Hematoxilin staining- For hematoxilin countersaining, slices were immersed in a hematoxilin solution (Sigma GHS316) for 3 minutes. After, slices were washed under running tap water for 15 minutes followed by dehydratation.

Measurement of Evans Blue leakage- The Evans blue dye is used to assess the permeability of the BBB to macromolecules. Briefly, animals were anesthetized and the Evans blue dye was injected into the bloodstream through the tail vein, where it rapidly binds to plasma albumin. After 30 minutes in circulation, animals were perfused with

citrate-buffered paraformaldehyde to clear the Evans blue from the bloodstream. Brains were removed and the cortex, hippocampus and cerebellum were dissected out. The dye extravasation was then removed from brain tissue by formamide. The concentration of the dye was then measured by spectrophotometry at 620 nm (Thermo Electron Corporation Type Helios gamma). Because serum albumin cannot cross the BBB, and virtually all Evans Blue is bound to albumin, normally the neural tissue remains unstained. However, when the plasma extravasates from blood vessels (vascular permeability) the Evans blue dye–albumin complex leaks into the surrounding tissues allowing the measurement of endothelial leakage.

Statistical analysis - Results are presented as mean ± SEM of the indicated number of experiments. Statistical significance was determined using the paired student *t*-test or Kurskal-Wallis test for multiple comparisons, followed by the posthoc Dunn test or using the Kurskal-Wallis test for multiple comparisons, followed by the post hoc Mann-Withney test.

3.3- In vitro studies

Brain microvascular endothelial cells isolation- Rat brain microvascular endothelial cells **(**RBMEC) were isolated from 10-week-old Sprague-Dawley rats and mouse brain microvascular endothelial cells (MBMEC) were isolated from 10-week-old $Lepr^{db}$ or $Dock7^{m}/Lepr^{db}$ mice, as previously described (Kis et al., 1999). Briefly, animals were decapitated under deep anesthesia, and the brain cortices were freed from meninges, homogenized, and digested. The homogenate was redistributed in 20% BSA and was centrifuged at 2100 rpm x 20 minutes (Sorvall RC5B Refrigerated Superspeed

Centrifuge) to yield cortical microvessels. The microvessels were washed in DMEM (Dulbecco's modified Eagle medium), further digested, layered on a continuous 33% Percoll gradient, and centrifuged again at 21000 rpm x 10 minutes (Internation Equipment Company - IECCR 6000 Centrifuge). The band of cerebral microvascular endothelial cells was aspirated, washed, and was then seeded onto collagen IV and fibronectin-coated glass-bottom culture dishes (MatTek, Ashland, MA, USA) and plates (BD Falcon, Bedford, MA). A 300 µl volume of cells was added to each well and let to seed for 24 hours. Then, the medium was changed and Puromycin (4 µg/ml) was added for 48 hours to avoid the proliferation of P-glycoprotein negative contaminating cells (Perriere et al., 2005). At day 3 the cells medium was replaced by fresh medium with different glucose concentrations and the medium was changed every 48 hours. The cell culture medium consisted of DMEM supplemented with 20% fetal bovine plasma-derived serum, 2 mM glutamine, 1 ng/ml basic fibroblast growth factor, 50 µg/ml endothelial cell growth supplement, 100 µg/ml heparin, 5 µg/ml vitamin C, and antibiotics. We have previously demonstrated the purity of our cultures (Domoki et al., 2008)

Cell culture and treatments - RBMEC were exposed to 5, 25 and 30 mM of glucose for 7 days, at 37 °C, and MBMEC were maintain in 5 mM or 25 mM glucose medium for 7 days, at 37ºC (**Figure 3.4**). 5 or 10 µM of $A\beta_{1-40}$ was added at day 6 (**Figure 3.4**). In some experiments (cell viability, $\Delta\Psi m$, total ROS and H_2O_2 levels), RBMECs were co-incubated with $A\beta_{1-40}$ and antioxidants (100 µM MitoTempo or 100 units/ml Peg-SOD) to prove the involvement of mitochondrial ROS in Aβ-induced toxicity (**Figure 3.4**). Osmotic controls in RBMEC were done with 25 mM mannitol and $A\beta_{40-1}$ was used as peptide negative control.

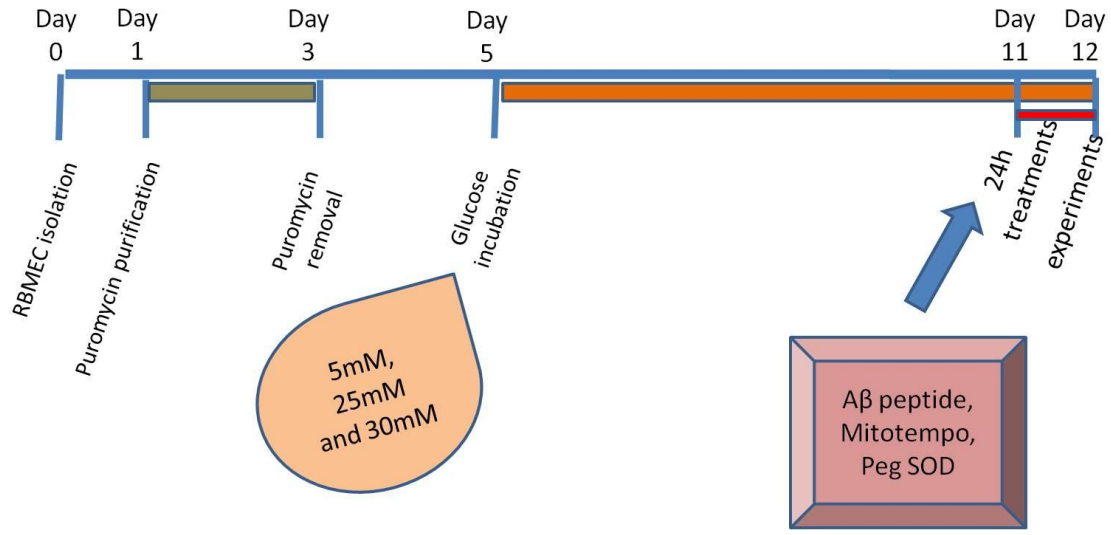


Figure 3.4- ***Experimental design used in chapter 9***. RBMEC were isolated from 10-weeks-old adult Sprague-Dawley rats and MBMEC were isolated from 10-weeks-old $Lepr^{db}$ or $Dock7m/Lepr^{db}$ mice. After 24 hours, cells were washed twice and fresh culture medium containing Puromycin was added. At day 5, RBMEC were exposed to 5, 25 or 30 mM of glucose for 7 days at 37 °C and MBMEC were maintain in 5 mM or 25 mM glucose for 7 days at 37ºC. At day 6 cells were exposed to 5 and/or 10 μM $A\beta_{1-40}$. In some experiments RBMECs were treated with a combination of Aβ peptide and antioxidants to evaluate the contribution of different ROS sources to cell death.

Assessment of cell viability - Cell viability was determined using the Alamar Blue assay. 1 hour before the end of cells treatment, a solution of Alamar blue was added to the culture medium in a final concentration of 10% (v/v) Alamar blue. After 1 hour incubation at 37 °C, the supernatant was collected and the absorbance was measured at 570 nm and 600 nm using a microplate reader (SpectraMax uQuant microplate Reader, BioTek, Winooski, VT) (Neves et al., 2006). Cell viability (% of control) was calculated according to the formula (A57 − A600) of treated cells × 100/(A570 − A600) of control cells.

Measurement of mitochondrial ROS production - Life staining of RBMEC for mitochondrial $O_2^{\bullet -}$ production was performed using MitoSOX (Molecular Probes, Eugene, OR), a cell permeable probe that accumulates in mitochondria and fluoresces following oxidation by $O_2^{\bullet -}$, as describe by Knorr et al. (2011). MitoSOX was dissolved in dimethyl sulfoxide (DMSO), and cells were incubated with a solution of MitoSOX in Dulbecco's Modified Eagle's Medium (DMEM) phenol-free at the final concentration of 5 µM, at 37ºC, for 20 minutes in a light-protected coverslip chamber. Then, cells were rinsed and fresh DMEM phenol-free was added. Cells were immediately examined by confocal microscopy. Images were obtained with a Leica SP2 AOB laser confocal microscope (Heidelberg, Germany) for a period no longer than 20 minutes.

Measurement of H_2O_2 levels - H_2O_2 levels were measured using the Amplex™ Red-horseradish peroxidase assay kit, as previously described (Muller et al., 2004). This assay utilizes horseradish peroxidase to catalyze the H_2O_2-dependent oxidation of non-fluorescent Amplex™ Red to fluorescent resorufin red. Briefly, 50 µM Amplex™ Red reagent and 0.1 U/ml peroxidase in DMEM phenol-free were added to cells and incubated for 1 hour at 37ºC protected from light (Santiago et al., 2008). Fluorescence was read at 565 nm wavelength, at 37 °C, in an automatic microplate reader (FLUOstar OPTIMA microplate reader, BMG Labtech, Offenburg, Germany) equipped with a thermally controlled compartment and results were expressed as % of control.

Measurement of (ΔΨm) - Rhodamine 123 (RHD123) (Molecular Probes, Eugene, OR), a fluorescent cationic dye, was used to monitor changes in ΔΨm. After

the treatment period, cells were washed with PBS and incubated with 2.5 μM RHD123 in DMEM phenol-free for 45 minutes at 37 °C. After the 45 minutes incubation with RHD123 the media was replaced with fresh DMEM phenol-free and fluorescence was read using a FLUOstar OPTIMA microplate reader (BMG Labtech, Offenburg, Germany). After incubation with rhodamine 123, basal fluorescence was monitored at 505 nm excitation and 525 nm emission wavelengths. Immediately after the initial reading, the mitochondrial uncoupler carbonylcyanide m-chlorophenylhydrazone (CCCP) (5 μM) was added to induce complete depolarization. The difference between the initial fluorescence and the final fluorescence (after CCCP exposure) was used to evaluate ΔΨm. The results were expressed as percentage of control fluorescence. Furthermore, the same protocol was performed for live imaging and cells were examined by confocal microscopy. Images were obtained with a Zeiss 7 Live laser scanning confocal microscope (Jena, Germany) for a period no longer than 10 minutes.

Statistical analysis - Results are presented as mean ± SEM of the indicated number of experiments. Statistical significance was determined using One-Way Anova test for multiple comparisons, followed by the post hoc Tukey test.

Chapter 4- Chronic hypoxia potentiates age-related oxidative imbalance in brain vessels and synaptosomes

4.1- Abstract

This study was aimed to evaluate and compare the effects of chronic hypoxia and aging in the oxidative status of brain vessels and synaptosomes. For this purpose we isolated brain vessels and synaptosomes from 3- and 12-month-old rats subjected to chronic hypoxia (10% O_2 for 7 days) or normoxia (21% O_2). Several parameters were evaluated: mitochondrial aconitase activity, H_2O_2 and MDA levels and enzymatic [SOD, catalase, GPx and GR] and non-enzymatic [GSH, GSSG and vitamin E] antioxidant defenses. Concerning brain vessels, we observed an age-dependent increase in MDA levels and SOD, catalase, GR and GPx activities. In vessels isolated from young animals, chronic hypoxia induced an increase in H_2O_2, GSSG and vitamin E levels and copper/zinc superoxide dismutase (CuZnSOD) and catalase activities and a decrease in GSH levels. In mature animals, hypoxia induced a decrease in GSH/GSSG ratio, vitamin E levels and mitochondrial aconitase, MnSOD and GR activities and an increase in H_2O_2 levels and CuZnSOD and catalase activities. Concerning synaptosomes we observed an age-dependent increase in MDA levels, CuZnSOD and GPx activities and a decrease in MnSOD activity. In synaptosomes from young animals, chronic hypoxia induced a decrease in mitochondrial aconitase activity and GSH levels and an increase in CuZnSOD activity and GSSG levels. In synaptosomes from mature animals, hypoxia induced a decrease in mitochondrial aconitase activity, GSH/GSSG ratio, GSH and vitamin E levels and an increase in GSSG levels. Our results show that chronic hypoxia promotes and potentiates age-dependent oxidative imbalance predisposing to neurodegeneration. Further, synaptosomes and brain vessels are differently affected by aging and chronic hypoxia supporting the idea of the existence of tissue-specific susceptibilities.

4.2-Introduction

It is well known that aging of CNS and the development of incapacitating neurological diseases are generally associated with a wide range of pathophysiological and histological changes eventually leading to a compromised cognitive status (Farkas and Luiten, 2001). Also, periods of chronic hypoxia, which can arise from numerous disorders and even aging, predispose individuals to the development of neurodegenerative conditions. Indeed, the reduction in brain blood flow leads to a decrease in O_2 levels (hypoxia) predisposing cells to death (Roy and Rauk, 2005). A decline in memory arising from brief hypoxic periods has been reported in experimental animals (Chleide et al., 1991). Prolonged or chronic hypoxia has also been shown to contribute to the establishment of neurological diseases by altering mitochondrial function and the excitability and functional expression of ion channels. Recent reports suggested that hypoxia induces oxidative stress, and apoptosis in cerebral cortex, hippocampus and striatum (Askew, 2002, Maiti et al., 2006, Maiti et al., 2007, Maiti et al., 2008a, Maiti et al., 2008b, Maiti et al., 2010). It was also showed that disruption of calcium homeostasis, following hypoxia, in central neurons may contribute to the neurotoxicity of Aβ and subsequent development of AD (Kawahara and Kuroda, 2000).

The aim of this study was to evaluate and compare the impact of chronic hypoxia in the oxidative status of brain vessels and synaptosomes obtained from young and mature rats. As far as we know, no consistent data exist concerning the effect of aging and chronic hypoxia on brain vessels. Since the brain is highly dependent on blood flow to sustain its energetic requirements, any alteration in blood vessels will impact severely brain function. Further, and due to the existence of tissue-specific susceptibilities (Surai et al., 1999, Venditti et al., 1999, Arguelles et al., 2004, Cattan et al., 2008), we also evaluate the effect of aging and chronic hypoxia in synaptosomes.

We evaluated several parameters: mitochondrial aconitase activity, H_2O_2 and MDA levels and enzymatic [SOD, catalase, GPx and GR activities] and non-enzymatic [GSH, GSSG and vitamin E levels] antioxidant defenses.

4.3-Results

4.3.1- Characterization of experimental animals

Hypoxia promoted a decrease in body and brain weight in both 3- and 12-month-old animals. However, only in the 12-month-old rats under chronic hypoxia a significant decrease in brain weight/body weight ratio was observed (**Table 4.1Table 4.1**).

Concerning blood glucose levels, hypoxia led to a significant decrease in this parameter in rats of both ages (**Table 4.1**).

We also observed that hypoxia-treated 12-month-old animals presented an increase in TG, CK and in CRP levels when compared with 12-month-old control animals. 3-month-old hypoxic animals only presented an increase in CRP levels (**Table 4.1**). Total blood analyses revealed that both 3- and 12-month-old animals subjected to chronic hypoxia presented a significant increase in red blood cells (RBC; blood cells that transport O_2 to the cells), hemoglobin (HGB; protein of RBC that transport O_2) hematocrit (HCT; measures % of RBC found in whole blood) and monocytes (**Table 4.1**). An age-dependent increase in red blood cells width (RDW) was also observed (**Table 4.1**).

Table 4.1- ***Characterization of hypoxic and normoxic animals***

	3-month-old		12-month-old	
	Control	**Hypoxia**	**Control**	**Hypoxia**
Body Weight (g)	314.7 ± 3.97	257.6 ± 5.07 ***	446.5 ± 11.48 ***	415.1 ± 9.77 $$$
Brain Weight (g)	2.6 ± 0.16	1.9 ± 0.06***	2.7 ± 0.13	2.1 ± 0.04 $$$
Brain Weight/Body Weight	0.008 ± 0.0004	0.008 ± 0.0002	0.006 ± 0.0003 ***	0.005 ± 0.0001 $$$
Glucose (mg/dl)	128.0 ± 6.44	94.4 ± 4.41 ***	124.2 ± 4.73	94.8 ± 4.50 $$$
TG (mg/dl)	67.5 ± 8.18	65.7 ± 9.7	74.3 ± 6.90	130.6 ± 9.83 $$$
CK (IU/L)	13170 ± 1680	13910 ± 1376	5114 ± 1629 *	5654 ± 1961
CRP (mg/dl)	3.35 ± 0.32	4.583 ± 0.40*	4.483 ± 0.30*	7.425 ± 0.41 $$$
Mo (%)	2.3 ± 0.48	3.9 ± 0.76 *	2.3 ± 0.36	3.9 ± 0.76 $
RBC (10^6/µl)	8.4 ± 0.31	9.6 ± 0.18 **	8.3 ± 0.16	9.38 ± 0.13 $$
HGB (g/dl)	154.4 ± 3.56	182.6 ± 3.43 ***	154.9 ± 2.04	175.1 ± 2.91 $$
HCT (%)	0.5 ± 0.02	0.5 ± 0.01 **	0.46 ± 0.01	0.5 ± 0.01 $$
RDW (%)	12.6 ± 0.30	12.6 ± 0.10	13.2 ± 0.28	13.9 ± 0.26 $

Data shown represent mean ± SEM from 5-6 independent experiments. *p<0.05 when compared with 3-month-old control animals; $p<0.05 when compared with 12-month-old control animals.

4.3.2- Chronic hypoxia potentiates oxidative stress

Mitochondrial aconitase activity is a sensitive redox sensor of ROS and RNS in cells. As shown in **Figure 4.1A** and **B**, aging did not significantly affect aconitase activity in brain vessels and synaptosomes. However, chronic hypoxia promoted a significant decrease in the activity of this enzyme in both vessels and synaptosomes isolated from 12-month-old animals and also in synaptosomes from 3-month-old rats.

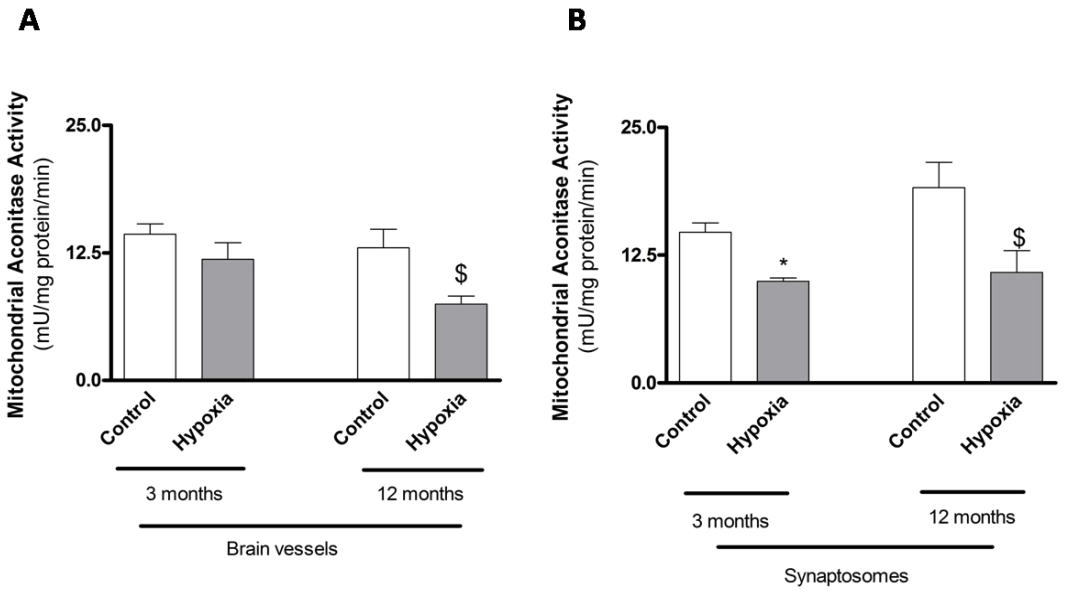


Figure 4.1- ***Effects of age and/or chronic hypoxia on mitochondrial aconitase activity*****.** Aconitase activity in brain vessels (A) and synaptosomes (B) was measured as described in the Material and methods section. Data shown represent mean ± SEM from 5-6 independent experiments. *$p<0.05$ when compared with 3-month-old control animals; \$$p<0.05$ when compared with 12-month-old control animals.

H_2O_2 production gives an indication about the predisposition of cells to originate and/or exacerbate oxidative stress. Aging did not increase the levels of H_2O_2 in brain vessels (**Figure** 4.**2A**). However, chronic hypoxia increased significantly the levels of H_2O_2 in both ages (**Figure 4.2A**). No significant alterations were observed in synaptosomes, although a slight age-dependent increase in H_2O_2 levels was observed (**Figure 4.2B**).

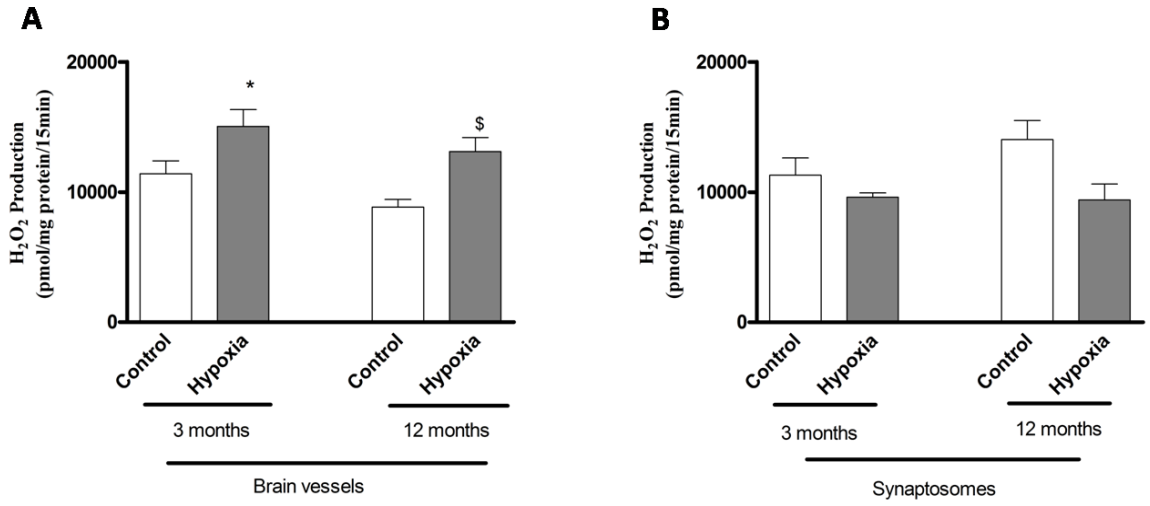


Figure 4.2- ***Effects of age and/or chronic hypoxia on hydrogen peroxide (H_2O_2) production***. H_2O_2 production in brain vessels (A) and synaptosomes (B) was measured as described in Material and methods section. Data are the mean ± SEM of 5-6 independent experiments. *P<0.05 when compared with 3-month-old control animals; $^{\$}$p<0.05 when compared with 12-month-old control animals.

4.3.3- Aging and chronic hypoxia affect the non-enzymatic antioxidant defenses

Endogenous antioxidants such as GSH and vitamin E belong to the first line of defense and act by scavenging potentially damaging free radical moieties. An age-dependent decrease in the levels of GSH and GSSG and GSH/GSSG ratio was observed in brain vessels, this effect being potentiated by chronic hypoxia (**Table 4.2**). In synaptosomes, aging did not affect GSH and GSSG levels (**Table 4.2**). Accordingly, GSH/GSSG ratio was not significantly affected by age (**Table 4.2**). However, chronic hypoxia induced a significant decrease in GSH and a significant increase in GSSG levels in synaptosomes isolated from both young and mature animals (**Table 4.2**). Chronic hypoxia promoted a decrease in GSH/GSSG levels, although only statistically significant in synaptosomes isolated from mature rats (**Table 4.2**).

Table 4.2- Effect of age and/or chronic hypoxia glutathione levels

	Brain vessels				**Synaptosomes**			
	3 months		**12 months**		**3 months**		**12 months**	
	Control	**Hypoxia**	**Control**	**Hypoxia**	**Control**	**Hypoxia**	**Control**	**Hypoxia**
GSH (nmol/mg protein**)**	1.68 ± 0.48	0.19 ± 0.09*	0.65 ± 0.21	0.49 ± 0.17	1.22 ± 0.16	0.49 ± 0.05*	1.27 ± 0.14	0.57 ± 0.09$^{\$\$}$
GSSG (nmol/mg protein**)**	0.64 ± 0.08	0.27 ± 0.14	0.39 ± 0.07	0.47 ± 0.04	0.29 ± 0.05	0.44 ± 0.02*	0.31 ± 0.03	0.71 ± 0.15$^{\$}$
GSH/GSSG	3.08 ± 0.55	2.31 ± 0.83	1.92 ± 0.34	0.57 ± 0.18$^{\$}$	3.55 ± 0.41	2.12 ± 0.61	4.25 ± 0.81	2.12 ± 0.62$^{\$}$

Data shown represent mean ± SEM from 5-6 independent experiments. *p<0.05 when compared with 3-month-old control animals; $p<0.05 when compared with 12-month-old control animals**.**

With regard to vitamin E, a significant increase was observed in vessels from 12-month-old rats (**Figure 4.3A**). Hypoxia induced a significant increase in vitamin E levels in vessels from young rats and a decrease of vitamin E in vessels from mature rats (**Figure 4.3A**). In synaptosomes, aging did not affect vitamin E levels and chronic hypoxia induced a slight decrease in both ages (**Figure 4.3B**).

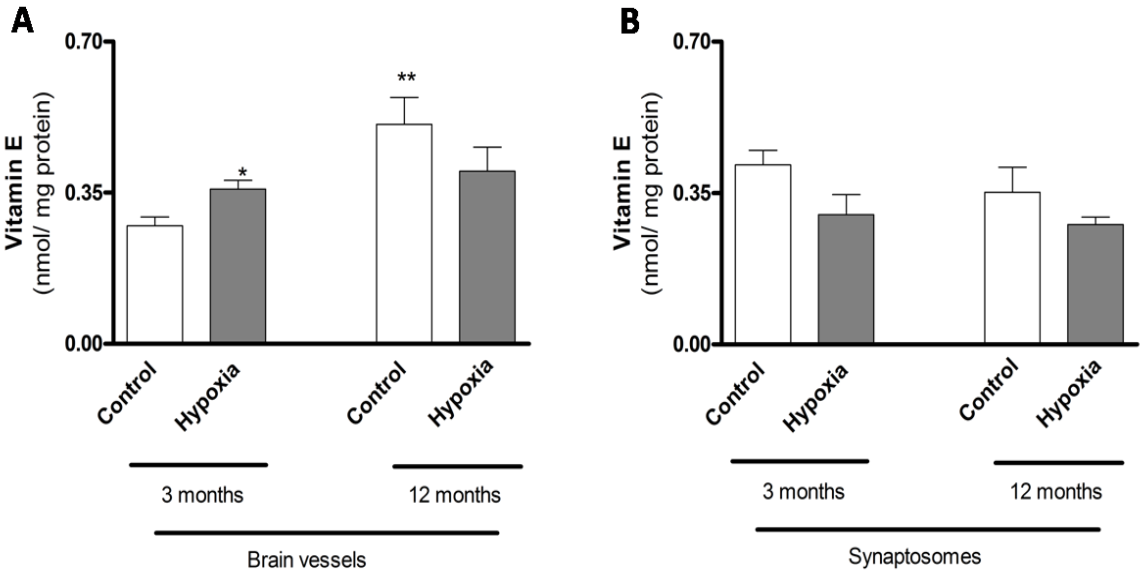


Figure 4.3- ***Effects of age and/or chronic hypoxia on vitamin E levels*****.** Vitamin E levels in brain vessels (A) and synaptosomes (B) were measured as described in Material and methods section. *P<0.05; **P<0.01 when compared with 3-month-old control animals.

4.3.4- Aging and chronic hypoxia affect the enzymatic antioxidant defenses

GPx and GR are two antioxidant enzymes involved in the detoxification of ROS. As shown in **Figure 4.4A and C** a significant age-dependent increase in the activity of both enzymes was observed in brain vessels. Additionally, GR activity of vessels from 12-month-old rats was significantly decreased by chronic hypoxia (**Figure 4.4C**). In synaptosomes, we observed an age-dependent increase in the activities of GPx (**Figure 4.4B**). Hypoxia induced a slight increase in GPx activity in both ages (**Figure 4.4B**) and a slight decrease in the activity of synaptosomal GR of young animals (**Figure 4.4D**).

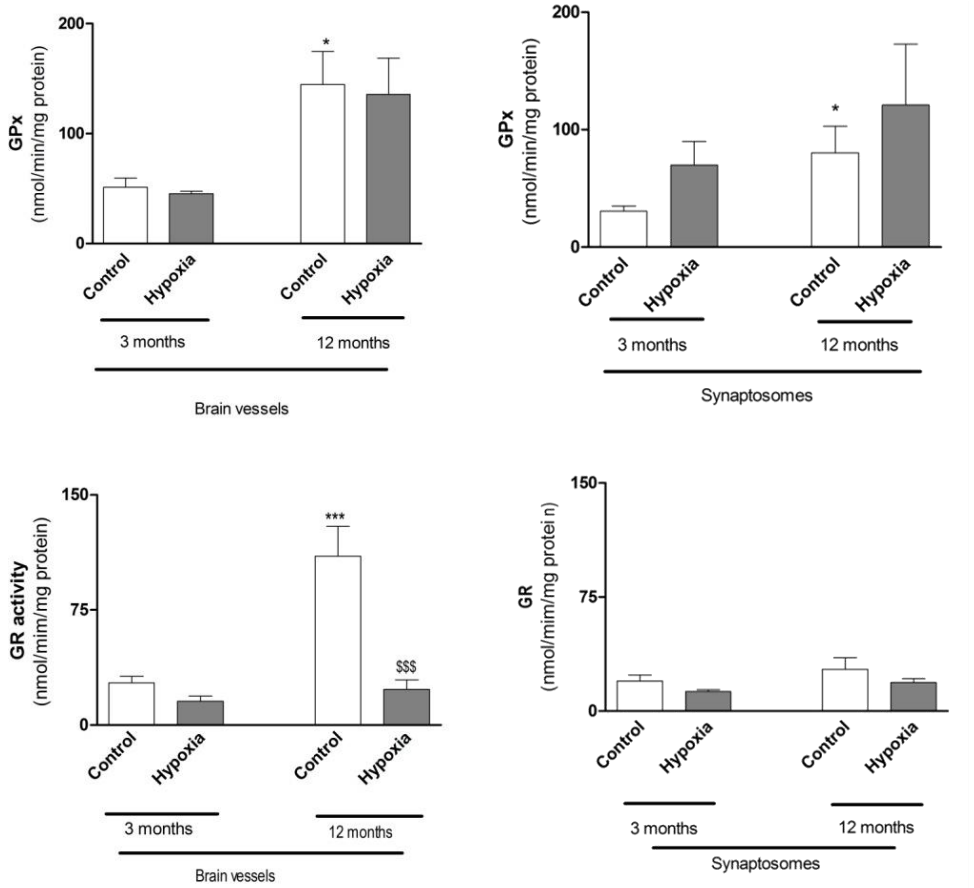


Figure 4.4- ***Effects of age and/or chronic hypoxia on glutathione peroxidase*** **(GPx) and glutathione reductase (GR) activities**. GPx activity on brain vessels (A) and synaptosomes (B) and GR activity on brain vessels (C) and synaptosomes (D) were determined as describe in Material and methods section Data are the mean ± SEM of 5-6 animals from each condition studied. Statistical significance: *p<0.05; ***p<0.001 when compared with 3-month-old control animals; $$$p<0.001 when compared with 12-months-old control animals.

With respect to SOD, we measured the activity of the two isoforms, MnSOD, the mitochondrial isoform, and CuZnSOD, the cytosolic isoform. In brain vessels, we observed a significant age-dependent increase in the activity of MnSOD. However, chronic hypoxia induced a significant decrease in the activity of this enzyme in vessels from mature rats (**Figure 4.5A**). The activity of the cytosolic isoform presents a slight age-dependent increase, this effect being also slightly increased by chronic hypoxia (**Figure 4.5C**). Concerning synaptosomes, we observed an age-dependent decrease in MnSOD, although not statistically significant (**Figure 4.5B**), and an age-dependent increase in CuZnSOD (**Figure 4.5D**). Chronic hypoxia also increased CuZnSOD in synaptosomes isolated from young animals (**Figure 4.5D**).

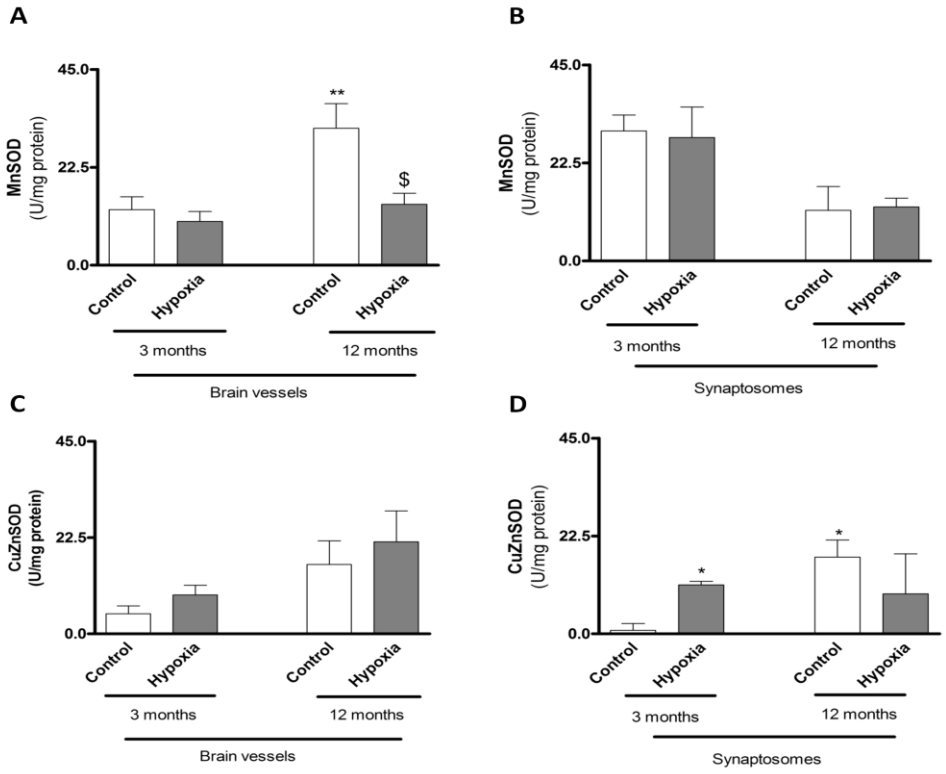


Figure 4.5 - ***Effects of age and/or chronic hypoxia on manganese superoxide dismutase (MnSOD) and copper-zinc superoxide dismutase (CuZnSOD) activities*****.** MnSOD (A) and CuZnSOD (C) activities on brain vessels and MnSOD (B) and CuZnSOD (D) activities on synaptosomes were determined as described in Material and methods section. Data are the mean ± SEM of 5-6 animals from each condition studied. Statistical significance: *p<0.05; **p<0.01 when compared with 3-month-old control animals; $p<0.05 when compared with 12-month- old control animals.

Catalase catalyzes the decomposition of H_2O_2 to H_2O. In brain vessels an age-dependent increase in the activity of this enzyme was observed (**Figure 4.6A**). Chronic hypoxia significantly increased the activity of catalase in the vessels from young rats (**Figure 4.6A**). No statistically significant alterations were observed in synaptosomes, although chronic hypoxia induced a slight increase in the activity of this enzyme (**Figure 4.6B**).

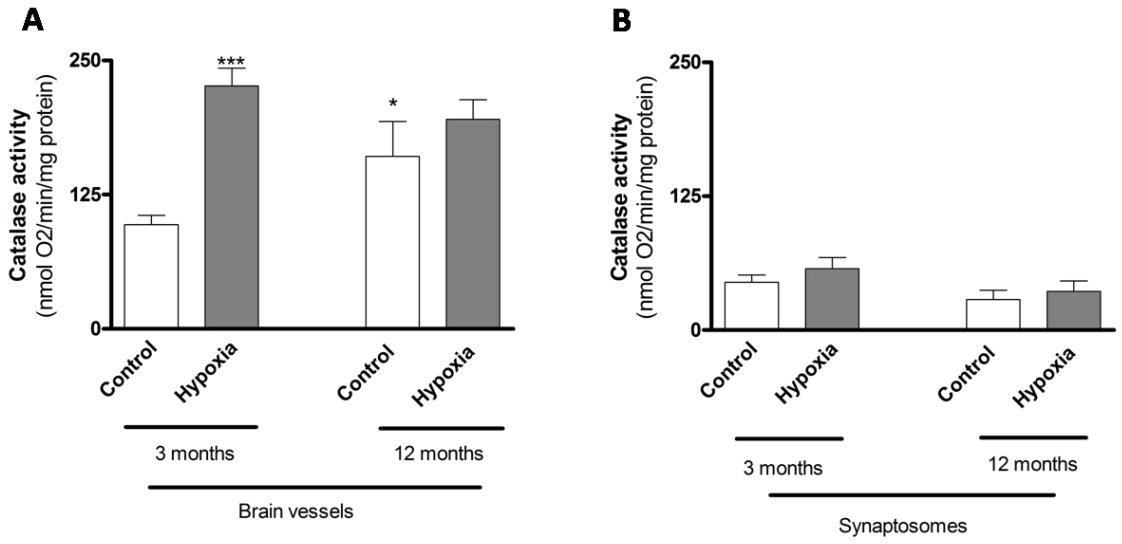


Figure 4.6- ***Effects of age and/or chronic hypoxia on catalase activity*.** Catalase activity in brain vessels (A) and synaptosomes (B) was determined as described in Material and methods section. Data are the mean ± SEM of 5-6 animals from each condition studied. Statistical significance: *$p<0.05$; ***$p<0.001$ when compared with 3-month-old control animals.

4.3.5- Aging and hypoxia increase lipid peroxidation

To quantify the extent of lipid peroxidation, the MDA levels were measured. An age-dependent increase in MDA levels was observed in both brain vessels and synaptosomes, although not statistically significant in synaptosomes. This effect was slightly increased by chronic hypoxia in synaptosomes isolated from mature animals (**Figure 4.7**).

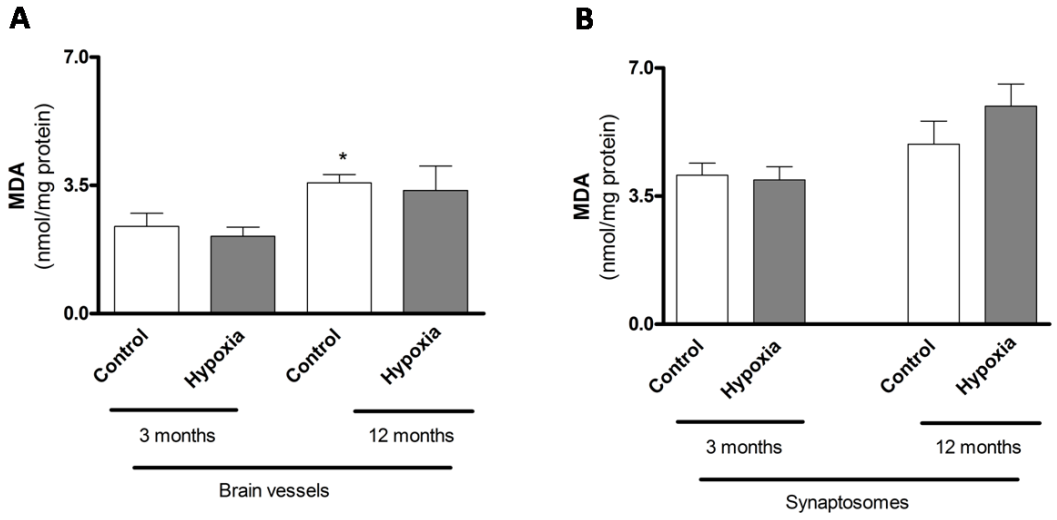


Figure 4.7- ***Effects of age and/or chronic hypoxia on lipid peroxidation*.** MDA levels in brain vessels (A) and synaptosomes (B) were measured as described in Material and methods section. Data shown represent mean ± SEM from 5-6 independent experiments. Statistical significance: *$p<0.05$ when compared with 3-month-old control animals.

4.4-Discussion

Here we demonstrated that aging leads to an increase in oxidative stress in rat brain vessels characterized by a decrease in GSH levels and an increase in lipid peroxidation and H_2O_2 and GSSG levels. Furthermore, an age-dependent increase in the activity of the antioxidant enzymes (GPx, GR and both SOD isoforms) was observed, probably representing a compensatory mechanism to counteract oxidative stress and

damage. Chronic hypoxia exacerbates age-associated oxidative imbalance in brain vessels by decreasing GSH/GSSG ratio, vitamin E levels and mitochondrial aconitase, MnSOD and GR activities and increasing H_2O_2 levels and CuZnSOD and catalase activities (**Figure 4.8**). Synaptosomes under the same experimental conditions present some tissue-specific differences, especially in what concerns glutathione and vitamin E, the two major cellular non-enzymatic antioxidants and the capacity to produce reactive species, namely H_2O_2 (**Figure 4.8**). These results indicate that aging and chronic hypoxia predisposes brain structures and cells to degeneration and death. Indeed, it was previously shown that alterations in blood flow leads to a decrease in O_2 and nutrients delivery to cells potentiating cell degeneration and death (Sharp et al., 2004). According to several authors, endothelial dysfunction is usually present in patients with AD (Dede et al., 2007, Hamel et al., 2008) and a direct link between hypoxia and AD has been established (Carvalho et al., 2009, Ogunshola and Antoniou, 2009, Zhang and Le, 2010). It was also demonstrated that the alteration in calcium homeostasis that follows hypoxia may contribute to the neurotoxicity of Aβ and subsequent development of AD (Kawahara and Kuroda, 2000). Sun and colleagues (2006) showed that hypoxia leads to increased β-secretase activity and production of Aβ. Similarly, Guglielmotto and collaborators (2009) demonstrated that hypoxia up-regulates β-secretase potentiating the production of Aβ, this effect being mediated by mitochondrial ROS. More recently, it has been demonstrated that hypoxia potentiates the phosphorylation of tau protein via extracellular-signal-regulated kinase (ERK) pathway (Fang et al., 2010).

It has been reported that hypoxia alters the neurovascular system (Wedzicha, 2000). This system is represented by the neurovascular unit that is composed by neurons, vascular cells and astrocytes working together to generate, coordinate and transduce the molecular signals that underlie the changes in CBF (Iadecola, 2004).

Under hypoxic conditions, usually associated with pathologies such as diabetes and hypertension, the neurovascular unit loses the ability to maintain blood flow homeostasis reducing protein synthesis crucial for learning and memory (Martin et al., 2000, Debiec et al., 2002, Iadecola, 2004). It was also shown that hypoxia alters synaptic plasticity and promotes mitochondrial dysfunction, oxidative stress, and apoptosis in the cerebral cortex, hippocampus, and striatum (Askew, 2002, Maiti et al., 2006, 2007, 2008a, 2008b). Hypoxia plays an important role in the pathophysiology of several diseases including cancer, myocardial infarction, and stroke (Yu et al., 1999). Clinical imaging, epidemiological and pharmacotherapy studies showed that cardiovascular disease in aged subjects could result in cerebrovascular dysfunction triggering dementia development (de la Torre, 2004, Bell and Zlokovic, 2009, Carvalho et al., 2009, Rocchi et al., 2009). Among the more than two dozen vascular risk factors already identified for AD, are cardiovascular disease and carotid artery atherosclerosis, which may exert their pathology by chronically lowering cerebral perfusion during aging (de la Torre, 2010b). Indeed, the physiological process of aging is associated with hypoxic episodes (Katschinski, 2006, Pirson et al., 2006) and is the main risk factor for several age-related degenerative diseases such as AD (Cardoso et al., 2009, Carvalho et al., 2009, Moreira et al., 2009a, 2010, Santos et al., 2010a).

We started by characterizing our experimental animals and it was observed that body and brain weight decreased in animals exposed to chronic hypoxia (**Table 4.1**). However, only 12-month-old animals presented a significant decrease in brain weight/body weight ratio (**Table 4.1**). Accordingly, Fike and collaborators (Fike et al., 1998) reported that chronically hypoxic piglets weighed less when compared to control animals. The same observation was made in Swiss mice submitted to chronic hypoxia (Deschodt-Arsac et al., 2010). We also observed an increase in TG and CRP levels in

12-month-old animals (**Table 4.1**) indicating an increased susceptibility to vascular disease and inflammation (Segura and Jurado, 2009, Bonaventure et al., 2010, Ferreira et al., 2010, Willems et al., 2010). 12-month-old rats also presented a decrease in CK levels (**Table 4.1**). CK is a kinase that has a pivotal role in mitochondrial respiration, controlling the metabolism of high energy compounds related with ATP production (Comim et al., 2008, Cassol et al., 2010). The decrease of CK levels in plasma suggest the occurrence of mitochondrial alterations in the tissues, resulting in an increased CK import from the blood (Comim et al., 2008, Cassol et al., 2010). Chronic hypoxia increased the levels of CRP and TG in both groups of experimental animals (**Table 4.1**). Additionally, chronic hypoxia decreased CK and increased RDW (**Table 4.1**), which is an indicator of vascular damaged (Risso et al., 2010). Chronic hypoxia also increased monocytes levels in both groups of experimental animals (**Table 4.1**), revealing an increased systemic inflammation. Ryan and coworkers (2009) showed that intermittent hypoxia activates pro-inflammatory transcription factors such as NF-kB and activator protein (AP)-1. These factors promote the activation of various cells involved in inflammatory processes, particularly lymphocytes and monocytes, leading to the expression of pro-inflammatory mediators that may lead to endothelial dysfunction (Ryan et al., 2009). Additionally, chronic hypoxia also increased RBC, HGB and HCT (**Table 4.1**), which prove the existence of a chronic hypoxia. Indeed, previous studies showed an increase in RBC, HGB and HCT in subjects under chronic hypoxia such as people living at high altitudes, smokers or chronic pulmonary diseases patients (Heinicke et al., 2003, Saldivar et al., 2003, Zubieta-Castillo et al., 2006) and also in some animal models of chronic hypoxia (Silkin Iu and Silkina, 2005, Baze et al., 2010, Zhang and Le, 2010).

Mitochondrial aconitase is a sensitive redox sensor of ROS in cells. Indeed, this enzyme contains a $[4Fe\text{-}4S]^{2+}$ cluster in its active site, which is oxidized by $O_2^{\bullet-}$ and related species, generating the inactive $[3Fe\text{-}4S]^{1+}$ aconitase (Vasquez-Vivar et al., 2000). Moreover, $O_2^{\bullet-}$-mediated mitochondrial inactivation leads to hydroxyl ($^{\bullet}OH$) radical formation increasing ROS formation, in a vicious cycle (Flint et al., 1993). In the present study we observed that chronic hypoxia decreases significantly the activity of mitochondrial aconitase in both brain vessels and synaptosomes (**Figure 4.1**). These results are in agreement with previous studies showing that chronic intermittent hypoxia is responsible for a reduction in aconitase activity (Powell and Jackson, 2003, Kumar et al., 2006, Yuan et al., 2008). Accordingly, chronic hypoxia increased H_2O_2 production levels in brain vessels of young and mature animals (**Figure 4.2A**). In opposite, chronic hypoxia promoted a slight decrease in the levels of H_2O_2 produced by synaptosomes (**Figure 2B**). These results are in accordance with a previous study showing that PC12 cells exposed to 5% O_2 presented a 40% decrease in H_2O_2 levels (Kroll and Czyzyk-Krzeska, 1998). These results illustrate that different cells and/or brain structures behave differently in the presence of injurious stimuli such as chronic hypoxia. We must take into account that our brain vessels preparation contains both pial and intraparenchymal vessels that are a mixture of arteries, arterioles, capillaries, veins and venules. Furthermore, vessels are formed by different cell types namely endothelial and smooth muscle cells. So, the effects concerning the brain vessels may result from one or more cell types.

Apparently, the H_2O_2 levels observed in synaptosomes seem to be in contradiction with those concerning aconitase activity (Figure 4.1B). However, it must be emphasized that other ROS species, rather than H_2O_2 interfere with mitochondrial aconitase activity. Furthermore, H_2O_2 levels are in accordance with the alterations

observed in SOD activity (Figure 4.5). SOD catalyzes the conversion of $O_2^{\bullet-}$ to H_2O_2 and its activity is undoubtedly important to the regulation of oxidative status (Correia et al., 2008a). In brain vessels from 12-month-old animals, an increase in MnSOD activity was observed (Figure 4.5A), probably representing an attempt to counteract age-induced oxidative stress. However, under chronic hypoxia, MnSOD has a lower activity (Figure 4.5A). In contrast, CuZnSOD activity increases during aging, this effect being potentiated by chronic hypoxia (Figure 4.5C). These effects are positively correlated with the increase in H_2O_2 levels observed in brain vessels isolated from hypoxic animals (Figure 4.2A). We also measured the activity of catalase, the enzyme responsible for the conversion of H_2O_2 to water. Catalase activity increased with age and also in 3-month-old hypoxic animals, probably in an attempt to compensate for the overproduction of H_2O_2 (Figure 4.2A). However, in brain vessels from 12-month-old hypoxic animals, the increase in catalase activity is not significant (Figure 4.6A). Under these conditions, vessels cells are not able to convert H_2O_2 to water so efficiently and, consequently, an increase in H_2O_2 levels is observed (Figure 4.2A). Accordingly, a previous study showed that in erythrocyte hemolysates an increase of catalase activity occurs with age, this effect being potentiated by hypoxia (Rauchova et al., 2005). Concerning synaptosomes, MnSOD activity has a tendency to decrease with age while the activity of the cytosolic isoform CuZnSOD, seems to increase (Figure 4.5C and D). Accordingly, Pritsos and Ma (2000) reported that tissues from older rats, including brain tissue, in general have less MnSOD activity than corresponding tissues from young rats. However, under chronic hypoxia no alterations were observed in MnSOD (Figure 4.5B) while the activity of the cytosolic isoform increases in synaptosomes from young rats (Figure 4.5D). Furthermore, we did not observe any statistical difference in catalase activity (Figure 4.6B). These results are in agreement with a recent study on

cultured rat embryos that shows that hypoxia induces minimal changes in catalase and SOD activities (Ornoy et al., 2010). The same study also shows a decrease in MnSOD activity that is compensated by the increase in CuZnSOD activity, without affecting catalase.

Oxidative stress is caused by an imbalance between ROS production and the ability of the biological system to readily detoxify the reactive intermediates or easily repair the resulting damage via several enzymatic and non-enzymatic antioxidant mechanisms (Carvalho et al., 2009, Syslova et al., 2009). One key antioxidant is GSH, which acts as a potent free radical scavenger and it is also the co-substrate of the antioxidant enzyme GPx. Intracellular GSH is converted into GSSG by GPx, which catalyzes the reduction of H_2O_2 and various hydroperoxides (Durmaz and Dikmen, 2007). Additionally, GR is responsible for regenerating GSH from GSSG using NADPH as an H^+ donor (Rauscher et al., 2001). The literature shows that GPx activity is incremented in aged animals (Sverko et al., 2004). Accordingly, we observed an age-dependent decrease in GPx activity in both brain vessels and synaptosomes (Figure 4.4A and B). We also observed an age-dependent increase in GR activity in synaptosomes and brain vessels (Figure 4.4C and D). Although some studies indicate that GR activity decreases with age (Juurlink et al., 1998, Shao et al., 2006), Leutner and co-workers, (Leutner et al., 2001) reported that antioxidant enzymes, including GR activity, present an age-dependent increase. This increase may represent an attempt to counteract the increase in GPx activity avoiding, consequently, GSH depletion. However, under chronic hypoxia, synaptosomes from 12-month-old animals present a significant decrease in GR activity while GPx activity is increased (Figure 4.4B and D**).**

The higher activity of GPx found in brain vessels from 12-month-old rats (**Figure 4.4A**) may be another reason to explain why the levels of H_2O_2 are not significantly increased in these animals when compared with 3-month-old control rats, since this enzyme has the capacity to catabolise H_2O_2 and lipid hydroperoxides, as discussed above.

It was also reported that GSH and total glutathione levels, and GSH redox index decreased ≈ 40%, 24%, and 52%, respectively, while GSSG levels showed a remarkable increase of ≈ 60% in synaptosomes of rat cortex with aging (Favilli et al., 1994). We also observed an age-dependent decrease of GSH levels in brain vessels, this effect being potentiated by chronic hypoxia (**Table 4.2**). Furthermore, GSH/GSSG ratio, a good indicator of the redox balance, also decreases with age (**Table 4.2**). Concerning synaptosomes, we observed no alterations in GSH/GSSG ratio with age (**Table 4.2**), probably because both GR and GPx are over-activated (**Figure 4.4B and D**). These tissue-specific differences are in accordance with a previous study showing that GSH content is tissue specific (Fokkelman et al., 2007). However, Rebrin and collaborators (Rebrin et al., 2003) reported an age-dependent decrease in GSH/GSSG ratio in brain mitochondria and homogenates from mice. However, this discrepancy may be due to the different experimental models and methodologies used in that study. Under chronic hypoxia a decrease in GSH and an increase in GSSG levels were observed in synaptosomes (**Table 4.2).** Consequently, a decrease in GSH/GSSG ratio also occurred, being more pronounced in 12-month-old animals, indicating that these animals are more susceptible to oxidative damage (**Table 4.2**). Similar effects were also reported by Sastre and collaborators (Sastre et al., 1999).

Concerning vitamin E, another powerful cellular antioxidant, we observed an increase in brain vessels from 12-month-old animals (**Figure 4.3A**), suggesting that these animals develop compensatory mechanisms to counteract aging-induced oxidative stress (Moreira et al., 2006). Chronic hypoxia promoted an increase in vitamin E levels in brain vessels from 3-month-old rats, which may be related with the decrease in the levels of GSH (**Table 4.2**) since two GSH molecules, are necessary for the regeneration of one molecule of vitamin E (Valko et al., 2007). Indeed, vitamin E seems to have a pivotal role in the response to hypoxic stimulus since the literature shows that vitamin E treatment is able to reverse hypoxia-induced mitochondrial damage in mouse skeletal muscle (Magalhaes et al., 2005). In synaptosomes, vitamin E levels slightly decrease during aging and chronic hypoxia (**Figure 4.3B**) reinforcing the idea that different brain structures and tissues behave differently under the same injurious conditions.

An age-dependent increase in MDA levels, a product of lipid peroxidation, in brain vessels and synaptosomes (**Figure 4.7**) was also observed. Accordingly, Akbulut and collaborators (2008) observed an age-dependent increased in MDA levels in the cortex and cerebellum of Wistar-albino rats. Catakay and collegues (2010) also reported an age-dependent increase in MDA levels. However, hypoxia does not affect lipid peroxidation (**Figure 4.7**). These results are in accordance with previous studies showing that chronic hypoxia does not affect the levels of MDA in rat cardiac tissue (Jun et al., 2008, Oka et al., 2008, Liu et al., 2010).

In summary, our results show that different brain cells and structures are differently affected by aging and/or chronic hypoxia. Further, the compensatory mechanisms developed by cells during aging are overcome by chronic hypoxia (**Figure 4.8**). These results could justify the high prevalence of neurodegenerative conditions in elderly people especially in those that have chronic vascular diseases.

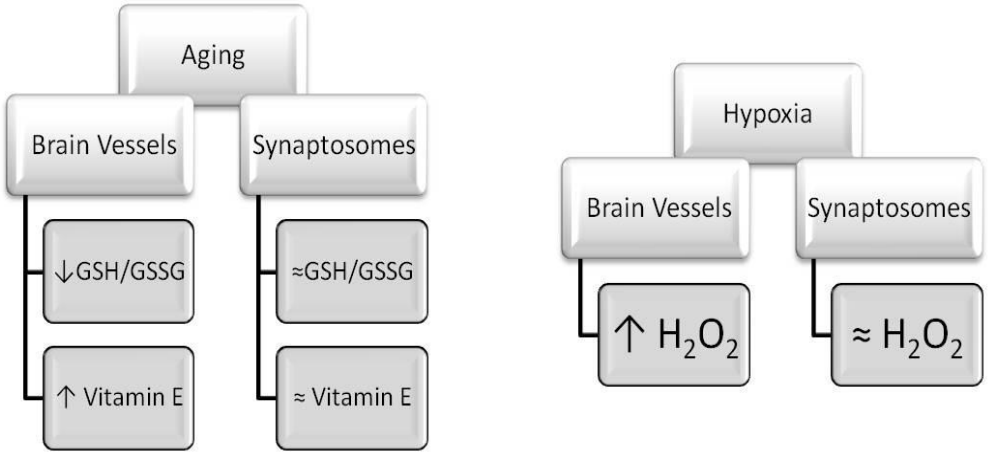


Figure 4.8- ***Tissue specific responses to aging and/or chronic hypoxia***. Aging and chronic hypoxia elicit some specific alterations in brain vessels and synaptosomes. Aging decreases glutathione (GSH) / glutathione disulfide (GSSG) ratio in brain vessels but does not affect this parameter in synaptosomes. However, the decrease in GSH/GSSG ratio that occurs in brain vessels is accompanied by an increase in vitamin E levels suggesting the existence of a compensatory mechanism. In contrast, age does not affect vitamin E levels in synaptosomes. Chronic hypoxia increases the generation of hydrogen peroxide (H_2O_2) in brain vessels but not in synaptosomes reinforcing the idea that tissue-specific responses to specific injurious stimuli do exist.

Chapter 5- Aging potentiates type 2 diabetes-associated brain vessels and synaptosomes alterations

5.1 - Abstract

T2D is a metabolic disorder affecting millions of people worldwide. The long-term complications of T2D include multiple organ damage, and the brain is no exception. However, the mechanism by which T2D leads to cognitive dysfunction and neuronal cell degeneration and death continues a matter of debate. Vascular alterations are emerging as major players in this process. This study was aimed to evaluate and compare the effects of age and T2D in brain vessels and synaptosomes. For this purpose we isolated brain vessels and synaptosomes from 3- and 12-month-old Wistar and GK rats, a spontaneous non-obese model for T2D. To achieve our goal we analyzed several parameters: mitochondrial aconitase activity, H_2O_2 and MDA levels and enzymatic [MnSOD, GPx and GR] and non-enzymatic [GSH, GSSG and vitamin E] antioxidant defenses. Brain vessels and synaptosomes from GK rats presented a significant increase in MnSOD activity and vitamin E levels and a significant decrease in aconitase and GR activities and GSH/GSSG, GSH and MDA levels, and these effects were not significantly affected by aging. However, an age-dependent increase in H_2O_2 levels occurred in diabetic rats. In addition, no significant alterations were observed in the activity of GPx and GR in both brain vessels and synaptosomes from diabetic animals. In contrast, an age-dependent increase in the activity of GPx and GR and MDA levels was observed in brain vessels from control animals. In brain vessels from control animals, aging decreased GSH levels and increased vitamin E levels and MnSOD activity. In contrast, a significant age-dependent increase in GSH levels and decrease in vitamin levels were observed in synaptosomes from control animals.

Altogether, our results show that T2D and aging differently affect brain vessels and synaptosomes. However, both conditions increase the vulnerability of brain structures to degenerative events.

5.2 - Introduction

T2D is the most common form of diabetes affecting a large percentage of people worldwide and continuous to increase due to the unhealthy lifestyles characterized by high-calorie intake and lack of physical exercise (Zhao et al., 2011).

Several studies report an increase in brain damage, including neuronal cell death, with concomitant increase in cognitive deficiencies in T2D however, the impact of this disease in CNS is not understood in detail (Baker et al., 2011). A vascular hypothesis linking the increase in neuronal cell degeneration and death and T2D is attracting the attention of scientists. Indeed, the endothelium plays a major role in maintaining energy balance and brain homeostasis through the regulation of brain metabolism, and minimal alterations in its normal function could be responsible for the development of vascular dementia (Weiss et al., 2009). T2D is responsible for several vascular alterations especially the damage of cortical microvasculature (Schwartz et al., 2010). Previously, we have showed that aging leads to several alterations that could be responsible for the increase in neuronal cell death present in several age-related disorders such as AD (Carvalho et al., 2010b). However, as far as we know, no consistent data exist concerning the effect of T2D on brain vessels and synaptosomes. Since the brain is highly dependent on blood flow to sustain its energetic requirements, any alteration in blood vessels will impact severely brain function. Because T2D is becoming a worldwide epidemic, it is of utmost importance to elucidate its effects on brain vessels and synaptosomes and to clarify the influence of age on the alterations promoted by this

metabolic disorder. So, the aim of this study was to evaluate and compare the impact of T2D in the oxidative status of brain vessels and synaptosomes obtained from young and mature rats. To achieve our purpose, several parameters were evaluated: mitochondrial aconitase activity, H_2O_2 and MDA levels and enzymatic [MnSOD, GPx and GR activities] and non-enzymatic [GSH, GSSG and vitamin E levels] antioxidant defenses.

5.3- Results

5.3.1-. Characterization of experimental animals

3- and 12-month-old GK rats presented a significant decrease in body weight and brain weight/body weight ratio when compared with 3- and 12-month-old control rats, respectively. A similar profile was observed in brain weight, although only statistically significant in mature GK rats. Aging increased body weight and decreased brain weight/body weight ratio of both control and GK rats when compared with the respective 3-month-old controls (**Table 5.1**). Concerning blood glucose levels, and as expected, GK animals presented significantly higher blood glucose levels, when compared with age-matched control animals (**Table 5.1**). GK animals also presented a significant increase in TG, cholesterol and HGB levels, these alterations being potentiated by aging. In 12-month-old GK rats, a significant increase in RBC, RDW and HCT levels was observed, which suggest that these animals are under a hypoxic state. Moreover, a decrease in CK levels was observed in GK and older Wistar rats, however in GK rats this decrease was not potentiated with aging. Diabetic animals also showed an increase in the percentage of monocytes (**Table 5.1**), this effect being more pronounced in older animals, which suggest an increased inflammatory state.

Table 5.1 - ***Animals characterization***

	3-month-old		12-month-old	
	Control	**GK**	**Control**	GK
Body Weight (g)	314.70 ± 3.97	304.00 ± 3.32*	446.50 ± 11.48***	399.30 ± 6.38$^{\#\#\$\$\$}$
Brain Weight (g)	2.64 ± 0.16	2.29 ± 0.11	2.72 ± 0.13	2.10 ± 0.12$^{\#}$
Brain Weight/Body Weight	0.0090 ± 0.00051	0.0071 ± 0.00021*	0.0061 ± 0.00032***	0.0050 ± 0.00028$^{\#\$\$\$}$
Glucose (mg/dl)	128.00 ± 6.44	327.80 ± 37.36***	124.2 ± 4.73	252.20 ± 13.52$^{\#\#\$\$}$
TG (mg/dl)	67.50 ± 8.18	111.90 ± 7.23*	74.33 ± 6.90	158.80 ± 21.71$^{\#\#\#\$}$
Chol (mg/dl)	32.63 ± 2.22	53.11 ± 0.73***	41.13 ± 4.11	157.50 ± 16.85$^{\#\#\#\$\$\$}$
CK (IU/L)	12930.00 ± 1336.00	6224 ± 876.80**	5114 ± 1629.00**	8974 ± 434.5
Mo (%)	2.32 ± 0.47	3.67 ± 0.28*	2.28 ± 0.36	7.20 ± 2.90$^{\#}$
RBC (10^6/µl)	8.45 ± 0.25	8.76 ± 0.068	8.33 ±0.16	9.31 ± 0.14$^{\#\#}$
HGB (g/dl)	155.60 ± 3.31	166.40 ± 1.37*	154.90 ± 2.04	183.00 ± 4.00$^{\#\#\#\$\$}$
HCT (%)	0.49 ± 0.015	0.49 ± 0.0037	0.46 ± 0.0096	0.54 ± 0.0074$^{\#\#\#}$
RDW (%)	12.68 ± 0.13	13.47 ± 0.088***	12.54 ± 0.23	14.47 ± 0.13$^{\#\#}$

Data are the mean ± SEM of 5-6 experiments from each condition studied. Statistical significance: *p<0.05; **p<0.01; ***p<0.001 when compared with 3- months-old control animals; #p<0.05; ##p<0.01; ###p<0.001 when compared with 12- months-old control animals; $^{\$}$p<0.05; $^{\$\$}$p<0.01; $^{\$\$\$}$p<0.001 when compared with 3- months-old GK animals.

5.3.2- Aging potentiates T2D-associated oxidative stress

Because the measurement of $O_2^{\bullet-}$ is not an easy task, we evaluated mitochondrial aconitase activity, as an indirect measure of oxidative stress given that the sulfur-iron cluster present in the enzyme active site is very sensitive and being rapidly inactivated by reactive species. A significant decrease in the activity of this

enzyme was observed in brain vessels and synaptosomes from diabetic animals, although this effect was not potentiated by aging (**Figure 5.1**).

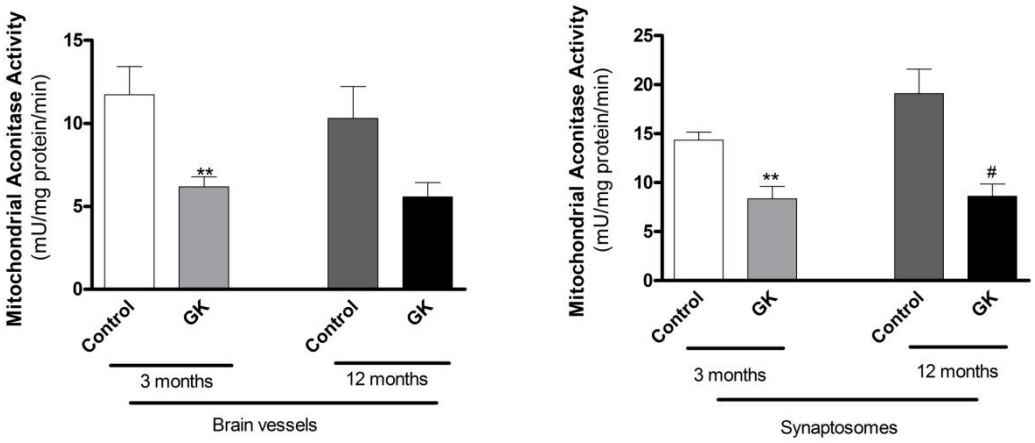


Figure 5.1 - ***Effects of age and/or type 2 diabetes on mitochondrial aconitase activity***. Aconitase activity in brain vessels (**A**) and synaptosomes (**B**) was measured as described in the Material and methods section. Data shown represent mean ± SEM from 5-6 independent experiments. **p<0.05 when compared with 3-month-old control animals; $^{\#}$p<0.05 when compared with 12 months-old control animals.

The analysis of H_2O_2 production gives a good indication about the predisposition of cells to originate and/or exacerbate oxidative stress. A significant increase in H_2O_2 levels was observed in brain vessels and synaptosomes from 12-month-old GK rats (**Figure 5.2**) but no significant changes were observed in 3-month-old GK animals.

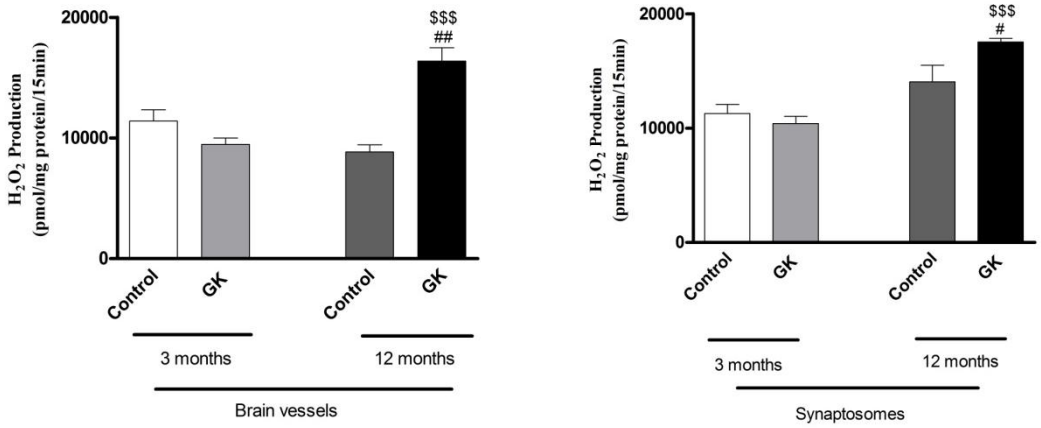


Figure 5.2 - ***Effects of age and/or type 2 diabetes on hydrogen peroxide (H_2O_2) production***. H_2O_2 production in brain vessels (A) and synaptosomes (B) was measured as described in Material and methods section. Data are the mean ± SEM of 5-6 independent experiments. #p<0.05; ##p<0.01 when compared with 12-month-old control animals; $^{\$\$\$}$p<0.001 when compared with 3-month-old control animals.

5.3.3- Aging and T2D differently affect lipid peroxidation

To quantify the extent of lipid peroxidation, the MDA levels were measured. In control animals, aging increased the levels of this oxidative marker, although only statistically significant in brain vessels (**Figure 5.3**). Surprisingly, GK rats presented a significant decrease in MDA levels compared with age-matched control animals (**Figure 5.3**).

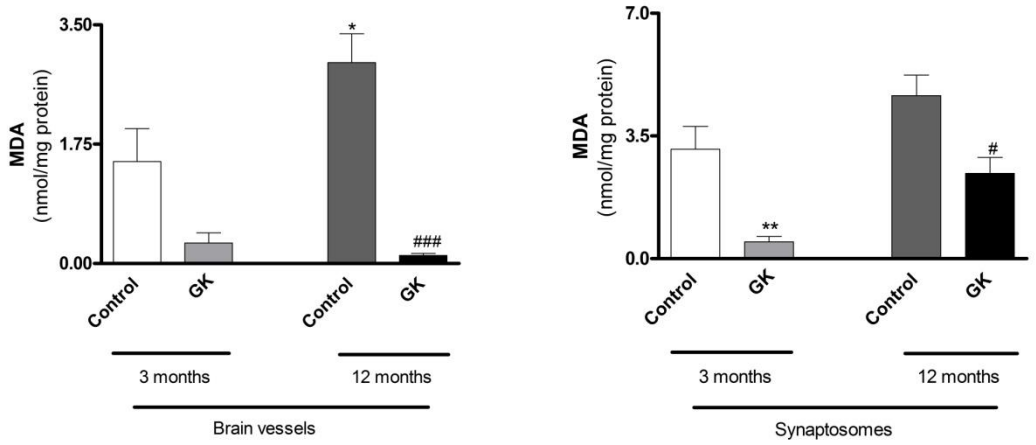


Figure 5.3 - ***Effects of age and/or type 2 diabetes on lipid peroxidation***. MDA levels in brain vessels (A) and synaptosomes (B) were measured as described in Material and methods section. Data shown represent mean ± SEM from 5-6 independent experiments. Statistical significance: *p<0.05; **p<0.01 when compared with 3-month-old control animals; #p<0.05; ##p<0.01 when compared with 12-month-old control animals.

5.3.4- Aging and T2D affect the antioxidant defenses

Endogenous antioxidants such as GSH and vitamin E belong to the first line of defense and act by scavenging potentially damaging free radical moieties.

In 12-month-old Wistar rats, a significant decrease in GSH levels occurred in brain vessels while an increase was found in synaptosomes revealing cell-specific alterations. Diabetic animals showed a decrease in GSH levels and GSH/GSSG ratio in both brain vessels and synaptosomes, these effects being more pronounced in older diabetic animals (except the GSH/GSSG ratio in synaptosomes) (**Table 5.2**).

Table 5.2 - ***Effects of age and/or type 2 diabetes in glutathione levels***

	Brain vessels				**Synaptosomes**			
	3 months		**12 months**		**3 months**		**12 months**	
	Control	GK	Control	GK	Control	GK	Control	GK
GSH (nmol/mg protein**)**	15.07 ± 2.12	2.81 ± 0.14**	9.64 ± 1.39*	1.45 ±0.38###	14.84 ± 0.92	6.62 ± 0.57**	17.94 ± 0.65*	5.13 ± 0.94###
GSSG (nmol/mg protein**)**	4.12 ± 0.67	5.49 ± 1.05	3.76 ± 0.28	4.51 ± 0.80	5.75 ± 0.19	5.64 ± 0.36	6.52 ± 0.74	4.50 ± 0.82
GSH/GSSG	2.66 ± 0.12	0.52 ± 0.078***	2.34 ± 0.19	0.33 ± 0.084##	2.18 ± 0.085	1.15 ± 0.077**	2.52 ± 0.20	1.44 ± 0.29##

Data shown represent mean ± SEM from 5-6 independent experiments. Statistical significance: *P<0.05 when compared with 3-month-old control animals; #p<0.05; ##p<0.01; ###p<0.01 when compared with 12-month-old control animals.

Curiously, a significant increase in vitamin E levels was observed in brain vessels and synaptosomes from GK animals when compared with the respective age-matched controls. Also brain vessels from older control animals presented a significant increase in vitamin E levels (**Figure 5.4**).

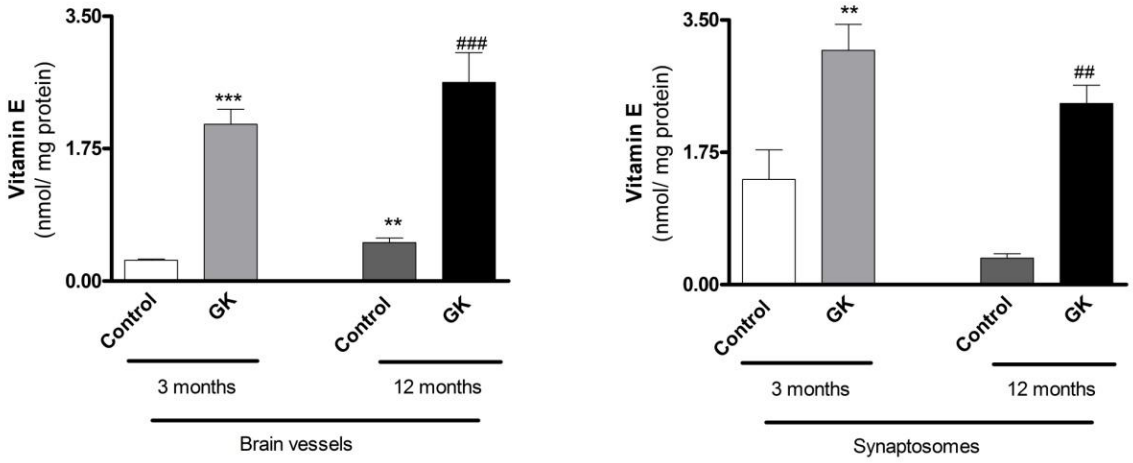


Figure 5.4- ***Effects of age and/or type 2 diabetes on vitamin E levels*.** Vitamin E levels in brain vessels (A) and synaptosomes (B) were measured as described in Material and methods section. *P<0.05; **P<0.01, when compared with 3-month-old control animals; $^{\#\#}$P<0.01; $^{\#\#\#}$P<0.001, when compared with 12-month-old control animals.

GPx and GR are two antioxidant enzymes involved in the detoxification of ROS. An age-dependent increase in the activity of GPx and GR was observed in both brain vessels and synaptosomes from control rats (**Figure 5.5**). No significant alterations were observed in the activity of GPx in brain vessels and synaptosomes from diabetic animals at both ages. However, compared to control animals, GR activity in brain vessels and synaptosomes from diabetic animals was significantly decreased. (**Figure 5.5**).

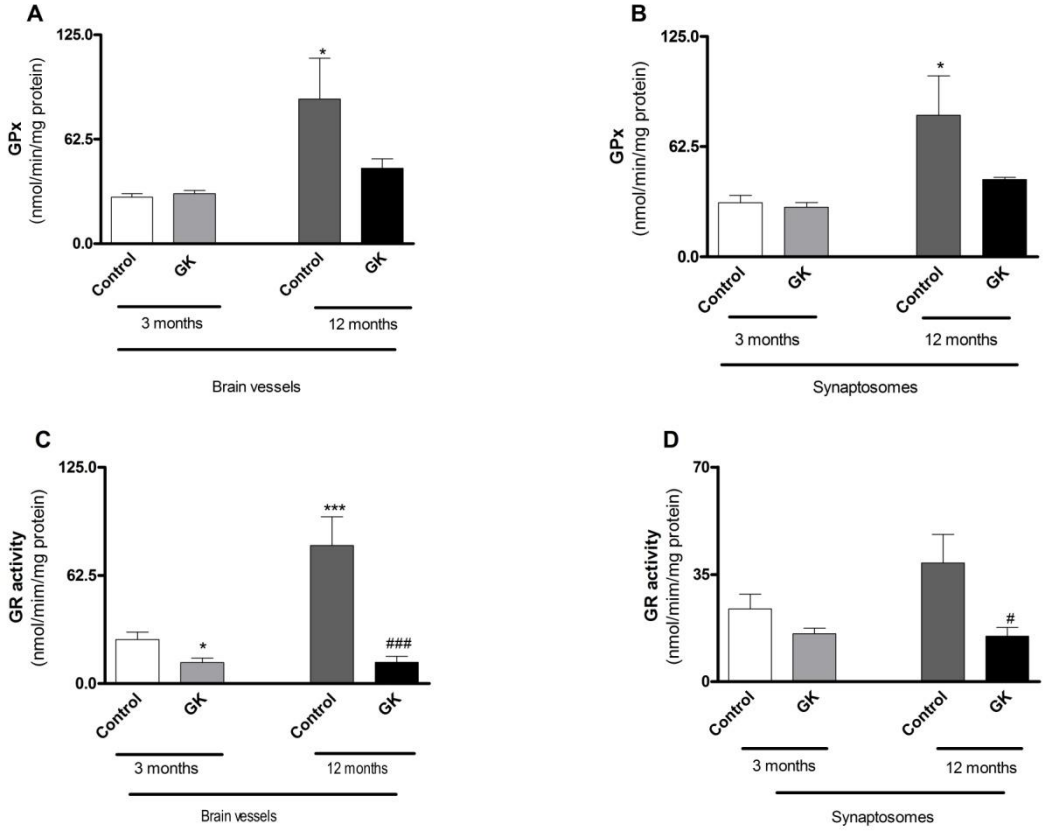


Figure 5.5- ***Effects of age and/or type 2 diabetes on glutathione peroxidase (GPx) and glutathione reductase (GR) activities*.** GPx activity on brain vessels (A) and synaptosomes (B) and GR activity on brain vessels (C) and synaptosomes (D) were determined as described in Material and methods section. Data are the mean ± SEM of 5-6 animals from each condition studied. Statistical significance: *p<0.05; ***p<0.001 when compared with 3-month-old control animals; #p<0.05; ###p<0.001 when compared with 12-month-old control animals.

With respect to MnSOD, an increase in the activity of this enzyme was found in GK rats, although only statistically significant in brain vessels. Aging also induced a

slight increase in MnSOD activity in brain vessels from diabetic rats (**Figure 5.6**). In control animals, aging promoted a significant increase in MnSOD in brain vessels and, in contrast, a significant decrease in the activity of this enzyme in synaptosomes (**Figure 5.6**), reinforcing the existence of cell-specific particularities.

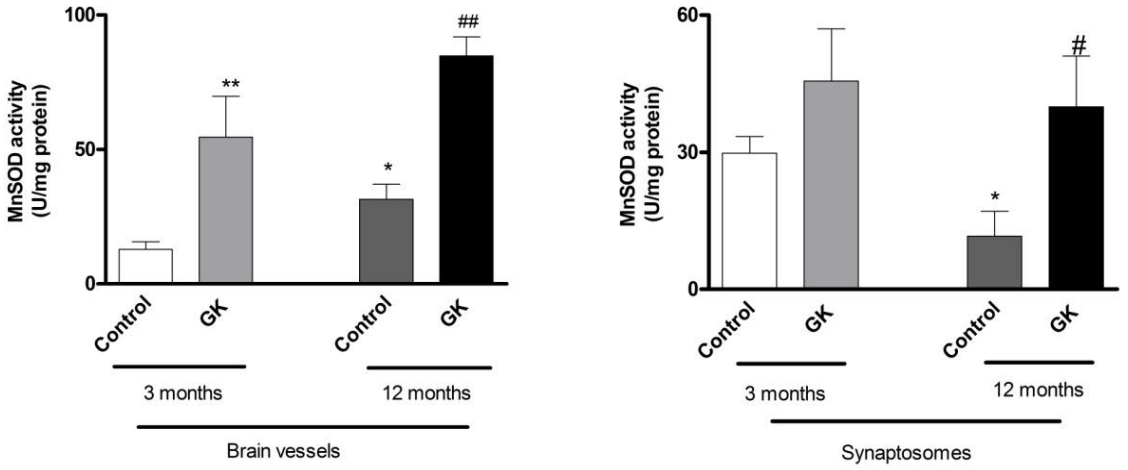


Figure 5.6 - ***Effects of age and/or type 2 diabetes on manganese superoxide dismutase (MnSOD) activity*****.** MnSOD activity on brain vessels (A) and synaptosomes (B) were determined as described in Material and methods section. Data are the mean ± SEM of 5-6 animals from each condition studied. Statistical significance: *p<0.05; **p<0.01 when compared with 3-month-old control animals; ##p<0.01 when compared with 12-month- old control animals.

5.4- Discussion

It is known that T2D is a risk factor for the development of several neurodegenerative diseases such as AD. Indeed, besides neuronal dysfunction, also endothelial dysfunction occur in patients with AD, which suggest that vascular pathology plays a key role in AD development (Dede et al., 2007, Hamel et al., 2008, Ogunshola and Antoniou, 2009, Carvalho et al., 2010b, Zhang and Le, 2010). Accumulating evidence also shows that the physiological process of aging and several pathological processes are characterized by increased oxidative stress and damage (Linnane et al., 1989, Miquel, 1991, Wallace, 1992, Moreira et al., 2008). In this study we showed that T2D is associated with an oxidative imbalance in both, brain vessels and synaptosomes, this effect being potentiated by aging.

Concerning the characterization of our experimental animals, a significant decrease in body weight of GK rats was observed, this decrease being more accentuated in 12-month-old animals when compared with age matched control animals. Similarly, a decrease in brain weight was also observed in GK rats, when compared with age-matched control animals, although only statistically significant in older animals (**Table 5.1**). A decrease in brain weight/body weight ratio was observed in 3-month-old GK rats and this decrease was potentiated by aging. Previous studies performed in a model of high fat diet-induced T2D reported a significant decrease in brain weight with the progression of the disease (Moroz et al., 2008, Lyn-Cook et al., 2009) but, as far as we know, this is the first study reporting a decrease in brain weight in a non-obese T2D model. Concerning blood and plasma biochemical parameters we observed a significant increase in blood glucose levels in both 3- and 12-month-old GK animals (**Table 5.1**), as previously observed in T2D animal models (Moreira et al., 2003, Correia et al., 2008a) and patients (Khan et al., 2003, Zhang et al., 2010). An increase in TG and

cholesterol levels was also observed in diabetic animals, which is also a typical characteristic of T2D (Rodrigues et al., 2011, Sharma et al., 2011). It was previously reported that high levels of TG levels in young men may indicate an increased risk to develop T2D (Tirosh et al., 2008). In fact, we observed high levels of TG in young diabetic mice, this alteration being exacerbated by aging (**Table 5.1**). An increase in monocytes levels was also observed in diabetic animals revealing an increased systemic inflammation in GK animals, which increase the predisposition to vascular disease, as previously described (Kristiansen et al., 2004). Interestingly, both 3- and 12-month-old GK animals showed alterations in HGB levels, with 12-month-old GK animals also presenting a significant increase in RBC, RDW and HCT levels (**Table 5.1**), these alterations being considered good indicators of chronic hypoxia, supporting the idea that aging and T2D are associated with vascular alterations (Silkin Iu and Silkina, 2005, Zubieta-Castillo et al., 2006, Baze et al., 2010, Carvalho et al., 2010b, Zhang and Le, 2010). CK is an enzyme that has a pivotal role in mitochondrial respiration, controlling the metabolism of high energy compounds related with ATP production (Comim et al., 2008, Cassol et al., 2010). The decrease of CK levels in plasma suggest the occurrence of mitochondrial alterations in the tissues, resulting in an increased CK import from the blood (Comim et al., 2008, Cassol et al., 2010). The decrease in CK in diabetic animals highlights the existence of tissue mitochondrial dysfunction (**Table 5.1**).

Mitochondrial aconitase activity, considered a good cellular oxidative stress sensor (Flint et al., 1993, Vasquez-Vivar et al., 2000), is significantly decreased in both brain vessels and synaptosomes of diabetic animals (**Figure 5.1A**). In addition, a significant increase in H_2O_2 levels occurred in 12-month-old GK animals (**Figure 5.2**). Although no significant alterations in H_2O_2 levels occurred in 3-month-old GK animals, we must take into consideration that other ROS species, such as $O_2^{\bullet-}$, rather than H_2O_2,

interfere with mitochondrial aconitase activity (Vasquez-Vivar et al., 2000). Indeed, it is well established that aconitase is inactivated in vivo by $O_2^{\bullet-}$ (Liochev, 1996). Furthermore, in brain vessels from diabetic animals, H_2O_2 levels are positively correlated with MnSOD activity (**Figure 5.6A**), but this correlation was not observed in diabetic synaptosomes (**Figure 5.6B**). Different brain structures and cells behave differently when exposed to adverse conditions. We believe that the alterations occurring in synaptosomes may represent an attempt to counteract the increased oxidative stress. In young diabetic animals, there is an increase in MnSOD activity as an attempt to detoxify $O_2^{\bullet-}$ without an increase in H_2O_2 levels. However, at 12-month-old this compensatory mechanism seemed to fail with a significant increase in H_2O_2 levels, possibly due to a reduction in catalase activity previously reported in T2D aging (Goth, 2008). However, further studies are necessary to corroborate our hypothesis.

In accordance with aconitase activity (**Figure 5.1**), a significant decrease in GSH/GSSG ratio in both diabetic and brain vessels and synaptosomes was observed (**Table 5.2**). Similarly, DeMattia and collaborators (2008) reported a decrease in GSH/GSSG ratio in T1D and T2D patients. The decrease in GSH/GSSG ratio could be due to a decrease in GSH production or an increase in its consumption. However, T2D did not promote significant alterations in GPx activity (**Figure 5.5A and B**), as previously described (Sekeroglu et al., 2000). In contrast, other studies reported a decrease in GPx activity induced by T2D (Zitouni et al., 2005, Rebolledo et al., 2008). In fact, Godin and coworkers (1988) demonstrated that GPx activity in diabetic rats differ in distinct tissues; no significant alterations in heart and pancreas and a significant increase in liver and kidney. Furthermore, an age-dependent decrease in GR activity was observed in brain vessels and synaptosomes from diabetic animals (**Figure 5C and D**) justifying the reduction in GSH levels observed in those animals. The decrease in

synaptosomes GSH levels can also result from the increase in vitamin E levels observed in diabetic animals (**Figure 5.4**) since GSH regenerates vitamin E. These alterations were also observed in diabetic liver (Ferreira et al., 1999). The increased levels of vitamin E observed in diabetic animals could also justify the significant decrease in MDA levels (**Figure 5.3**), a marker of lipid peroxidation, since vitamin E is the major protector of cells against lipid peroxidation. Those results are in agreement with previous studies from our laboratory showing a decreased susceptibility of diabetic rat synaptosomes to membrane lipid peroxidation, this decrease being correlated with a 1.3-fold increase in synaptosomal vitamin E levels (Santos et al., 2000).

In summary, our results show that both brain vessels and synaptosomes of GK animals are under an oxidative imbalance increasing the susceptibility of the brain cells damage. Particularly, the alterations observed in brain vessels may compromise BBB integrity and brain homeostasis predisposing to cerebral diseases. These results may help better understand how cerebrovascular dysfunction underlies the higher incidence of cognitive dysfunction and neurodegenerative diseases, particularly in elderly T2D patients.

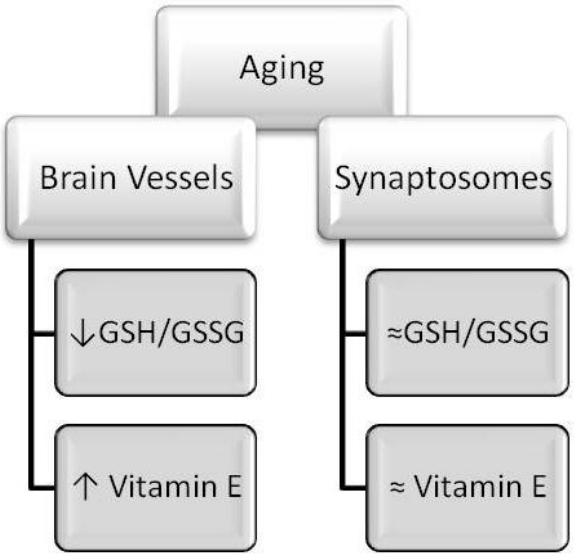


Figure 5.7- ***Tissue specific responses to aging***. Aging in control animals elicits some specific alterations in brain vessels and synaptosomes. Normal aging decreases glutathione (GSH) / glutathione disulfide (GSSG) ratio in brain vessels but does not affect this parameter in synaptosomes. However, the decrease in GSH/GSSG ratio that occurs in brain vessels is accompanied by an increase in vitamin E levels suggesting the existence of a compensatory mechanism. In contrast, age does not affect vitamin E levels in synaptosomes.

Chapter 6- Type 2 diabetic and Alzheimer disease mice present similar vascular, behavioral and cognitive anomalies

6.1- Abstract

T2D is considered a major risk factor for AD. To elucidate the links between both pathological conditions we compared behavioral and cognitive functions, cerebral Aβ levels and vasculature integrity of 11-month-old T2D and AD mice. For this purpose, we performed behavioral tests (open field, object recognition, Y-maze, and elevated plus maze tests), ELISA to assess plasma markers of endothelial/vascular dysfunction, spectrophotometric assays to evaluate cerebral vascular permeability and enzymatic activities and immunohistochemistry for the assessment of Aβ levels. Both T2D and AD showed similar behavioral and cognitive anomalies characterized by increased fear and anxiety and decreased learning and memory abilities. Interestingly, both groups of animals presented increased plasma markers of endothelial/vascular dysfunction and permeability of cerebral vasculature and impaired mitochondrial enzymatic activities. In addition, a significant increase in Aβ levels was observed in the cortex and hippocampus of T2D mice. These results support the notion that T2D predisposes to cerebrovascular alterations, cognitive decline and development of AD.

6.2- Introduction

AD, the most common age-related neurodegenerative disease, affects more than 25 million individuals worldwide. Neuropathologically, AD is characterized by the presence of extracellular senile plaques mainly composed of Aβ, the intracellular deposition of neurofibrillary tangles mostly constituted by hyperphosphorylated tau protein, and massive neuronal loss, while the hallmark clinical symptom is memory impairment (Carvalho et al., 2009).

T2D is a metabolic disorder characterized by insulin resistance and poor insulin secretion, as a compensatory mechanism, resulting in impaired glucose regulation, and

subsequent hyperglycemic state (Baker et al., 2010). This metabolic disease can have deleterious consequences on target tissues and several studies linked T2D to an increased risk of cognitive impairment and AD (Toro et al., 2009).

Although the mechanisms linking T2D and AD remain under debate, recent studies suggest that cerebrovascular mechanisms are likely involved (Cheng et al., 2011). In fact, functional vascular abnormalities are one of the earlier clinical manifestations in both sporadic and familial forms of AD and many cardiovascular risk factors, such as T2D, are also risk factors for this neurodegenerative disease, which suggest that vascular abnormalities may contribute to AD pathology (Paris et al., 2003). Vascular abnormalities are also associated with mitochondrial impairment and oxidative stress (Liu and Zhang, 2012). Mitochondria are intracellular organelles mainly devoted to energy production however, they are also involved in ROS production, as an inevitable by-product of cellular respiration. Several studies showed that increased ROS production, as a result of mitochondrial malfunction, are partially responsible for the progressive decline in biological function predisposing to age-related diseases such as AD (Buizza et al., 2012). The present study is aimed to evaluate and compare the impact of AD and T2D on mice cerebral vasculature, putting focus on mitochondrial enzymatic activities, and correlate those alterations with behavioral and cognitive changes and deposition of Aβ.

6.3- Results

6.3.1- Characterization of experimental animals

Compared to WT mice, 3xTg-AD animals presented a significant decrease in body and brain weight, and consequently, a decrease in brain weight/body weight ratio (**Table 6.1**). These animals also presented an increase in HbA1c and occasional glucose

levels (**Table 6.1**). Diabetic mice presented an increase in body weight and a decrease in brain weight and brain weight/body weight ratio (**Table 6.1**). An increase in plasma insulin, $HbA1_C$ and occasional blood glucose levels was also observed in diabetic mice (**Table 6.1**). Concerning plasma markers of endothelial dysfunction and inflammation, 3xTg-AD animals presented a significant increase in CRP and ICAM-1 levels and diabetic animals showed a significant increase in CRP, ICAM, e-selectin and VCAM levels when compared to WT mice (**Table 6.1**).

Table 6.1- ***Animals characterization*****.**

	WT control	**T2D**	**3xTg-AD**
Body weight **(g)**	34.74 ± 0.91	39.45 ± 0.95**	29.19 ± 1.06**
Brain weight **(g)**	1.01 ± 0.08	0.71 ± 0.07*	0.67 ± 0.07*
Brain/Body weight	0.029 ± 0.002	0.018 ± 0.002**	0.022 ± 0.002*
Occasional glycemia **(mg/dl)**	123.20 ± 5.55	143.20 ± 4.61*	119.90 ± 6.54
Insulin levels **(µg/l)**	1.42 ± 0.25	4.25 ± 0.86**	0.87 ± 0.17
HbA1c %	3.35 ± 0.14	3.75 ± 0.09*	3.85 ± 0.12*
ANP (ng/ml)	40.39 ± 3.84	32.33 ± 5.80	40.00 ± 3.90
CRP (ng/ml)	7.75 ± 2.38	16.62 ± 2.85*	20.28 ± 2.39**
e-Selectin (pg/ml)	26970 ± 1290	35020 ± 1322**	27550 ± 1083
ICAM (ng/ml)	245.40 ± 10.31	365.7 ± 44.79**	317.10 ± 30.95**
VCAM (ng/ml)	554.70 ± 24.23	733.20 ± 45.40**	626.7 ± 27.38

Data shown represent mean ± SEM from 6-8 animals. Statistical significance: *$p<0.05$; **$p<0.01$ when compared with WT control animals; Plasma levels of C-reactive protein (CRP), E-selectin, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and atrial natiuretic peptide (ANP).

6.3.2- Alzheimer's disease and diabetes promote similar behavioral and cognitive deficits

Compared to WT mice, both 3xTg-AD and T2D animals presented significantly higher levels of anxiety, characterized by an increased freezing behavior and thigmotaxis when exposed to the open field test and a decrease in the number of entries and permanence time in the open arms in the elevated plus maze test (**Figures 6.1C and 6.1D**). 3xTg-AD mice also presented a significant decrease in the number of central crosses (representing the predatory susceptibility area) (**Figure 6.1B**). A decrease in grooming and rearing behavior was also observed in both diabetic and 3xTg-AD mice.

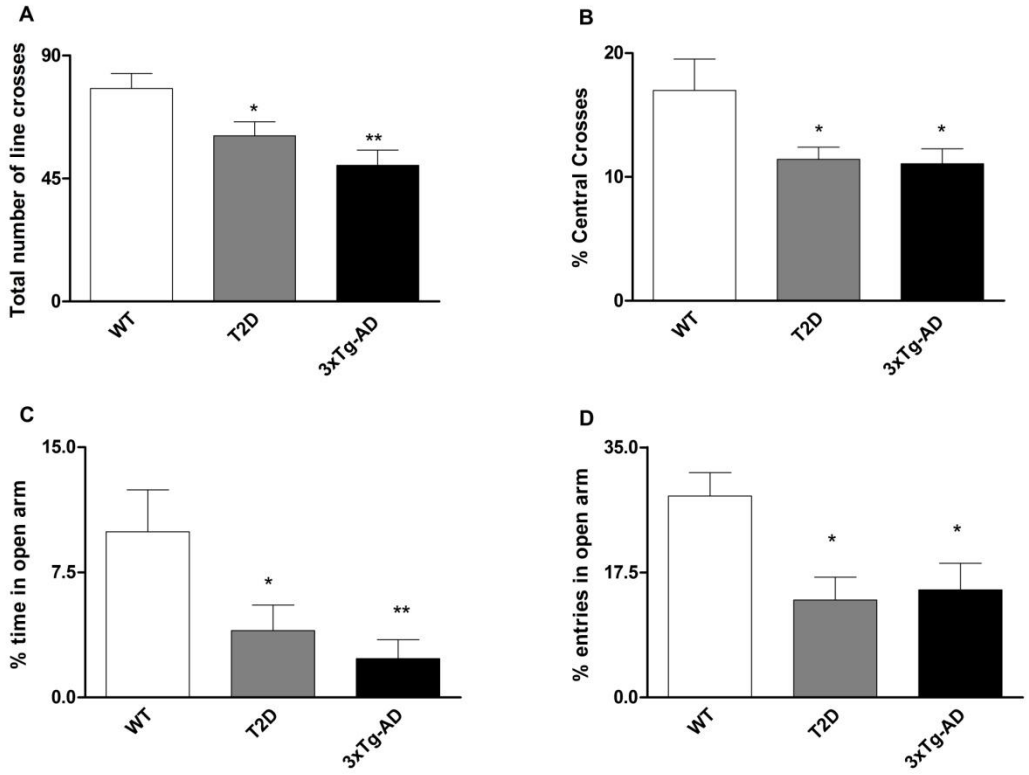


Figure 6.1- ***Effect of T2D and Alzheimer's disease on exploratory behavior and anxiety.*** **(**A) Total number of line crosses and (B) % of central crosses in open field; (C) % time and (D) % entries in open arm in elevated plus maze test. Data shown represent mean ± SEM from 10-12 animals. Statistical significance: *p<0.05; **p<0.01 when compared with WT control animals.

The object recognition test is based on the natural tendency of rodents to investigate a novel object, instead of a familiar object, reflecting the use of learning and recognition memory processes and, usually, it is sensitive to age-related deficits and is very suitable to test AD-related deficits. This test revealed that both 3xTg-AD and T2D animals had a significant impairment in their capability to recognize new objects, although more pronounced in 3xTg-AD mice (**Figure 6.2A**). However, the Y-maze test revealed that hippocampal-dependent memory was more affected in T2D animals, since they spent less time in the new arm, when compared with WT animals (**Figure 6.2B**).

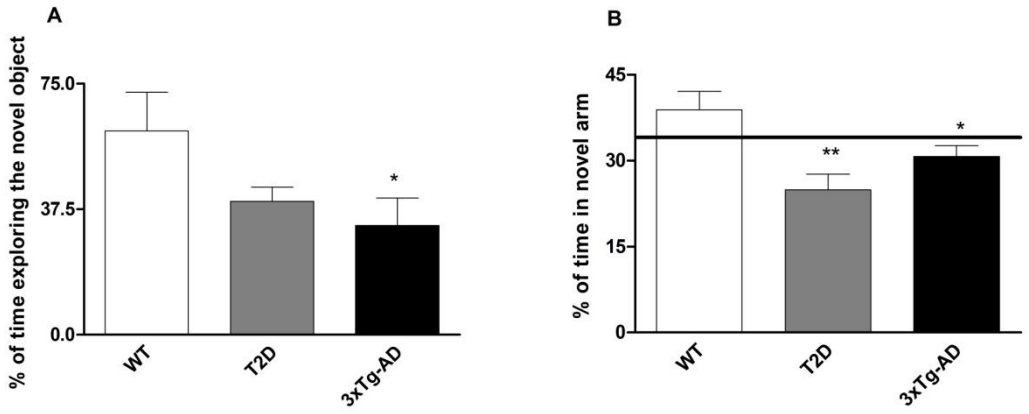


Figure 6.2- ***Effect of T2D and Alzheimer's disease on memory and learning*****.** (A) Object recognition test and (B) Y-maze test. Data shown represent mean ± SEM from 8-12 animals. Statistical significance: *p<0.05; **p<0.01 when compared with wt control animals.

6.3.3 - Alzheimer's disease and diabetes promote an increase in Aβ levels both in cortex and hippocampus.

As previously described by Oddo and coworkers (2003), 3xTg-AD mice presented a significant increase in Aβ levels in both cortex and hippocampus (**Figures 6.E-H**). Interestingly, and in accordance with previous findings from our lab (Carvalho et al., 2012), T2D mice also presented a significant increase in Aβ levels in brain cortex and hippocampus (**Figures 6.3A-D)**.

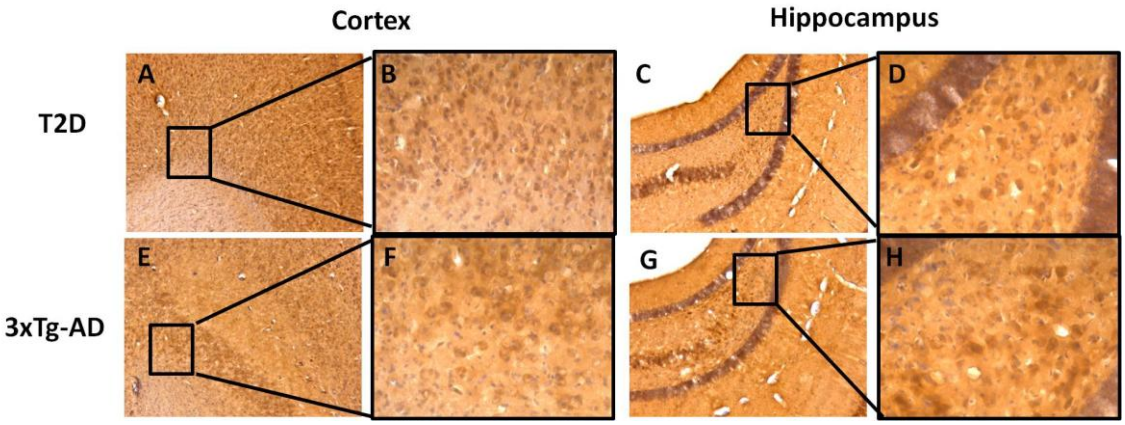


Figure 6.3 - *Aβ peptide distribution in brain cortex and hippocampus*. A clear staining of Aβ (visualized through DAB reaction product) is observed in brain cortex and hippocampus of both T2D (A, B, C, D) and 3xTg-AD mice (E, F, G, H). Data shown represent typical images of 3 animals per group. Hematoxilin counterstaining was performed.

6.3.4 - Alzheimer's disease and diabetes are associated with an increased permeability of brain blood vessels

The Evans Blue dye is widely used to study blood vessels and cellular membrane permeability. Because the Evans Blue dye is non-toxic, it can be administered into the bloodstream where it binds to serum albumin. The Evans Blue dye-albumin conjugate can then be quantified in tissue homogenates by spectrophotometry. This assay revealed that both 3xTg-AD and T2D mice presented an increase in blood vessel permeability in brain cortex and hippocampus (**Figures 6.4A and B**). The cerebellum was used as negative control and no significant alterations were observed in this brain area, as expected (**Figure 6.4C**)

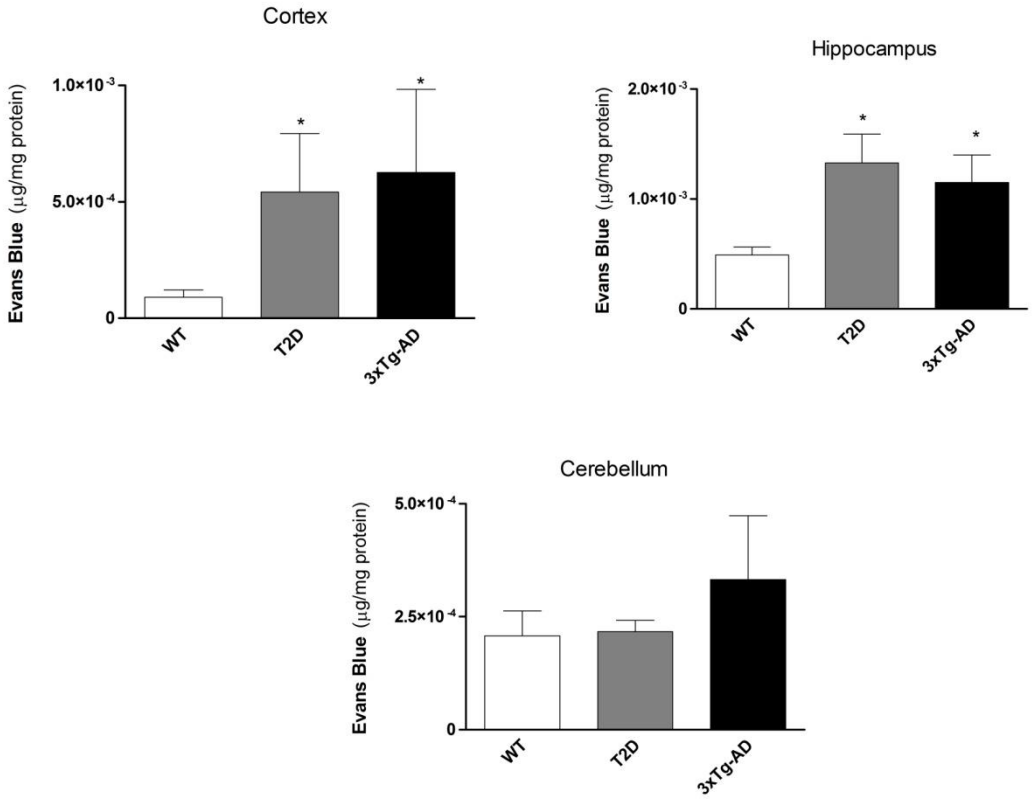


Figure 6.4 - ***Effect of T2D and Alzheimer's disease on blood-brain barrier (BBB) leakage*****.** BBB permeability is given by Evans blue dye leakage in (A) cortex, (B) hippocampus and (C) cerebellum. Data shown represent mean ± SEM from 6-8 animals. Statistical significance: *$p<0.05$ when compared with wt control animals.

6.3.5- Alzheimer's disease and diabetes promote oxidative stress

It is well known that vascular dysfunction is associated with oxidative stress. In this line, we evaluated the activity of aconitase, an enzyme highly susceptible to oxidative stress. This enzyme comprises an iron-sulfur cluster in its active site, and the increase in ROS and RNS negatively affects the activity of aconitase. Interestingly, brain vessels from 3xTg-AD and T2D mice presented a significant and similar decrease in aconitase activity (**Figure 6.5A**). The same observation was made in synaptosomes from the same groups of animals (**Figure 6.5B**).

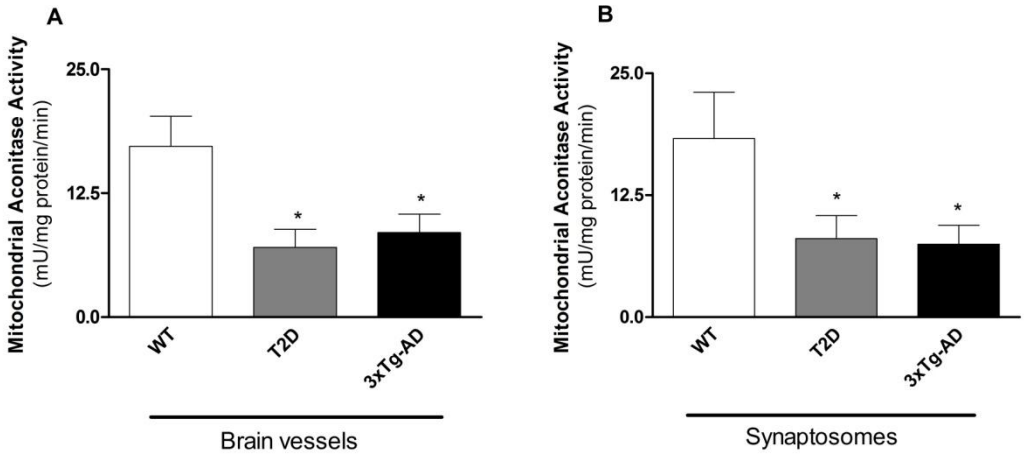


Figure 6.5- ***Effect of T2D and Alzheimer's disease on mitochondrial aconitase activity.*** Aconitase activity was measured in (A) brain vessels and (B) synaptosomes. Data shown represent mean ± SEM from 6-8 animals. Statistical significance: *$p<0.05$ when compared with WT control animals.

6.3.6- Alzheimer's disease and diabetes impair mitochondrial enzymatic complexes activity

Because oxidative stress is intimately associated with mitochondrial impairment, we evaluated the activity of mitochondrial enzymatic complexes. Compared to control animals, brain vessels from 3xTg-AD and T2D animals presented a significant decrease in mitochondrial complexes I-III (**Figure 6.6A**). A similar observation was made in synaptosomes from both groups of animals (**Figure 6.6A**)

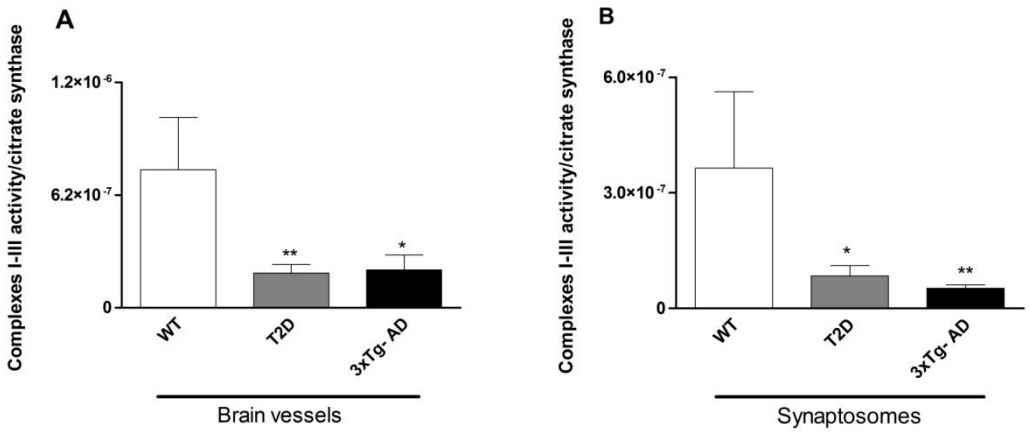


Figure 6.6- ***Effect of T2D and Alzheimer's disease on complexes I-III activities.*** Complexes I-III activities were measured in (A) brain vessels and (B) synaptosomes. Data shown represent mean ± SEM from 6-8 animals. Statistical significance: *p<0.05; **p<0.01 when compared with wt control animals.

No significant alterations were observed in the activity of mitochondrial complexes II-III in brain vessels (**Figure 6.7A**), although a significant decrease was observed in synaptosomes from T2D mice (**Figure 6.7B**).

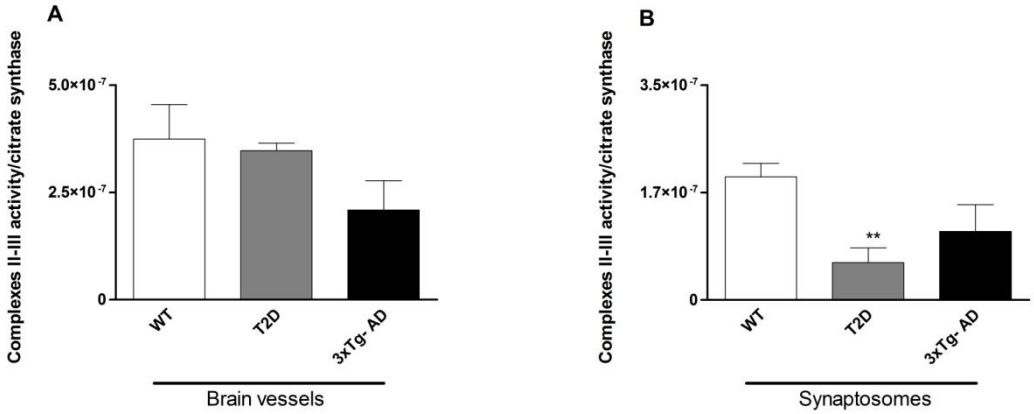


Figure 6.7- ***Effect of T2D and Alzheimer's disease on complexes II-III activities*****.** Complexes II-III activities were measured in (A) brain vessels and (B) synaptosomes. Data shown represent mean ± SEM from 6-8 animals. Statistical significance: **p<0.01 when compared with WT control animals.

T2D mice also presented a significant decrease in complex IV activity in brain vessels (**Figure 6.8A)** and synaptosomes (**Figure 6.8B**), while a significant decrease in synaptosomal complex IV activity (**Figure 6.8B**) was observed in 3xTg-AD mice.

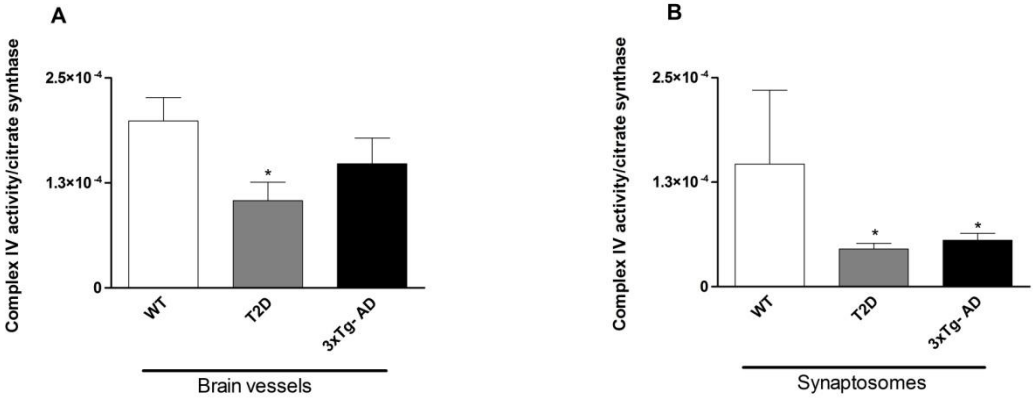


Figure 6.8- ***Effect of T2D and Alzheimer's disease on complex IV activity*****.** Complex IV activity was measured in (A) brain vessels and (B) synaptosomes. Data shown represent mean ± SEM from 6-8 animals. Statistical significance: *p<0.05 when compared with WT control animals.

6.4- Discussion

This study supports the idea that diabetes is a main risk factor for AD since both 3xTg-AD and T2D mice present marked vascular abnormalities that culminate in a similar profile of behavioral and cognitive deficits. In fact, several studies suggest a strong link between T2D and AD and, according to the vascular hypothesis, aging and vascular risk factors are responsible for a reduction in CBF that results in brain hypoperfusion and consequent neurodegeneration (Roy and Rauk, 2005). In fact, a recent study showed that multiple vascular risk factors are associated with a greater rate of decline in cognition, function, and regional CBF in AD patients, which highlights the contribution of vascular risk factors on the progression of AD (Kume et al., 2011). Further, phenotypes associated with obesity and/or alterations on insulin homeostasis are at increased risk for developing cognitive decline and dementia, namely vascular dementia and AD (Luchsinger et al., 2004). Accordingly, we found that both 3xTg-AD and T2D mice presented significant vascular anomalies associated with learning and memory deficits.

In our study we showed that 3xTg-AD mice presented a significant decrease in body and brain weight and, consequently, in brain weight/body weight ratio (**Table 6.1**), as previously described (Carvalho et al., 2012). These characteristics are also present in AD patients, weight loss being a frequent complication of AD that occurs in 40% of patients at all disease stages (Power et al., 2001). Sucrose intake led to the development of T2D characterized by an increase in body weight, blood glucose, $HbA1_C$ and insulin levels (**Table 6.1**), as previously reported (Carvalho et al., 2012).

Increasing evidence shows that T2D (Natali and Ferrannini, 2012) and AD (Salmina et al., 2010) are intimately associated with endothelial/vascular dysfunction and inflammation. In fact, when endothelial cells undergo inflammatory activation the

increased expression of selectins, VCAM-1, and ICAM-1 promotes the adherence of monocytes. Adhesion molecule expression is induced by proinflammatory cytokines such as IL-1β and TNF-α, and the acute-phase protein CRP that is produced by the liver in response to IL-6, among others (Szmitko et al., 2003). In our study, both T2D and 3xTg-AD mice presented a significant increase in CRP and ICAM levels (**Table 6.1**). ICAM-1 is an adhesion molecule that allows the attachment of leukocytes to the endothelium and may permit their subsequent transmigration into peripheral tissue while increases microvascular permeability (Frank and Lisanti, 2008)**.** An increase in ICAM-1 levels was reported to occur in the plasma (Reale et al., 2012) and brain tissue of AD subjects (Rozemuller et al., 1989), which suggests that adhesion molecules may play a role in the pathogenesis of neurological diseases (e.g. AD) that involve the interaction between immune system and the brain (Frohman et al., 1991). Rentzos and coworkers (2004) showed an increase in ICAM but not in e-selectin levels in plasma of AD patients. Accordingly, our 3xTg-AD mice presented an increase in ICAM levels but no significant alterations in e-selectin levels were observed (**Table 6.1**).

An increase in CRP levels was observed in AD affected brain areas (Blasko et al., 2007) and plasma CRP was positively correlated with a more rapid functional decline on the Weintraub Activities of Daily Living Scale (Locascio et al., 2008), which evaluates the functional abilities of dementia patients over time. Several studies also showed a positive correlation between e-selectin, an inducible, 117 kDa, type I transmembrane glycoprotein that is transiently and principally expressed on endothelial cells after activation by cytokines (Weller et al., 1992) and VCAM levels, expressed on vascular endothelium upon induction by a number of inflammatory stimuli (Carter and Wicks, 2001) and the progression of T2D. Accordingly, our T2D mice presented a significant increase in e-selectin and VCAM levels (**Table 6.1**).

Interestingly, the increase in plasma inflammatory and endothelial dysfunction markers was positively correlated with an increase in brain vascular permeability (namely in cortex and hippocampus) in both 3xTg-AD and diabetic mice (**Figures 6.4A and 6.4B**), which is in accordance with previous findings showing an increased BBB permeability in Tg2576 mice, an animal model of AD (Atiea et al., 1995) and in diabetic db/db mice (Yan et al., 2012).

It is well known that vascular anomalies are closely associated to oxidative stress and mitochondrial impairment (Liu and Zhang, 2012). Aconitase is a sensitive redox sensor and its activity is inhibited in the presence of ROS and NOS, especially $O_2^{\bullet -}$ ions. Brain vessels (**Figure 6.5A**) and synaptosomes (**Figure 6.5B**) from 3xTg-AD and T2D mice presented a significant decrease in aconitase activity suggesting that, like neuronal cells, also cells from brain blood vessels are under an oxidative environment, a phenomenon that is usually related with mitochondrial alterations. In fact, 3xTg-AD mice presented a significant decrease in the activity of complexes I-III in both brain vessels (**Figure 6.6A)** and synaptosomes **(Figure 6.6B**). However, and in contrast to the significant decrease observed in synaptosomal complex IV activity (**Figure 6.8B)**, no significant alterations in the activity of this complex were found in 3xTg-AD brain vessels **(Figure 6.8A**). In diabetic mice, a significant decrease in the activity of complexes I-III and IV was found in both brain vessels (**Figures 6.6A and 6.8A**) and synaptosomes (**Figures 6.6B and 6.8B)**. Concerning synaptosomes from T2D mice, a significant decrease in the activity of complexes II-III (**Figure 6.7B**) was observed. In fact, several animal models of diabetes (e.g. db/db mice and STZ- and alloxan-induced diabetic rodents) showed reduced activities of mitochondrial complexes I, II and/or IV in several tissues such as heart, skeletal muscle, kidney and liver (Remor et al., 2011, Zhang et al., 2011). Furthermore, defects in mitochondrial complexes I, III and IV

activities have been reported in AD animal models and human brains (Hauptmann et al., 2009, Rhein et al., 2009). These results show that like neuronal cells, also cells from blood vessels are characterized by mitochondrial abnormalities. However, some tissue/cell-specific differences in mitochondrial complexes activities were observed, as described above. In fact, while in neuronal cells the most important role of mitochondria is the production of energy, in endothelial cells, major components of brain vessels, mitochondria are mainly responsible for maintaining the Ca^{2+} concentration in the cytosol and the generation of ROS which act as second messengers (Davidson and Duchen, 2007). Nevertheless, the impairment of endothelial and neuronal mitochondria will culminate in ROS overproduction and, potentially, cell death.

3xTg-AD mice presented increased thigmotaxis, i.e., the animals spent more time in close proximity to the walls, than in the unprotected center area (Bailey and Crawley, 2009)**,** when compared with WT mice that were more prone to explore the open arena (**Figure 6.1B**). These animals also exhibited significantly higher freezing behavior and limited exploratory behavior, spending more time in the center before the exploration of the field (**data not shown**). These observations are in agreement with the study by Sterniczuk and coworkers (Sterniczuk et al., 2010) performed in 3xTg-AD mice. Interestingly, it was previously reported that anxiety and depression are common and occur in an early stage in AD patients (Chung and Cummings, 2000, Di Iulio et al., 2010) and are associated with a more rapid cognitive decline (Drevets and Rubin, 1989, Levy et al., 1996). Interestingly, a similar profile of behavioral alterations was observed in diabetic mice (**Figure 6.1B**). Accordingly, it was previously shown that db/db and STZ-induced diabetic mice exhibited a significant increase in anxiety levels (Jung and Lee, 2010, Dinel et al., 2011).

Concerning learning and memory abilities, both T2D and 3xTg-AD mice presented learning and memory deficits (**Figures 6.2A-C**). Dinel and coworkers (2011) showed that db/db mice presented a decrease in learning ability, spending less time exploring the new arm in Y-maze test. Also, several studies showed that people suffering from T2D had increased cognitive dysfunction compared to age-matched control subjects (Atiea et al., 1995, Mogi et al., 2001). In addition, several studies performed in AD animal models (Sterniczuk et al., 2010) and patients (Kessels et al., 2011) confirm the existence of learning and memory deficits. Interestingly, these cognitive defects were associated with an increase in brain cortical and hippocampal levels of Aβ (**Figure 6.3**), which is in accordance with previous studies (Oddo et al., 2003, Li et al., 2007, Carvalho et al., 2012).

Altogether our results show that vasculature anomalies, closely associated to mitochondrial alterations and oxidative stress, are a common denominator between T2D and AD. In fact, both pathological conditions present similar cellular and structural changes that culminate in a similar profile of behavioral and cognitive changes. In conclusion, this study gives support to the idea that T2D may trigger AD-like pathology.

Chapter 7- Metabolic alterations induced by sucrose intake and Alzheimer's disease promote similar brain mitochondrial abnormalities

7.1- Abstract

Evidence shows that diabetes increases the risk of developing AD. Many efforts have been done to elucidate the mechanisms linking diabetes and AD. To demonstrate that mitochondria are a functional link between both pathologies, we compared the effects of AD and sucrose-induced metabolic alterations on brain mitochondrial bioenergetics and oxidative status. For this purpose, brain mitochondria were isolated from WT, 3xTg-AD and WT mice subjected to 20% sucrose-sweetened water for 7 months. Polarography, spectrophotometry, fluorimetry, high-performance liquid chromatography and electron microscopy were used to evaluate mitochondrial function, oxidative status and ultrastructure. Western blotting was performed to determine the AD pathogenic protein levels. Sucrose intake caused metabolic alterations like those found in type 2 diabetes (T2D). Mitochondria from 3xTg-AD and T2D mice presented a similar impairment of the respiratory chain and phosphorylation system, decreased capacity to accumulate calcium, ultrastructural abnormalities and oxidative imbalance. Interestingly, T2D mice presented a significant increase in Aβ levels, a hallmark of AD. These results support the idea that diabetes increases the risk of developing AD, mitochondrial abnormalities representing a link between both disorders.

7.2- Introduction

AD is a progressive neurodegenerative disorder that leads to dementia and affects approximately 10% of the population older than 65 years of age. AD is characterized by a severe neuronal loss and the presence of two brain lesions, senile plaques and neurofibrillary tangles, which are mainly constituted by Aβ and hyperphosphorylated tau protein, respectively (Khachaturian, 1985).

T2D is a well known metabolic disorder that usually occurs in people over 30 years old and affects more than 7% of the global population. This disorder is characterized by a relative insulin deficiency, reduced insulin action and insulin resistance of glucose transport, especially in skeletal muscle and adipose tissue. There is a cluster of risk factors for T2D and vascular disease that include high blood glucose, obesity, increased blood triacylglycerols and insulin resistance. All these factors, both individually and collectively, increase the risk of AD and vascular dementia. Epidemiological studies corroborate the idea that diabetes is a risk factor for vascular dementia and AD (Kalaria et al., 2008, Roriz-Filho et al., 2009). Both AD and T2D share similar demographic profiles, risk factors and, perhaps more importantly, clinical and biochemical features (Selkoe, 1997)

Previous studies from our laboratory demonstrated that mitochondria isolated from the brains of T2D rats are more susceptible to Aβ protein exposure (Moreira et al., 2003) suggesting that mitochondria are a functional link between diabetes and AD. Mitochondria play a critical role in the regulation of both cell survival and death (Moreira et al., 2010). These organelles are essential for the production of ATP through oxidative phosphorylation and regulation of intracellular Ca^{2+} homeostasis. Thus, dysfunction of mitochondrial energy metabolism culminates in ATP production and Ca^{2+} buffering impairment and exacerbates the generation of ROS. High levels of ROS cause, among other things, damage of cell membranes through lipid peroxidation and accelerate the high mutation rate of mtDNA. Accumulation of mtDNA mutations enhances oxidative damage, causes energy depletion and increases ROS production, in a vicious cycle (Correia et al., 2010b). Moreover, the brain is especially prone to oxidative stress-induced damage due to its high levels of polyunsaturated fatty acids,

high oxygen consumption, high content in transition metals, and poor antioxidant defenses.

The literature shows that mitochondrial dysfunction and oxidative stress play an important role in the early pathology of AD. Indeed, there are strong indications that oxidative stress occurs prior to the onset of symptoms in AD and oxidative damage is found not only in the vulnerable regions of the brain affected in disease but also peripherally (Nunomura et al., 2001). Moreover, it has been shown that oxidative damage occurs before Aβ plaque formation (Nunomura et al., 2001) supporting a causative role of mitochondrial dysfunction and oxidative stress in AD.

Since we believe that brain mitochondria are a link between diabetes (and pre-diabetic states) and AD, this study aimed to evaluate and compare the impact of T2D and AD on brain mitochondria bioenergetics and oxidative status. For this purpose three groups of experimental animals were used: 1) wild type (WT) control mice; 2) T2D mice and 3) 3xTg-AD mice. The use of sucrose solution was based on compelling evidence showing that excessive consumption of sugars plays a key role in the epidemic of obesity and T2D (Cao et al., 2007). Several parameters were evaluated: mitochondrial respiratory chain [respiratory states 2, 3 and 4, RCR and ADP/O index], phosphorylation system [ΔΨm, repolarization level, repolarization lag phase and ATP/ADP ratio], Ca^{2+}-induced permeability transition pore (Ca^{2+} fluxes and mitochondrial ultrastructure), mitochondrial aconitase activity, H_2O_2 levels and non-enzymatic [vitamin E levels, GSH to GSSG ratio] and enzymatic [GR, GPx and MnSOD activities] antioxidant defenses. The levels of Aβ and p-tau protein were also evaluated.

7.3- Results

7.3.1- Characterization of experimental animals

Compared to WT mice, 3xTg-AD animals presented a significant decrease in body and brain weight, and consequently, a decrease in brain weight/body weight ratio (**Table 7.1**).

Table 7.1 - ***Animals characterization***

	WT control	**T2D**	**3xTg-AD**
Body weight (g)	35.20 ± 1.09	42.86 ± 1.27***	31.14 ± 0.7485**
Brain weight (g)	1.06 ± 0.07	0.75 ± 0.08*	0.76 ± 0.09*
Brain/Body weight	0.03 ± 0.002	0.02 ± 0.002**	0.02 ± 0.002**
Postprandial glycemia (mg/dl)	100.70 ± 2.19	116.60 ± 3.72*	121.30 ± 8.53*
Occasional glycemia (mg/dl)	122.40 ± 7.90	144.00 ± 5.58*	117.10 ± 7.86
Insulin levels (µg/l)	0.44 ± 0.08	0.68 ± 0.23	0.36 ± 0.02
HbA1c %	3.36 ± 0.07	3.77 ± 0.08**	3.99 ± 0.11***
Triglycerides (TG) levels (mg/dl)	159.80 ± 19.16	277.40 ± 30.60**	168.20 ± 17.80
Cholesterol (chol) levels (mg/dl)	161.30 ± 2.81	160.10 ± 1.91	160.60 ± 2.48

Data shown represent mean ± SEM from 10-12 animals. Statistical significance: *$p<0.05$; **$p<0.01$; ***$p<0.001$ when compared with wt control animals; $^{\$}p<0.05$; $^{\$\$}p<0.01$ when compared with 3xTg-AD control animals.

These animals also presented an increase in HbA1c and postprandial glucose levels (**Table 7.1**). In WT mice, sucrose intake promoted an increase in body weight, a decrease in brain weight and, consequently, a decrease in brain weight/body weight ratio when compared with WT mice under basal conditions. In addition, T2D promoted an increase in HbA1c, blood glucose, insulin and triglycerides levels (**Table 7.1**) levels and a decrease in glucose tolerance in WT mice when compared with the respective control mice (**Figure 7.1**). No alterations in cholesterol levels were observed (**Table 7.1**).

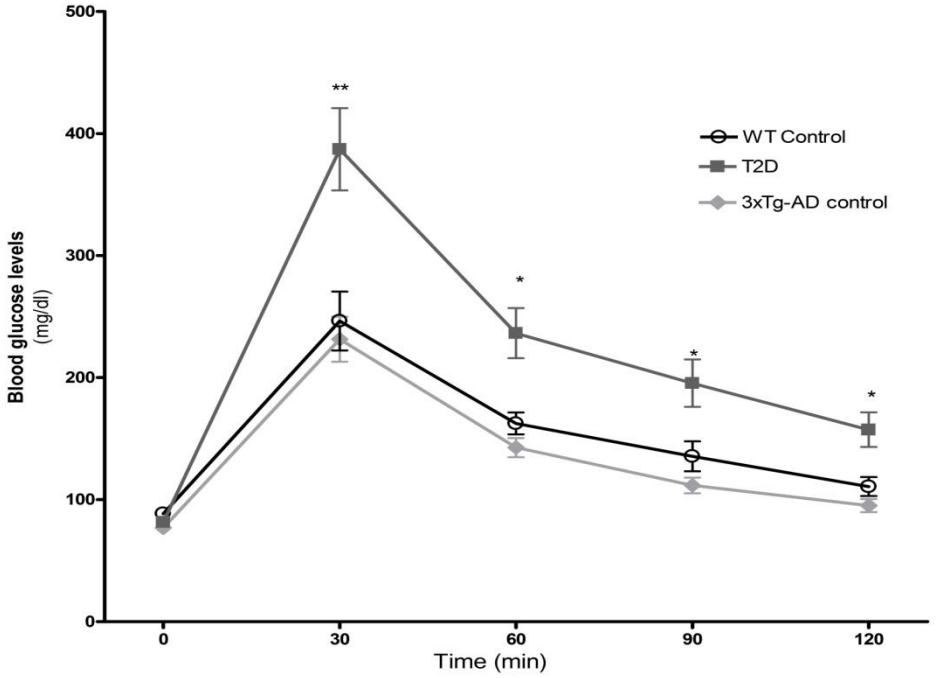


Figure 7.1- ***Glucose tolerance test.*** Data shown represent mean ± SEM from 10-12 animals. Statistical significance: *p<0.05; **p<0.01; ***p<0.001 when compared with wt control animals; $^{\$}$p<0.05; $^{\$\$}$p<0.01 when compared with 3xTg-AD control animals.

7.3.2- Alzheimer's disease and T2D impair mitochondrial respiratory chain and oxidative phosphorylation system

T2D animals present an increase in respiratory states 2 (≈ 35% and ≈ 41%, respectively) and 4 (≈ 46% and ≈ 54%, respectively) and a decrease in respiratory state 3 (≈ 33% and ≈ 31%, respectively) and RCR (≈ 15% and ≈ 17%, respectively) (**Figure 7.2**). No significant changes were observed in ADP/O index (**Figure 7.2**).

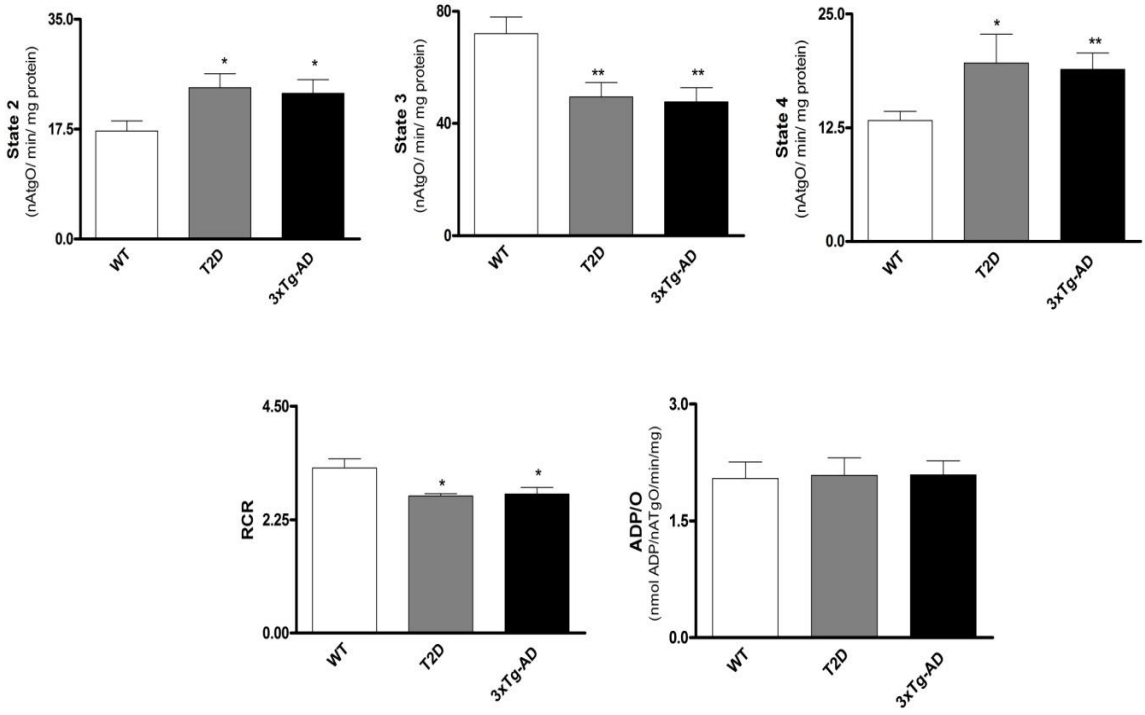


Figure 7.2- ***Effects of AD and T2D on mitochondrial respiration***. The respiratory states 2, 3, and 4, respiratory control ratio (RCR) and ADP/O index were evaluated in freshly isolated brain mitochondrial fractions (0.5 mg) in 1 ml of the reaction medium energized with 5 mM succinate in the presence of 2 μM rotenone. Data shown represent mean ± SEM of 5-6 independent experiments. nAtgO/min/mg = nAtom-gram oxygen/min/mg. Statistical significance: *p<0.05; **p<0.01; ***p<0.001 when compared with WT control animals.

The $\Delta\Psi_m$ is fundamental for the phenomenon of oxidative phosphorylation, which results in the conversion of ADP to ATP via ATP synthase. When compared with mitochondria isolated from WT control mice, mitochondria from both 3xTg-AD and T2D mice presented a significant decrease in $\Delta\Psi_m$ (≈ 9% and ≈ 7%, respectively), ADP-induced depolarization (≈ 23% in both groups of animals) and ATP/ADP ratio (≈ 48% and ≈ 42%, respectively) and a significant increase in the repolarization lag phase, the time needed to phosphorylate exogenous ADP (≈ 37% and ≈ 40%, respectively) (**Table 7.2**).

Table 7.2- ***Effects of AD and T2D on the mitochondrial oxidative phosphorylation system [mitochondrial transmembrane potential (ΔΨm), ADP-induced depolarization, repolarization lag phase, and ATP/ADP].***

	WT	**T2D**	**3xTg-AD**
ΔΨm (-mV)	219.90 ± 4.11	202.90 ± 2.95**	199.10 ± 1.69***
ADP-induced depolarization (-mV)	30.60 ± 1.99	23.23 ± 2.19*	23.56 ± 2.51*
Repolarization lag phase (min)	1.64 ± 0.19	2.30 ± 0.23*	2.24 ± 0.17*
ATP/ADP	8.59 ± 1.02	4.95 ± 1.13**	4.45 ± 1.33*

The oxidative phosphorylation parameters were evaluated in freshly isolated brain mitochondrial fractions (0.5 mg) in 1 ml of the reaction medium supplemented with 3 μM TPP^+ and energized with 5 mM succinate in the presence of 2 μM rotenone. Adenine nucleotide levels were determined by HPLC, as described in Materials and methods section. Data shown represent mean ± SEM from 5-6 independent experiments. Statistical significance: *p<0.05; **p<0.01; ***p<0.001 when compared with WT control animals; $p<0.05; $$p<0.01 when compared with 3xTg-AD control animals.

7.3.3- Alzheimer's disease and T2D potentiate the opening of the mitochondrial permeability transition pore induced by calcium

The mitochondrial PTP is characterized by an increase in mitochondrial membrane permeability that leads to the loss of $\Delta\Psi_m$, alteration in Ca^{2+} fluxes, mitochondrial swelling, and rupture of mitochondrial membranes and cristae (Vatassery and Younoszai, 1978, Flohe and Gunzler, 1984). In the presence of 80 nmol Ca^{2+} mitochondria isolated from 3xTg-AD (**Figure 7.3, trace 6**) and T2D (**Figure** Figure **7.3, trace 5**) animals accumulated and retained less Ca^{2+} compared with WT control mitochondria (**Figure 7.3, trace 4**). The pair oligomycin/ADP (**Figure 7.3, traces 1, 2 and 3**), which is more effective than cyclosporin A (CsA) in preventing PTP opening in brain mitochondria (Flohe and Gunzler, 1984), significantly increased the capacity of mitochondria to accumulate and retain Ca^{2+}.

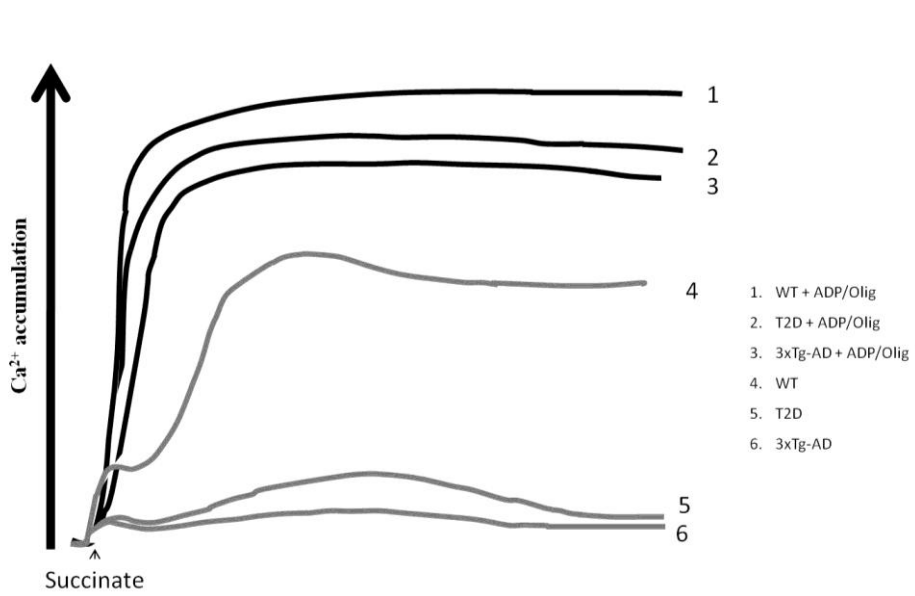
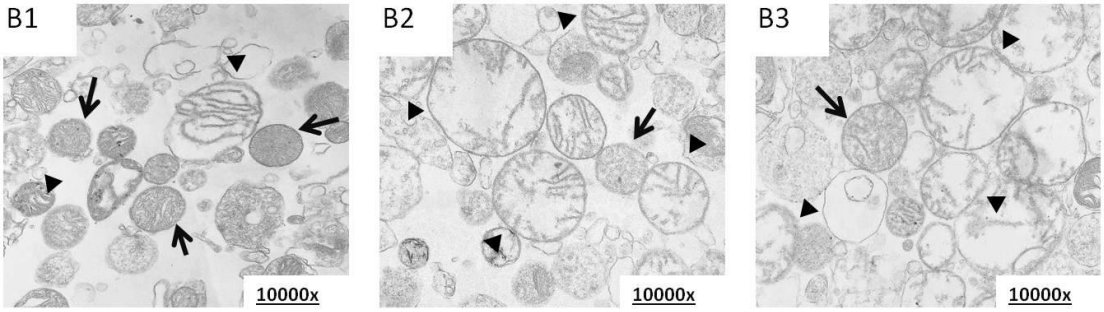
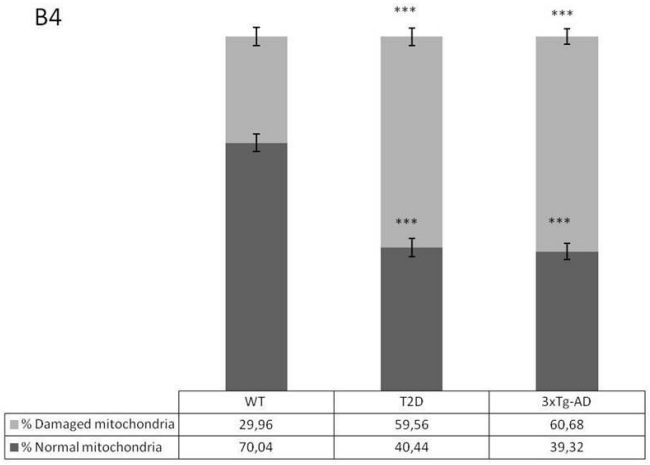


Figure 7.3- ***Effects of AD and T2D on mitochondrial Ca^{2+} fluxes and ultrastructure.*** (A) Freshly isolated brain mitochondria (0.5 mg) in 1ml of the reaction medium were energized with 5mM succinate. Ca^{2+} (80 nmol/mg protein) was added 1 min before mitochondria energization. Oligomycin (0.2 μg/ml) plus ADP (100 μM) were added 2 min before Ca^{2+} addition. The traces are typical of 5-6 independent experiments. Trace 1 - WT control mitochondria in the presence of oligomycin plus ADP; trace 2 – T2D mitochondria in the presence of oligomycin plus ADP; Trace 3 - 3xTg-AD mitochondria in the presence of oligomycin plus ADP; Trace 4 - WT control mitochondria**;** Trace 5 – T2D mitochondria; Trace 6 – 3xTg-AD mitochondria.

Concerning ultrastructure, mitochondria from T2D (**Figure 7.4.2**) and 3xTg-AD (**Figure 7.4.3**) mice present a high % of damaged mitochondria characterized by swollen mitochondria with disrupted mitochondrial membranes and cristae, when compared with WT control mitochondria (**Figure 7.4.1**).





	WT	T2D	3xTg-AD
% Damaged mitochondria	29,96	59,56	60,68
% Normal mitochondria	70,04	40,44	39,32

Figure 7.4- ***Effects of AD and T2D on mitochondrial ultrastructure*****.** After Ca^{2+} experiments, mitochondria were fixed for electron microscopy. Images represent (B.1) WT control mitochondria in the presence of Ca^{2+} (80 nmol/mg protein) (B.2) T2D mitochondria in the presence of Ca^{2+} (80 nmol/mg protein) (B.3) 3xTg-AD mitochondria in the presence of Ca^{2+} (80 nmol/mg protein) and (B.4) graphic representation of normal/damaged mitochondria. → - Normal mitochondria; ▲ Damaged mitochondria.

7.3.4- Alzheimer's disease and T2D promote oxidative stress and damage

Mitochondrial aconitase activity is a sensitive redox sensor of ROS and RNS in cells. As shown in **Figure 7.5A**, brain mitochondria isolated from 3xTg-AD mice presented a significant decrease (≈ 54%) in aconitase activity when compared with WT control mitochondria. Interestingly, T2D animals showed a similar decrease (≈ 51%) in aconitase activity compared to 3xTg-AD mice.

Accordingly, a significant increase in H_2O_2 levels was observed in mitochondria from both 3xTg-AD and T2D mice (≈ 25% and 18%, respectively) (**Figure 7.5B**).

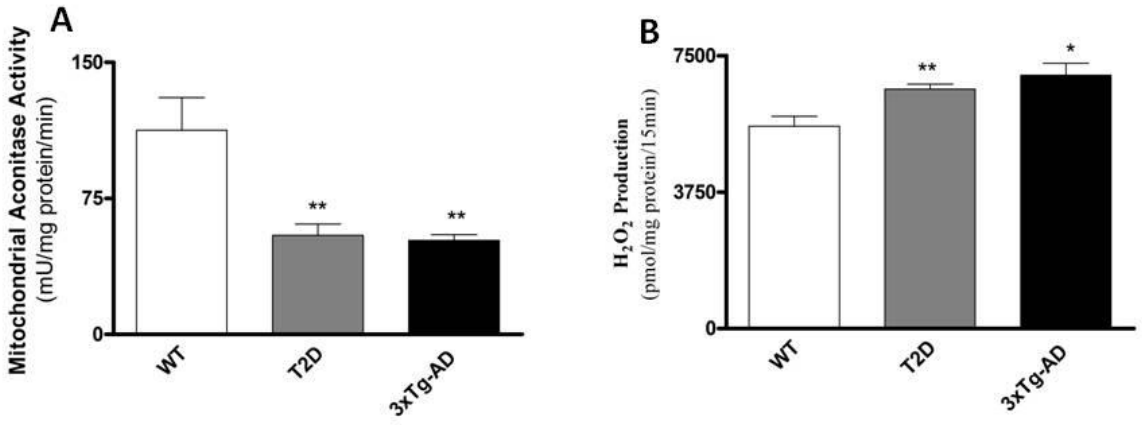


Figure 7.5- ***Effects of AD and T2D on mitochondrial oxidative stress*.** Aconitase activity (A) and H_2O_2 levels (B). Data shown represent mean ± SEM from 5-6 independent experiments. Statistical significance:*p<0.05; **p<0.01 when compared with WT control animals.

7.3.5- Alzheimer's disease and T2D impair antioxidant defenses

Glutathione and vitamin E are important intracellular antioxidants, acting as free radical scavengers and, consequently, protecting cells against oxidative damage. Brain mitochondria isolated from 3xTg-AD and T2D animals present a significant decrease in GSH/GSSG ratio (≈ 60% and 64%, respectively) (**Figure 7.6A**) and vitamin E levels (≈ 60% and 64%, respectively) (**Figure 7.6B**) when compared with WT mitochondria.

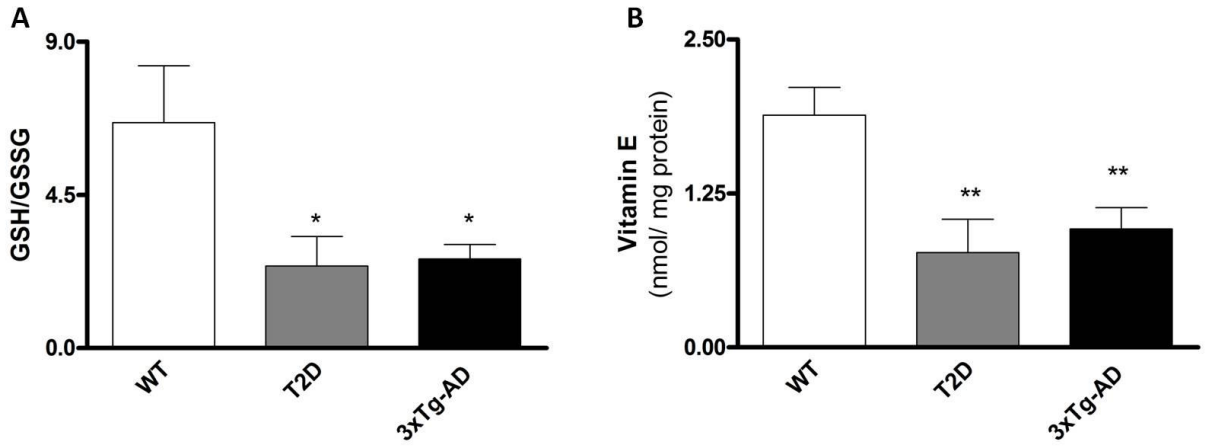


Figure 7.6- ***Effects of AD and T2D on non-enzymatic antioxidant defenses***. Glutathione/glutathione disulfide ratio (GSH/GSSG) (A) and vitamin E levels (B). Data shown represent mean ± SEM from 5-6 independent experiments. Statistical significance: *p<0.05; **p<0.01 when compared with WT control animals.

GPx and GR are two antioxidant enzymes involved in the detoxification of ROS. GPx catalyzes the reduction of H_2O_2 and various hydroperoxides to water. Additionally, GR is responsible for regenerating GSH from GSSG using NADPH as an H^+ donor (Bernardi et al., 1994). When compared with WT mice, mitochondria isolated from both 3xTg-AD and T2D animals present a significant increase in the activity of GPx (≈ 38% and ≈ 48%, respectively) and a decrease in GR activity (≈ 70% and ≈ 62%, respectively) (**Figures 7.7A and B**). MnSOD activity is significantly increased in 3xTg-AD and T2D animals (≈ 375% and ≈ 318%, respectively) when compared with WT control animals (**Figure 7.7C**).

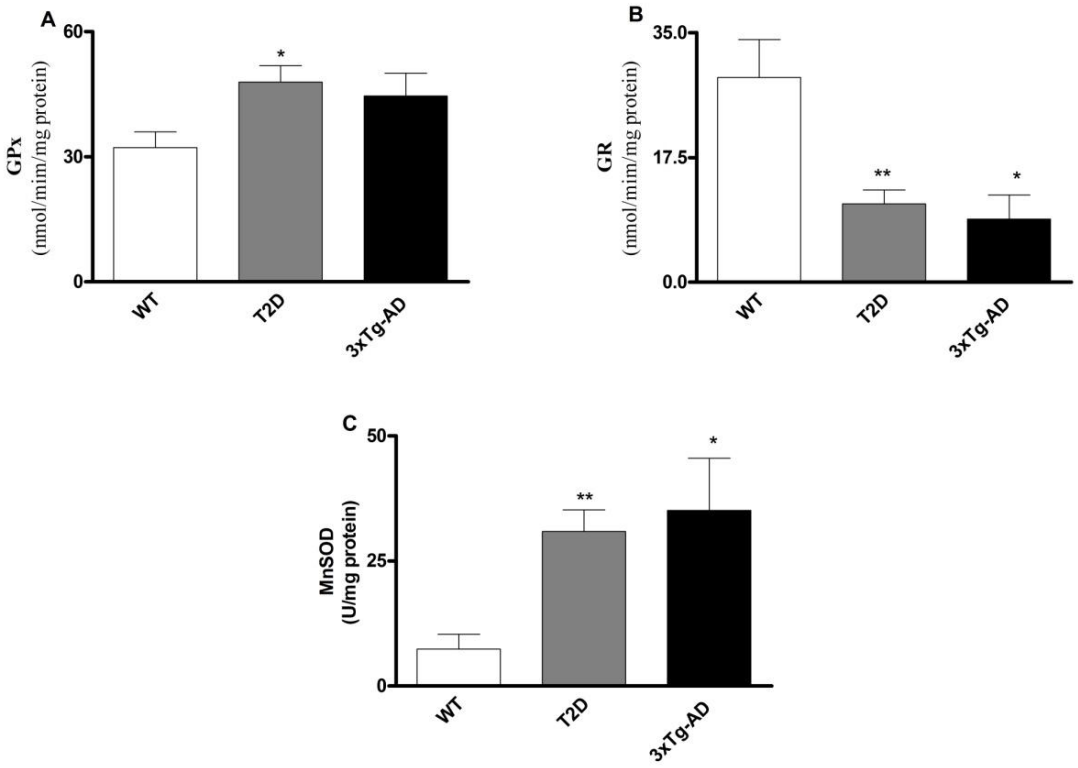


Figure 7.7- ***Effects of AD and T2D on enzymatic antioxidant defenses.*** Glutathione peroxidase (GPx; A), glutathione reductase (GR; B) and manganese superoxide dismutase (MnSOD; C) activities. Data shown represent mean ± SEM from 5-6 independent experiments. Statistical significance: *p<0.05; **p<0.01 when compared with WT control animals.

7.3.6- T2D increases Aβ levels

Not surprisingly, the highest levels of Aβ were found in 3xTg-AD. However, T2D also presented a significant increase in the levels of Aβ protein, particularly in the cortex. An increase in ρ-tau protein levels was also observed although not statistically significant (**Figure 7.8**).

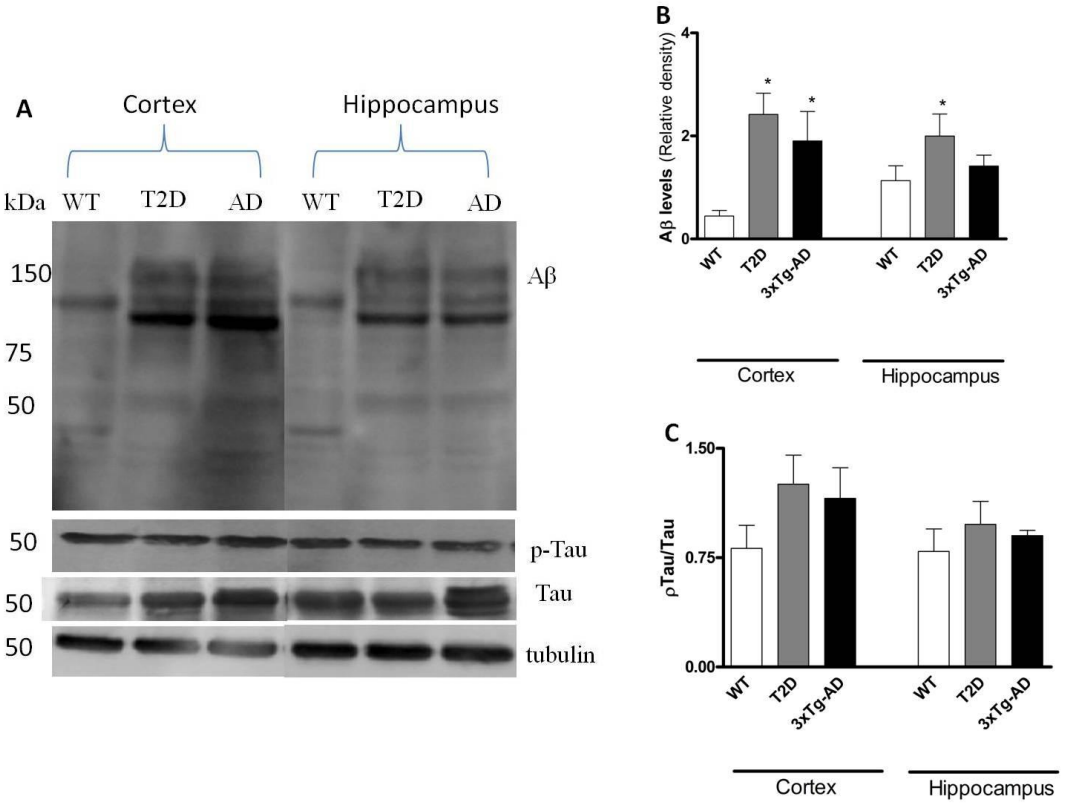


Figure 7.8- ***Effects of AD and T2D on Aβ and phosphorylated tau (ρ-Tau) protein levels.*** Western blot detection (A) and densitometry of bands (B) corresponding to amyloid β (Aβ) and total and phosphorylated tau proteins. WT- WT control animals; T2D- T2D animals; AD- 3xTg-AD control animals.

7.4- Discussion

This study supports the idea that brain mitochondria are a functional link between metabolic abnormalities associated to (pre)diabetes and AD. Sucrose intake-associated metabolic alterations promoted an impairment of mitochondrial respiratory chain, OXPHOS and Ca^{2+} homeostasis and an oxidative imbalance similar to those observed in AD. Furthermore, the metabolic alterations induced by sucrose intake significantly increased the levels of Aβ, a hallmark of AD.

Sucrose intake in WT mice impaired glucose tolerance (**Figure 7.1**) and increased blood glucose, HbA1c, insulin and triglycerides levels (**Table 7.1**). A significant increase in body weight and a decrease in brain weight and brain weight/body weight ratio (**Table 7.1**) were also observed in T2D and 3xTg-AD mice. Previous studies show that diabetes is associated to a body weight gain and a decrease in overall brain weight (Wuarin et al., 1996). The alterations induced by sucrose intake indicate that these animals are in a diabetic state. Several studies showed that phenotypes associated with obesity and/or alterations on insulin homeostasis are at increased risk for developing cognitive decline and dementia, namely vascular dementia and AD (Luchsinger et al., 2004). A recent study also showed that multiple vascular risk factors are associated with a greater rate of decline in cognition, function, and regional CBF in AD patients, which highlights the contribution of vascular risk factors on the progression of AD (Kume et al., 2011). Furthermore, there is published literature showing that diabetes influences survival of AD patients (Magierski et al., 2010).

Interestingly, an increase in HbA1c levels in 3xTg-AD mice under basal conditions was also observed, which may represent a consequence of the altered glucose metabolism occurring in AD. This idea is reinforced by the observation that these AD

mice presented an increase in postprandial blood glucose levels (**Table** 7.1). HbA1c is an early glycation product and one precursor of AGEs (Makita et al., 1992). Previous studies demonstrated that the interaction of AGEs with their receptor, named RAGE, elicits the formation of ROS that are also believed to be an early event in AD pathology (Carvalho et al., 2009).

3xTg-AD animals under basal conditions also presented a decrease in body and brain weight and, consequently, in brain weight/body weight ratio (**Table 7.1**), characteristics also observed in AD patients (Power et al., 2001). Indeed, weight loss is a frequent complication of AD and occurs in 40% of patients at all disease stages (Power et al., 2001).

To prove that brain mitochondria are a link between diabetes (and prediabetic states) and AD, we evaluated and compared the impact of T2D and AD in mitochondria bioenergetics and oxidative status. Previous studies from our laboratory showed that the synthetic $A\beta_{25-35}$ and $A\beta_{1-40}$ peptides impair the respiratory chain, uncouple the OXPHOS and decrease ATP levels of isolated brain mitochondria (Moreira et al., 2001, Moreira et al., 2002). More recently, Dragicevic and collaborators (Dragicevic et al., 2010) evaluated the function of mitochondria isolated from several brain regions obtained from 12 months APPsw and APP+PS1 mouse models of AD. The authors observed an impairment of the respiratory chain and a decrease in $\Delta\Psi m$ in both animal models, these defects being more pronounced in hippocampal and cortical mitochondria (Dragicevic et al., 2010). Accordingly, we observed that mitochondria from 3xTg-AD animals present an impairment of the respiratory chain (**Figure 7.2**) and phosphorylation system culminating in lower production of ATP (**Table 8.2**). This ATP deficit was also observed in mitochondria isolated from AD platelets (Cardoso et al., 2004). The low levels of cellular ATP may result in the loss of synapses and synaptic

function leading to cognitive decline (Butterfield et al., 2006). Interestingly, mitochondria isolated from T2D animals present a similar pattern of respiratory chain and OXPHOS impairment (**Figure 7.2, Table 7.2**), supporting the idea that mitochondrial dysfunction is a common denominator between diabetes (and pre-diabetic states) and AD.

The PTP is a non-selective, high-conductance channel that spans the inner and outer mitochondrial membranes (Bernardi et al., 1994). Mitochondria can tolerate a certain amount of Ca^{2+}, but ultimately their capacity to adapt to Ca^{2+} loads is overwhelmed, and mitochondria depolarize completely due to a profound change in the inner membrane permeability, reflecting PTP induction (Carvalho et al., 2008). Although Ca^{2+} is considered to be the most important inducer, matrix pH, $\Delta\Psi m$, Mg^{2+}, Pi, cyclophilin D, oxidative stress and adenine nucleotides are also effective regulators (Fontaine et al., 1998). In addition, PTP plays an important role in the apoptotic process by releasing several apoptogenic factors such as cytochrome C (Liu et al., 1996). In the present study, mitochondria from both 3xTg-AD and T2D mice presented a decreased capacity to accumulate and retain Ca^{2+} (**Figure 7.3**). These results are in accordance with previous studies from our laboratory showing that isolated brain mitochondria exposed to synthetic Aβ peptides present a lower capacity to accumulate and retain Ca^{2+} (Moreira et al., 2001, Moreira et al., 2002). Du and collaborators (Du et al., 2010) reported that synaptic mitochondria from mAPP mice show an age-dependent accumulation of Aβ and mitochondrial alterations characterized by a decrease in cytochrome oxidase activity and respiration and an increase in oxidative stress and mitochondrial permeability transition. Furthermore, it was shown that the overactivation of NMDA and AMPA receptors, mitochondrial Ca^{2+} overload and mitochondrial damage underlie the neurotoxicity induced by Aβ oligomers (Alberdi et al., 2010). We

also showed that brain mitochondria isolated from diabetic rats exposed to Aβ peptides are more susceptible to Ca^{2+}-induced PTP opening when compared with mitochondria from control animals (Moreira et al., 2005a, Moreira et al., 2005b). The capacity of mitochondria to accumulate and retain Ca^{2+} was significantly increased by the presence of ADP plus oligomycin (**Figure 7.3**). Although CsA is considered the specific inhibitor of PTP, previous studies demonstrated that the pair ADP plus oligomycin is more effective in preventing PTP in brain mitochondria (Brustovetsky and Dubinsky, 2000, Moreira et al., 2002). In accordance with Ca^{2+} fluxes data, our electron microscopy analyses revealed that both 3xTg-AD and T2D animals have a high percentage of damaged mitochondria characterized by mitochondrial swelling and rupture of mitochondrial membranes and cristae (**Figure 7.4**).

Because mitochondria are major intracellular sources of ROS, we also evaluated the oxidative status of our brain mitochondrial preparations. We observed an increased production of H_2O_2 in 3xTg-AD and T2D mice (**Figure 7.5B**), which is positively correlated with the increased susceptibility to PTP opening (**Figure 7.3**). Indeed, it was previously reported that a rise in the production of endogenous mitochondrial ROS, including H_2O_2, facilitates PTP opening (Kowaltowski et al., 2001). Manczak and coworkers (2006) also observed a significant increase in the levels of H_2O_2 in Tg2576 mice compared to age-matched WT littermates prior to the appearance of Aβ plaques. The increase in mitochondrial ROS production is positively correlated with the decrease in the activity of mitochondrial aconitase in both 3xTg-AD and T2D animals (**Figure 7.4A**). It has been proposed that the reaction between mitochondrial aconitase and $O_2^{\bullet-}$ plays a major role in mitochondrial oxidative damage (Gardner et al., 1995). Aconitase has an iron-sulfur cluster in its active center highly sensitive to $O_2^{\bullet-}$ and other reactive species, which inactivates the enzyme (Vasquez-Vivar et al., 2000). A decrease in

aconitase activity was also demonstrated in several experimental models of neurodegenerative diseases (Patel et al., 1996).

In view of the fact that oxidative stress is caused by an imbalance between ROS production and the ability of the biological system to readily detoxify the reactive intermediates or easily repair the resulting damage via antioxidant defenses (Carvalho et al., 2009), we also evaluated several enzymatic and non-enzymatic antioxidant defenses. One key cellular antioxidant is GSH, a potent free radical scavenger and the co-substrate of the antioxidant enzyme GPx. Intracellular GSH is converted into GSSG by GPx, which catalyzes the reduction of H_2O_2 and various hydroperoxides (Durmaz and Dikmen, 2007). Additionally, GR is responsible for regenerating GSH from GSSG using NADPH as an H^+ donor (Rauscher et al., 2001). Several studies already reported alterations in glutathione levels in AD and diabetes (Liu et al., 2004, Mastrocola et al., 2005). Accordingly, we observed that brain mitochondria from both 3xTg-AD and T2D animals present a significant decrease in GSH/GSSG ratio (**Figure 8.6A**). Also, a decrease in GR activity and an increase in GPx in these two groups of experimental animals (**Figure 7.7A and B**) were observed, justifying the decrease in GSH levels (**Figure 7.6A**). We have previously shown that brain tissue from 3-5-month-old female 3xTg-AD mice present lower levels of GSH and vitamin E and an increased activity of SOD and GPx (Resende et al., 2008). Accordingly, we also observed a significant decrease in vitamin E levels in 3xTg-AD and T2D animals (**Figure 7.6B**). A decrease in vitamin E levels was also observed in plasma of diabetic (Peerapatdit et al., 2006) and AD (Baldeiras et al., 2008) patients. An increase in MnSOD activity in 3xTg-AD and T2D mice (**Figure 7.7C**) was also observed, which is consistent with the increase in H_2O_2 levels observed in these animals (**Figure 7.5B**). SOD catalyzes the conversion of $O_2^{\bullet-}$ to H_2O_2 and its activity is undoubtedly important to the regulation of oxidative

status. These results are in accordance with previous results obtained in AD fibroblasts cell lines where MnSOD activity was significantly elevated by 30% when compared to normal euploid cell lines (Zemlan et al., 1989).

Another interesting finding is that metabolic alterations induced by T2D increase the levels of Aβ and (slightly) ρ-tau proteins (**Figure 7.8**), hallmarks of AD, which supports the idea that (pre)diabetes is a risk factor for AD. Accordingly, it was previously shown that type 2 diabetic BBZDR/Wor rats and type 1 diabetic BB/Wor rats present brain accumulation of Aβ and p-tau protein, this accumulation being more pronounced in type 2 diabetic rats (Li et al., 2007).

In summary, our results show that metabolic alterations associated to diabetic or pre-diabetic conditions induce mitochondrial abnormalities and an oxidative imbalance similar to those found in AD brains supporting the idea that brain mitochondria are a functional link between AD and (pre)diabetes. Furthermore, these oxidative and mitochondrial alterations are associated with an increase in the AD pathogenic proteins corroborating that (pre)diabetes increases the risk of developing AD.

Chapter 8- Increased susceptibility to β-amyloid toxicity in rat brain microvascular endothelial cells under hyperglycemic conditions

8.1 – Abstract

We hypothesized that hyperglycemia-induced mitochondrial dysfunction and oxidative stress are closely associated with β-amyloid peptide (Aβ) toxicity in endothelial cells. Brain microvascular endothelial cells from rat (RBMEC) and mice (MBMEC) were isolated from adult Sprague-Dawley rats and homozygous db/db (Leprdb/Leprdb) and heterozygous (Dock7m/Leprdb) mice, and cultured under normo- and hyperglycemic conditions for 7 days followed by 24 hours exposure to $A\beta_{1-40}$. Some experiments were also performed with two mitochondrial $O_2^{\bullet-}$ scavengers, MitoTempo and Peg-SOD. Cell viability was measured by the Alamar blue assay and ΔΨm by confocal microscopy. Mitochondrial $O_2^{\bullet-}$ and (H_2O_2) production was assessed by fluorescence microscopy and H_2O_2 production was confirmed by microplate reader. Hyperglycemia or $A\beta_{1-40}$ alone did not affect cell viability in RBMEC. However, the simultaneous presence of high glucose and $A\beta_{1-40}$ reduced cell viability and ΔΨm, and enhanced mitochondrial $O_2^{\bullet-}$ and H_2O_2 production. MitoTempo and Peg-SOD prevented $A\beta_{1-40}$ toxicity. Interestingly, MBMEC presented a similar pattern of alterations with db/db cultures presenting higher susceptibility to $A\beta_{1-40}$. Overall, our results show that high glucose levels increase the susceptibility of brain microvascular endothelial cells to Aβ toxicity supporting the idea that hyperglycemia is a major risk factor for vascular injury associated with AD.

8.2- Introduction

Modern improvements in health care, increased life expectancy, and the proportion of the aged population in the developed world (Taqui et al., 2007) led to an

abrupt rise in the prevalence of age-associated diseases such as AD. Sporadic AD, which represents the majority of AD cases, is a late-onset disease primarily affecting people over 65 years old. This disease is characterized by a progressive cognitive decline with behavioral changes culminating in a complete loss of control of bodily functions and death (Chaturvedi and Beal, 2008). The deposition of Aβ peptide as senile plaques and neurofibrillary tangles formed mainly by hyperphosphorylated tau protein are characteristic neuropathologic hallmarks of AD. Although research has focused primarily on Aβ peptide and the mechanisms underlying its production and toxic effects on neurons (Arab et al., 2011), evidence shows that cerebral vascular endothelium is an early target in AD (Merlini et al., 2011).

T2D is one of the major risk factors for AD with chronic hyperglycemia being one of its hallmarks. High levels of glucose activate several deleterious pathways culminating in several metabolic and cellular abnormalities (Correia et al., 2008b). Indeed, under a hyperglycemic state high levels of ROS are produced activating several deleterious pathways, such as the activation of the transcription factor NF-κB enhancing monocytes adhesion to the vessel walls, which may initiate an atherosclerotic state (Yorek, 2003). Furthermore, an increase in ET-1 and a decrease in $NO^{\bullet}$ levels can contribute to increased blood-brain barrier permeability, inflammation, vasoconstriction and, consequently, neuronal damage (Carvalho et al., 2009).

Since T2D is a risk factor for AD and chronic hyperglycemia is a hallmark of diabetes, we postulated that elevated blood glucose could compromise the cerebral vascular endothelium and enhance the toxicity of Aβ. Furthermore, we hypothesized that endothelial mitochondria might be adversely affected by hyperglycemia and initiate, via mechanisms involving enhanced production ROS, the destructive effects of Aβ. Therefore, in this work we investigated whether high glucose potentiates the effects

of $A\beta_{1-40}$ in RBMEC and MBMEC derived from adult Sprague Dawley (SD) rats and diabetic db/db mice, respectively.

8.3 - Results

8.3.1- Hyperglycemia increases the susceptibility of endothelial cells to Aβ peptide toxicity.

Under chronic hyperglycemia, RBMEC did not show any significant alteration in cell viability (**Figure 8.1**). However, under hyperglycemic conditions, the viability of RBMEC decreased by approximately 40% when exposed to 10 μM $A\beta_{1-40}$ whereas cells under normoglycemic conditions (5 mM glucose) did not show any significant alterations (**Figure 8.1A**). Interestingly, MBMEC from homozygous but not heterozygous db/db mice presented an increased susceptibility to $A\beta_{1-40}$ at both 5 and 10 μM (**Figure 8.1B**) showing that endothelial cells isolated from mice under an in vivo hyperglycemic state are more vulnerable to $A\beta_{1-40}$ toxicity. For RBMEC, MitoTempo and Peg-SOD were equally effective in protecting cell viability (**Figure 8.1C**). Peg-Catalase and L-NAME were also able to modestly reverse cell death, but with less efficacy (**data not shown**).

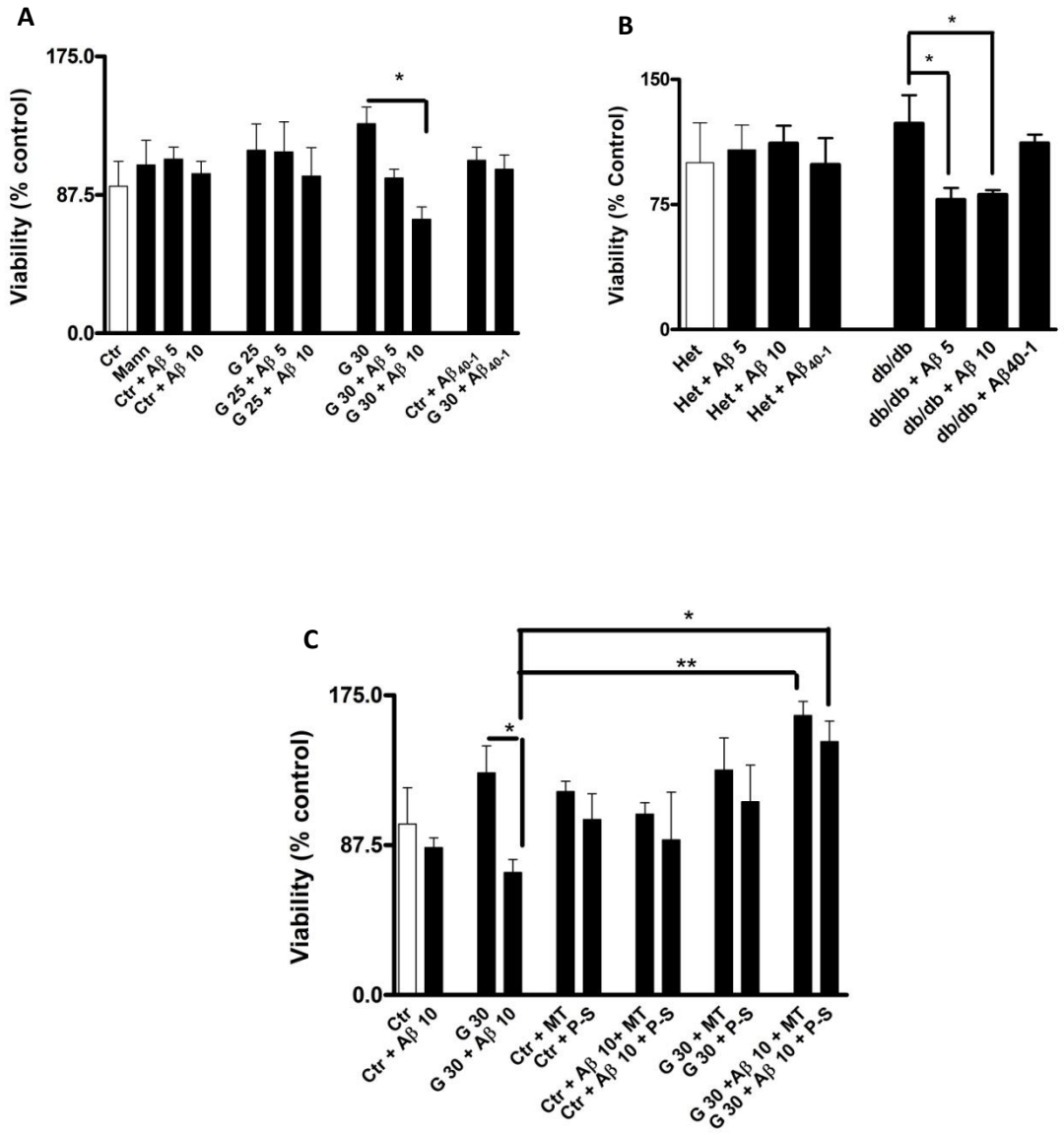


Figure 8.1- ***Effects of glucose and Aβ on viability of RBMEC and MBMEC.*** (A) High levels of glucose alone did not affect cell viability however, high glucose increased susceptibility to Aβ toxicity. (B) In MBMEC from db/db mice but not from lean mice, both high and low glucose increased Aβ toxicity. (C). When treated with MitoTempo and Peg-SOD, cell viability was restored to values close to control treated cells. Ctrl = Control, Mann = Mannitol, Aβ 5 and 10 = Aβ 1-40 (5 and 10 µM, respectively), G 25 and and 30 = Glucose (25 and 30 mM, respectively), MT = MitoTempo, P-S is Peg-SOD, Aβ40-1 = Aβ40-1 (10 µM), Het = heterozygous; db/db = diabetic mice, HG = high glucose medium and LG = low glucose medium. Data are combined from at least 5 independent cultures. Statistical significance: *p<0.05; **p<0.01 and ***p<0.001.

8.3.2- Hyperglycemia-induced ROS production is enhanced by Aβ exposure

The RBMEC under hyperglycemic conditions presented higher levels of mitochondrial $O_2^{\bullet-}$ production (**Figure 8.2G**), which were exacerbated in the presence of Aβ1-40 (**Figure 8.2J**). As expected, MitoTempo and Peg-SOD effectively prevented the increase in $O_2^{\bullet-}$ levels (**Figure 8.2H, I, K, and L**).

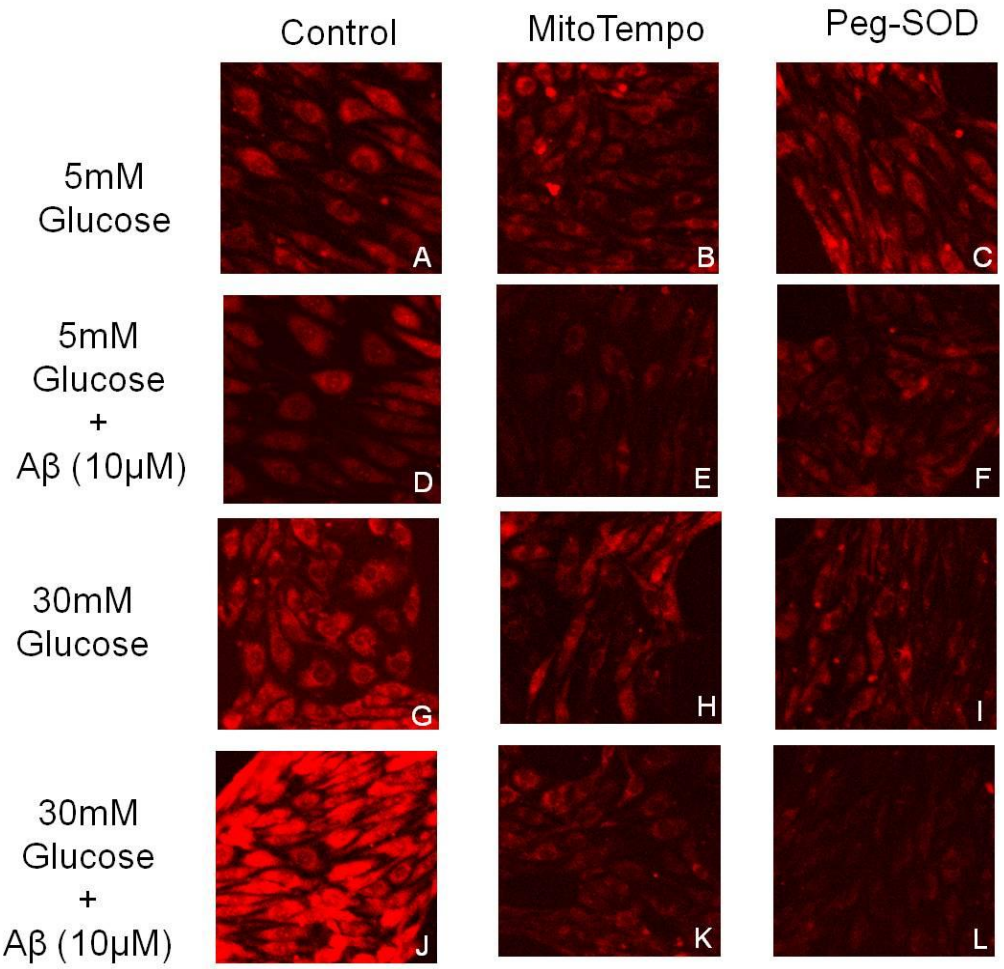


Figure 8.2 - ***Role of ROS in promoting toxicity of Aβ.*** Representative MitoSOX fluorescence images of RBMEC treated with 5 mM (A-F) or 30 mM (G-L) glucose in the presence (D-F; J-L) or absence (A-C; G-I) of Aβ are shown. Furthermore, MitoTempo (B,E,H,K) and Peg-SOD (C,F,I,L) representative fluorescence images are also shown.

Hyperglycemia alone did not change H_2O_2 levels (**Figure 8.3**). However, $A\beta_{1-40}$ promoted an increase in H_2O_2 levels, in both, normo- and hyperglycemic conditions, an effect that was prevented by MitoTempo and Peg-SOD (**Figure 8.3**).

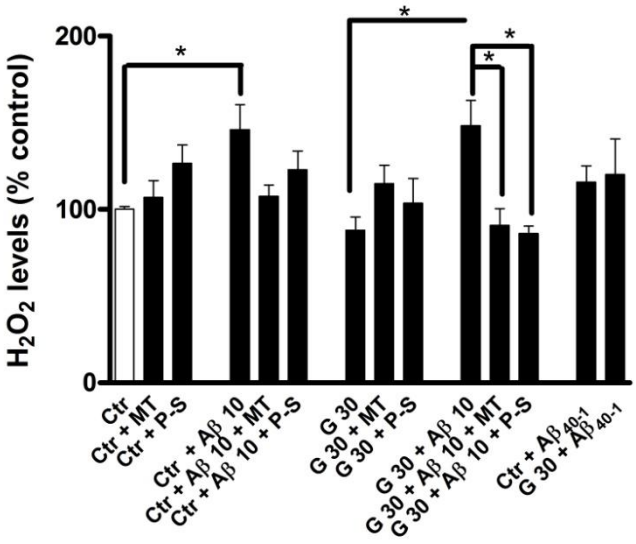


Figure 8.3- ***Effects of hyperglycemia and Aβ on H_2O_2 levels.*** High glucose did not increase H_2O_2 levels however, Aβ led to a significant increase in H_2O_2 levels in the presence of both 5 or 30 mM glucose. When treated with MitoTempo and Peg-SOD, H_2O_2 levels reverted to values near control cells values. Data are combined from five independent cultures. Statistical significance: *p<0,05.

8.3.4- Aβ leads to a decrease in mitochondrial membrane potential under chronic hyperglycemia.

The plate reader assay showed that RBMEC exposed to high glucose did not present significant alterations in ΔΨm. However, those cells co-incubated with $A\beta_{1-40}$ showed a significant loss of ΔΨm (**Figure 8.4**). The antioxidants MitoTempo and Peg-SOD prevented the loss of ΔΨm however Peg-SOD was more effective in preserving ΔΨm (**Figure 8.4**).

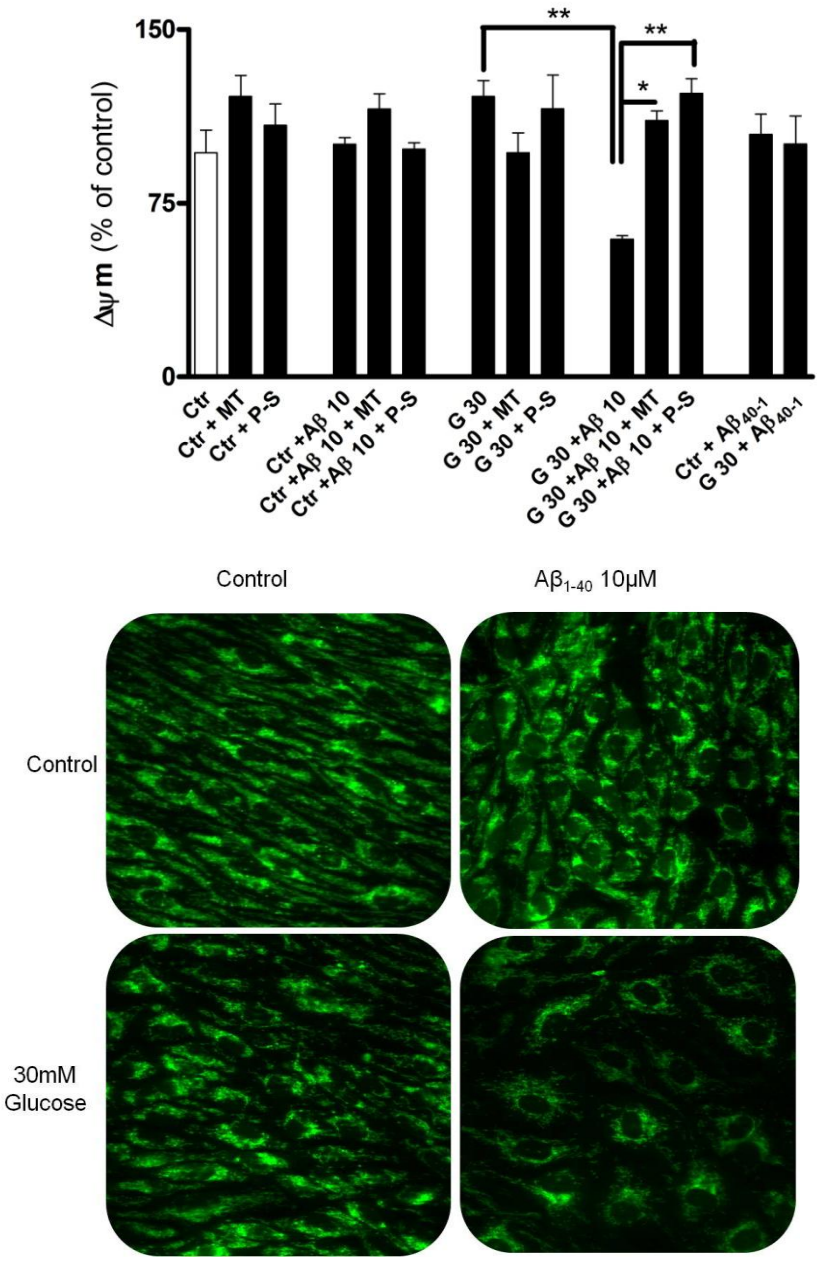


Figure 8.4- ***Effects of hyperglycemia and Aβ on ΔΨm.*** (A) High levels of glucose did not affect mitochondrial membrane potential (ΔΨm). However, in the presence of Aβ a significant drop in ΔΨm was observed. MitoTempo and Peg-SOD restored ΔΨm in 30 mM glucose. Data are combined from five independent cultures. Statistical significance: **p<0,01. Also representative images of RHD123 fluorescence images of RBMEC treated with 5 mM (B1, B2) or 30 mM (B3, B4) glucose in the presence (B2, B4) or absence (B1, B3) of Aβ (10 μM) are shown. 30 mM glucose + Aβ condition present a loss in fluorescence with a mitochondrial network breakage and perinuclear accumulation.

8.4 - Discussion

The major finding of our study is that chronic hyperglycemia enhances brain microvascular endothelial cells susceptibility to Aβ peptide exposure through mitochondrial changes that seem to be initiated by increased mitochondrial $O_2^{\bullet-}$ production. Additionally, the results obtained with MitoTempo and Peg-SOD seemed to corroborate this hypothesis, since these antioxidants revealed to be effective in preventing high glucose and Aβ toxicity. Thus, our study provides a mechanistic basis for the linkage between T2D and the enhanced damage to cerebral endothelial cells as a cause for AD.

T2D is one of the major risk factors for the development of neurodegenerative diseases. Over 60% of T2D patients are estimated to suffer from neurological disorders (Ristow, 2004, Zhu et al., 2010, Yan et al., 2012). Furthermore, studies report that people with T2D are 1.39 times more likely to develop AD (Lu et al., 2009). In fact, previous studies from our laboratory showed that diabetic and AD mice present a similar profile of behavioral, cognitive, vascular and mitochondrial abnormalities (Carvalho et al., 2012, 2013). Interestingly, diabetic mice presented a significant increase in Aβ levels in brain cortex and hippocampus (Carvalho et al., 2012, 2013). Accordingly, Kolluru and coworkers (2012) reported that vascular dysfunction is a major player in the establishment of diabetes-associated complications. However, studies on the cerebral microvasculature exposed to hyperglycemia are not as advanced as those on peripheral vessels (Cerbone et al., 2009).

Hyperglycemia *per se* did not affect viability or other parameters in RBMEC. However, the combination of high glucose plus $A\beta_{1-40}$ peptide induced a significant decrease in cell viability in a dose-dependent manner, whereas the viability of RBMEC

cells was not significantly changed when exposed to $A\beta_{1-40}$ alone. The absence of $A\beta_{1-40}$ toxicity seems, in some extent, contradictory with most published studies describing the toxic effects of the peptide. However, when we analyzed those studies, we realize that the control maintenance medium is supplemented with high levels of glucose hiding the possible non-toxic, physiological roles of $A\beta_{1-40}$ (Broersen et al., 2011, Manzoni et al., 2011). To prove that chronic hyperglycemia increases the susceptibility of brain endothelial cells to $A\beta_{1-40}$ we also tested the effects of this peptide in MBMEC isolated from diabetic db/db mice. The db/db mice are polyphagic, polydipsic, and polyuric and demonstrate an uncontrolled rise in blood sugar, severe depletion of the insulin-producing β-cells of the pancreatic islets, and death by 10 months of age. Interestingly, a substantial effect on db/db MBMEC viability occurred regardless of glucose level in culture when exposed to Aβ peptide. Additionally, the experiments on MBMEC demonstrate that these endothelial cells retain a distinct phenotype in culture consistent with chronic exposure to hyperglycemia. Previous studies also reported that Aβ neurotoxicity was exacerbated during hypoglycemia/hyperglycemia (≦ 2 mM/≧30 mM) (Wang et al., 2010). Hyperglycemia alone did not interfere with $\Delta\Psi m$ but, in the presence of $A\beta_{1-40}$, we observed a significant decrease in $\Delta\Psi m$ (**Figure 8.4**). These results are in agreement with a study performed by Moreira et al. (2003) showing that brain mitochondria isolated from T2D Goto-Kakizaki rats did not show significant alterations in $\Delta\Psi m$, but a significant drop in this parameter occurred when mitochondria were exposed to $A\beta_{1-40}$ peptide.

Furthermore, we showed that the increased susceptibility of RBMEC under high glucose exposure to Aβ is mediated by mitochondrial $O_2^{\bullet-}$. Indeed, it was previously suggested that mitochondrial overproduction of ROS due to hyperglycemia may increase the susceptibility of endothelial cells to injury (Giacco and Brownlee, 2010). In

fact, we observed a significant increase in mitochondrial $O_2^{\bullet-}$ when cells were exposed to 30 mM glucose and this increase was exacerbated by $A\beta_{1-40}$. A significant increase in H_2O_2 levels was also observed in both normoglycemic and hyperglycemic cells exposed to Aβ peptide but it is unclear whether this ROS was produced directly or via dismutation of $O_2^{\bullet-}$. These results are consistent with previous studies showing that, under physiologic conditions, cells maintain the redox balance through the generation and elimination of ROS, however, when redox homeostasis is disturbed, oxidative stress may lead to aberrant cell death and contribute to disease development including neurodegenerative diseases (Trachootham et al., 2008). To confirm that mitochondrial ROS play a key role in the alterations observed in cells exposed to 30 mM glucose and Aβ we tested several antioxidants: MitoTempo, Peg-SOD, Peg-catalase, L-NAME, and apocynin (an inhibitor of NADPH oxidases). The MitoTempo and Peg-SOD completely prevented the loss of cell viability induced by high glucose plus $A\beta_{1-40}$, contrasting with the other antioxidants which only exerted a partially protective effect (**data not shown**). As expected, MitoTempo and Peg-SOD normalized $O_2^{\bullet-}$ levels in cells exposed to high glucose or high glucose plus $A\beta_{1-40}$. These observations suggest that mitochondrial $O_2^{\bullet-}$ has a key role in mediating high glucose and Aβ toxicity. Previous studies showed that MitoTempo is a SOD mimetic with a mechanism of action similar to SOD in $O_2^{\bullet-}$ detoxification, which is however specifically targeted to mitochondria (Hoehn et al., 2009). Furthermore, both MitoTempo and Peg-SOD were able to prevent the drop in $\Delta\Psi m$ and the increase in H_2O_2 levels in cells exposed to high glucose plus $A\beta_{1-40}$. Interestingly, Peg-SOD was more effective in restoring $\Delta\Psi m$ than MitoTempo. This may be due to the fact that Peg-SOD is also able to detoxify the $O_2^{\bullet-}$ produced at other locations besides mitochondria. Although mitochondria are major sources of ROS we cannot exclude the potential contribution of other cytosolic sources (Lee et al., 2011),

which may potentiate mitochondrial dysfunction and ROS production (Park et al., 2011a). Concerning H_2O_2 levels, no alterations were observed in the presence of Peg-SOD in control conditions (**Figure 8.3**). However, Peg-SOD reversed Aβ-induced increase in H_2O_2 levels under hyperglycemic conditions. The reduction in H_2O_2 levels seem contradictory since Peg-SOD converts $O_2^{\bullet-}$ to H_2O_2. Liochev and Fridovich (1991) suggested that an increase in $O_2^{\bullet-}$ dismutation would prevent the formation of H_2O_2 by other reactions. This is supported by studies performed in cell lines overexpressing CuZnSOD, which show reduced levels of H_2O_2 (Teixeira et al., 1998). It was also reported that Peg-SOD treatment increased catalase activity in cancer cells (Sen et al., 2012), an additional mechanism that could justify the decrease in H_2O_2 levels. Additionally, MitoTempo was able to decrease H_2O_2 levels (**Fig. 4**). Dikalova and coworkers [34] showed that in addition to $O_2^{\bullet-}$ scavenging, MitoTempo has the capacity to reduce mitochondrial ROS production by normalizing mitochondrial respiration. In agreement with our results, Liang et al. [35] also showed that MitoTempo was able to recover $\Delta\Psi m$ through the inhibition of the mitochondrial permeability transition pore.

In summary, our results show that brain endothelial cells under chronic hyperglycemia are more susceptible to Aβ toxicity, an effect that seems to be mediated by mitochondrial ROS. Furthermore, we have shown that our *in vitro* model of chronic hyperglycemia mimics diabetic conditions. This study supports the idea that diabetes is a risk factor for AD.

Chapter 9- General conclusions

9.1- General conclusions

This work showed that the aging process, chronic hypoxia, T2D and AD affect the cerebral vasculature, particularly endothelial cells, and synaptosomes. These alterations are closely associated with mitochondrial abnormalities and oxidative stress predisposing to brain cells damage and, eventually, death. However, it is also shown that different brain structures or cells present a distinct pattern of alterations when exposed to similar injurious situations reflecting the existence of a tissue -or structure- specific network of defenses. The *in vivo* studies also show that mitochondrial abnormalities associated to T2D predispose to Aβ overproduction in brain cortex and hippocampus and the in vitro study shows that hyperglycemia increases the susceptibility of brain endothelial cells to Aβ, a phenomenon closely related to mitochondrial superoxide production. In fact, these results suggest that mitochondrial alterations promote Aβ production and vice-versa. Finally, these findings reinforce the idea that the alterations promoted by T2D provoke cerebrovascular and neuronal changes culminating in AD development.

9.2- Clinical relevance

Diabetes is recognized as an epidemic of the 21st century and it is expected to rise in the coming years due to the population aging and Western lifestyles characterized by sedentary habits and unhealthy diets. Because diabetes is a main risk factor for cognitive impairment and dementia, particularly AD, these pathologies represent an increasing social and economic burden. The clarification of the cellular and molecular links between these pathological conditions may help design more effective

diagnostic and therapeutic strategies. We believe our studies represent a step forward in the search for effective therapies. In the meanwhile, prevention is the key!

9.3- Future directions

It would be interesting to perform in vivo studies comparing the alterations in CBF and brain oxygen delivery in T2D and AD models.

Because mitochondrial abnormalities are key participants in T2D and AD, testing the in vivo efficacy of MitoTempo against T2D-induced endothelial and the development of AD-like pathology is of maximum importance.

It would be also interesting to analyze the role of T2D in the activity and expression of enzymes involved in Aβ processing and clearance.

In addition, the identification of the factors released by endothelial cells under these pathological conditions is of utmost importance, which may bring new clues about the association between endothelial and neuronal dysfunction.

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