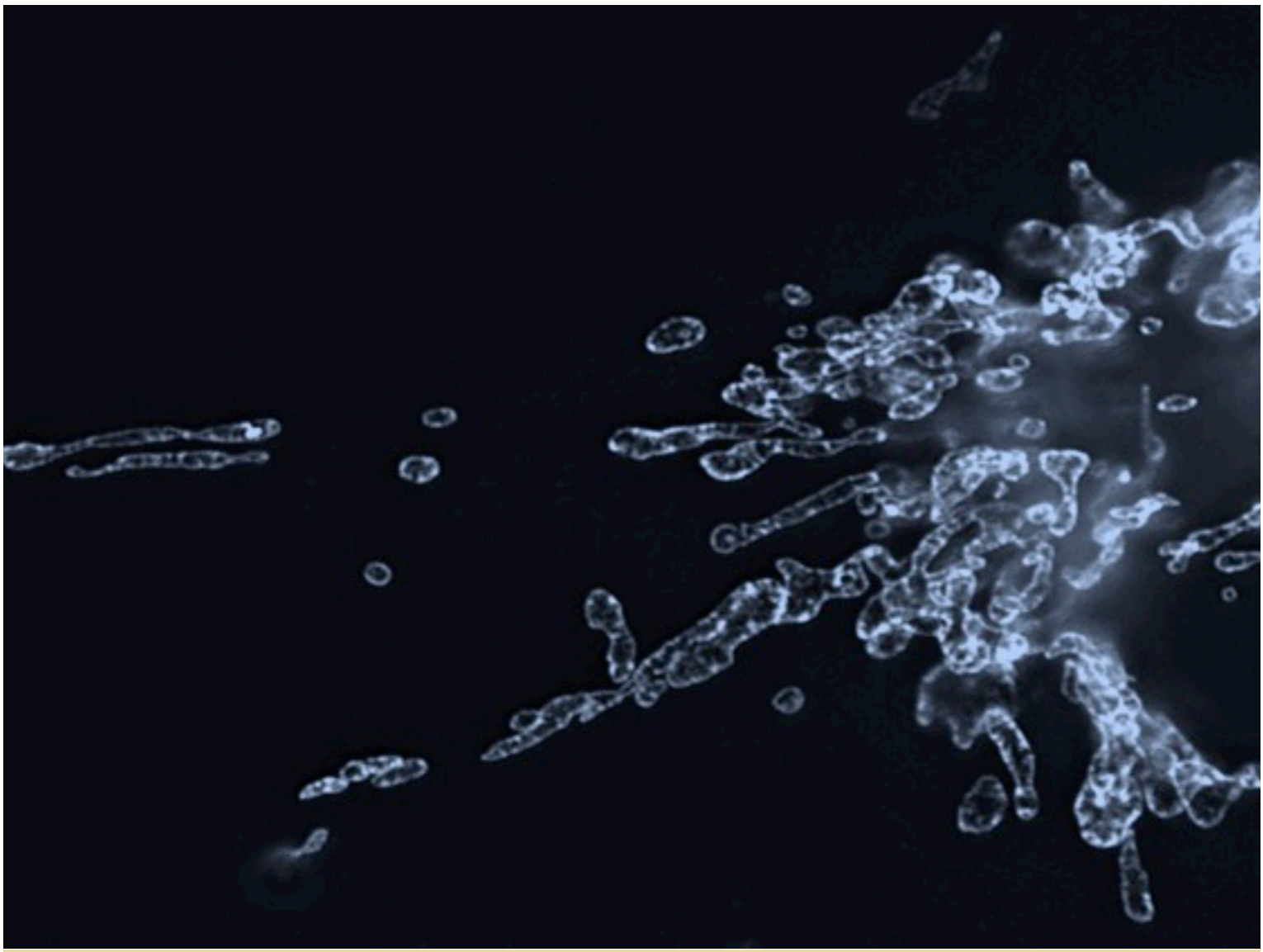

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Dissertação apresentada ao departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra para prestação de provas de Doutoramento em Biologia, na especialidade de Biologia Celular

“The saddest aspect of life right now is that science gathers knowledge faster than society gathers wisdom.”

Isaac Asimov

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Table of Contents

List of abbreviations

AD – Alzheimer's disease

ADA- American diabetes association

ADP – adenosine diphosphate

AIF – apoptosis-inducing factor

ASP-I – insulin aspart

ATP – adenosine triphosphate

AVs – autophagic vacuoles

Aβ – amyloid β

AβPP – amyloid β precursor protein

BBB – blood-brain barrier

BCA – bicinchoninic acid

BSA – bovine serum albumin

Ca^{2+} – calcium ion

cAMP – cyclic adenosine monophosphate

CNS – central nervous system

CoQ – coenzyme Q

CREB – cAMP response-element-binding protein

CSF – cerebrospinal fluid

Cu/Zn-SOD – copper/zinc-containing superoxide dismutase

CypD – cyclophilin D

DM – diabetes mellitus

DNA – deoxyribonucleic acid

DRG – dorsla root ganglia

DRP1 or DLP1 – dynamin-like protein 1

DTT – dithiothreitol

ECF – enhanced chemifluorescence

ELISA – enzyme-linked immunosorbent assay

ETC – electron transport chain

FCCP – carbonyl cyanide-p-trifluoromethoxyphenylhydrazone

Fis1 – fission protein 1

FPG – fasting plasma glucose

GK – Goto-Kakizaki

GLUTs – glucose transporters

GSH – glutathione

GSK3β – glycogen synthase kinase-3β

GTP – guanosine triphosphate

H_2O_2 – hydrogen peroxide

HbA1c- haemoglobin A1c (glycated haemoglobin)

IDE – insulin-degrading enzyme

IGF – insulin-like growth factor

INS – insulin

IR – insulin receptor

IRS1 – insulin receptor substrate 1

JNK – c-Jun N-terminal kinase

LTP – long-term potentiation

MAPK – mitogen-activated protein kinase

MCI – mild cognitive impairment

MCU – mitochondrial calcium uniporter

MFF – mitochondrial fission factor

Mfn1 – mitofusin 1

Mfn2 – mitofusin 2

MIEF1 – mitochondrial elongation factor 1

mRNA – messenger ribonucleic acid

mtDNA – mitochondrial deoxyribonucleic acid

Na^+ - sodium ion

NADPH – nicotinamide adenine dinucleotide phosphate

nDNA – nuclear deoxyribonucleic acid

NF-kB – nuclear factor-kB

NFTs – neurofibrillary tangles

$NO^{\bullet}$ - nitric oxide

NO_2 – nitrogen dioxide radical

NOD – non-obese diabetic

NRF1 – nuclear respiratory factor 1

NRF2 – nuclear respiratory factor 2

$O_2^{\bullet -}$ – superoxide anion

OGTT – oral glucose tolerance test

$ONOO^-$ - peroxynitrite

OPA1 – optic atrophy 1

Oxphos – oxidative phosphorylation

PCR – polymerase chain reaction

PE – phosphatidylethanolamine

PGC1α – peroxisome proliferator activator receptor gamma-coactivator 1α

PI3K – phosphatidylinositol-3-kinase

PI3P – phosphatidylinositol 3-phosphate

PKB – protein kinase B

PMSF – phenylmethylsulfonyl fluoride

PP2A – protein phosphatase 2A

PRX – peroxiredoxin

PS1 – presenilin 1

PS2 – presenilin 2

PSD95 – postsynaptic density protein 95

PTP – permeability transition pore

PTP1B – protein tyrosine phosphatase 1B

PVDF – polyvinylidene difluoride

RCR – respiratory control ratio

RH-I – regular human insulin

RIPA – radioimmunoprecipitation assay

ROS – reactive oxygen species

rRNA – ribosomal ribonucleic acid

SDS – sodium dodecyl sulfate

Ser – serine

Shc – src-homology-2-containing protein

SNAREs – soluble N-ethylmaleimide-sensitive factor attachment protein receptors

SOD – superoxide dismutase

STZ – streptozotocin

T1D – type 1 diabetes

T2D – type 2 diabetes

TBS – tris-buffered saline

TCA – tricarboxylic acid

TFAM – mitochondrial transcription factor A

Thr – threonine

TOR – target of rapamycin

TPP^+ - tetraphenylphosphonium ion

tRNA – transfer ribonucleic acid

TRX – thioredoxin

Tyr – tyrosine

ULK1 – unc-51-like kinase 1

WHO – world health organization

Δp – electrochemical potential

$\Delta\Psi_m$ – mitochondrial membrane potential

Abstract

Diabetes mellitus has become a global epidemic. Etiologically, diabetes can be classified in two main forms, type 1 and type 2 diabetes (T1D and T2D, respectively). Both forms of diabetes have been associated with a number of complications including neurodegeneration. Additionally, increasing evidence supports the idea that diabetes increases the risk of cognitive decline and dementia. Insulin, a widely used therapeutic agent in the treatment of diabetes, which assumes critical importance in the treatment of T1D, has also been proved to exert beneficial effects in the brain.

Mitochondria sit at a strategic position in brain cells, particularly in neurons, that present a high energy demand. Besides energy production, mitochondria play other key roles such as cell life or death decision and homeostasis of second messengers (Ca^{2+} and reactive oxygen species). The maintenance of a healthy mitochondrial pool requires an equilibrium between mitochondrial fission/fusion, biogenesis and degradation by autophagy (mitophagy).

Although previous studies demonstrated the involvement of mitochondrial dysfunction in diabetes-associated brain damage, the knowledge concerning the role of mitochondrial fission/fusion and biogenesis and autophagy in diabetic brains is insufficient. So, the main aim of this work was to elucidate these mitochondrial parameters in T1D and T2D rat brain.

Studies were conducted in brain cortices of six-month-old Wistar (control) and Goto-Kakizaki (GK) rats (Chapter 4). GK rats are a model of nonobese T2D considered by some authors a good animal model of prediabetes or initial stages

of T2D since it does not present the full clinical setting of this pathology. In fact, these rats are characterized by mild hyperglycemia and insulin resistance. Concerning brain cortical mitochondria, no significant differences were observed in respiratory chain function and oxidative phosphorylation efficiency. Despite the inexistence of functional alterations, increased mitochondrial fission was observed in T2D rats. Furthermore, autophagy was significantly decreased, whereas mitochondrial biogenesis remained unaltered in the brain cortex of T2D rats. These observations suggest the occurrence of compensatory mechanisms that may help prevent alterations in mitochondrial function.

Insulin is critical for the survival of T1D individuals and has proven to have beneficial effects in the brain. Therefore, to evaluate the effects of T1D and insulin treatment in mitochondrial function, fission/fusion and biogenesis, autophagy and tau protein phosphorylation we used streptozotocin (STZ)-induced T1D rats (3 months of diabetes duration) and STZ rats treated with a daily injection of insulin during the last month of the experimental protocol (Chapter 5). Vehicle-treated Wistar rats were used as control animals. STZ-induced T1D and insulin treatment did not affect mitochondrial function. No significant changes were observed in brain cortical levels of glucose and pyruvate in T1D rats. However, insulin treatment increased significantly brain cortical glucose levels, despite no significant alterations in pyruvate levels occurred in the brains of these animals. An increase in mitochondrial biogenesis as well as a shift towards increased mitochondrial fission were observed in the brain cortex of T1D animals. T1D animals also presented, an increased phosphorylation of the tau protein at Ser396 residue, this effect being partially reversed by insulin treatment. This effect of insulin was associated with a

modest decrease in the active form of glycogen synthase kinase 3β (GSK3β) and significant increase in protein phosphatase 2A (PP2A) activity. Insulin was also able to slightly decrease LC3-II levels, a marker of autophagy. No significant alterations were found in apoptotic cell death and synaptic integrity.

Overall the results presented in this thesis suggest the existence of mitochondrial readjustments in brain cortex of diabetic rats in order to preserve mitochondrial function and, consequently, the integrity and functionality of brain cells. In general, insulin therapy was shown to have positive effects against T1D-induced brain cortical alterations reinforcing the idea that insulin is an effective therapeutic agent against diabetes-associated complications. Further research must be done to evaluate the behavior of the brain mitochondrial network in later stages of diabetes since it has been described that mitochondrial dysfunction is intimately associated with diabetes-associated neurodegenerative conditions.

Resumo

A diabetes mellitus já atingiu proporções epidémicas. Etiologicamente, a diabetes pode ser classificada em duas formas principais, diabetes tipo 1 e diabetes tipo 2. A diabetes tipo 2 é a forma mais comum da doença. Ambas as formas de diabetes têm sido associadas a várias complicações, incluindo eventos neurodegenerativos. Adicionalmente, um número crescente de evidências apoia a ideia de que a diabetes aumenta o risco de declínio cognitivo e demência. A administração de insulina, um agente terapêutico amplamente usado no tratamento da diabetes, sendo essencial no tratamento da diabetes tipo 1, mostrou exercer efeitos benéficos no cérebro.

As mitocôndrias assumem uma posição de particular importância nas células cerebrais, particularmente nos neurónios devido à sua elevada exigência energética. Além da produção de energia, as mitocôndrias também são determinantes na decisão de vida ou morte celular e na homeostase de segundos mensageiros (Ca^{2+} e espécies reactivas de oxigénio). Para que as células mantenham uma população mitocondrial "saudável" é necessário haver um equilíbrio entre fusão/fissão e biogénese mitocondrial e mitofagia (degradação das mitocôndrias por autofagia).

Apesar de estudos anteriores mostrarem o envolvimento da disfunção mitocondrial no dano cerebral associado à diabetes, o conhecimento acerca do papel da fusão/fissão e biogénese mitocondrial e da autofagia no cérebro diabético é insuficiente. Desta forma, este trabalho teve como objectivo elucidar o envolvimento destes parâmetros mitocondriais nos cérebros diabéticos tipo 1 e tipo 2.

Foram feitos estudos em córtex cerebral de ratos Goto-Kazikaki (GK) com seis meses de idade e Wistar controlo da mesma idade (Capítulo 4). Os ratos GK são um modelo não obeso de diabetes tipo 2 considerado por alguns autores um modelo animal de pré-diabetes uma vez que não apresentam todas as características da diabetes tipo 2. De facto, estes animais apresentam hiperglicemia moderada e resistência à insulina. No que diz respeito às mitocôndrias de córtex cerebral, não se observaram alterações significativas na função da cadeia respiratória e na eficiência da fosforilação oxidativa. No entanto, verificou-se um aumento significativo da fissão mitocondrial e uma diminuição significativa da autofagia nos cérebros dos ratos GK. A biogénese mitocondrial não sofreu qualquer alteração. Estas observações sugerem a ocorrência de mecanismos compensatórios que podem contribuir para a manutenção da função mitocondrial.

A insulina é essencial para a sobrevivência dos pacientes com diabetes tipo 1 e vários estudos têm descrito os efeitos benéficos da insulina no cérebro. Como tal, para avaliar os efeitos da diabetes tipo 1 e do tratamento com insulina na função, fissão/fusão e biogénese mitocondrial, autofagia e fosforilação da proteína tau, usámos ratos injectados com estreptozotocina (STZ), de forma a induzir diabetes tipo 1 (diabetes com duração de 3 meses), e ratos diabéticos tipo 1 injectados diariamente com insulina durante o último mês do protocolo experimental (Capítulo 5). Ratos Wistar tratados com a solução veículo foram usados como animais controlo. Tanto a diabetes tipo 1 como o tratamento com insulina não alteraram significativamente a função mitocondrial. Os níveis cerebrais de glicose e piruvato em ratos diabéticos tipo 1 também permaneceram inalterados. Contudo, o tratamento com insulina aumentou os

níveis cerebrais de glicose, não alterando os níveis cerebrais de piruvato. Também se observou um aumento significativo da biogénese e da fissão mitocondrial no córtex cerebral de animais diabéticos tipo 1. Relativamente à proteína tau, foi observado um aumento da fosforilação do resíduo Ser396 em ratos diabéticos tipo 1, sendo este efeito parcialmente revertido pelo tratamento com insulina. Este efeito da insulina mostrou estar associado a um decréscimo ligeiro da forma activa da cinase GSK3β e a um aumento significativo da actividade da proteína fosfatase 2A. O tratamento com insulina também reduziu ligeiramente os níveis de LC3-II, um marcador de autofagia. Não se encontraram alterações significativas nos marcadores de morte celular por apoptose e de integridade sináptica.

Globalmente, os resultados apresentados nesta tese sugerem uma remodelação da rede mitocondrial no córtex cerebral de ratos diabéticos de forma a preservar a função mitocondrial e, consequentemente, a integridade e a funcionalidade das células cerebrais. No geral, a terapia com insulina demonstrou ter efeitos positivos nas alterações que ocorrem no córtex cerebral de animais diabéticos tipo 1, reforçando a ideia de que a insulina é um agente terapêutico eficaz contra as complicações associadas à diabetes nomeadamente a nível cerebral. No entanto, mais estudos devem ser feitos para avaliar o comportamento da rede mitocondrial cerebral em fases mais avançadas da diabetes uma vez que está descrito que a disfunção mitocondrial desempenha um papel chave na neurodegenerescência associada à diabetes.

CHAPTER 1

General Introduction

1.1. Diabetes mellitus: epidemiology, etiology, and clinical symptoms

Between 2010 and 2030 there will be a 69% increase in the number of adults with diabetes mellitus (DM) in developing countries and a 20% increase in developed countries (Shaw et al., 2010). These increases are much higher than the increase in total number of adult population in the same period, which will be of 36% for developing countries and 2% for developed countries (Shaw et al., 2010). Therefore, it is not surprising that DM is gaining epidemic proportions worldwide. Globally, the number of people with DM by the year of 2030 is predicted to be 439 million, which represents 7.7% of the total adult population of the world aged 20–79 years (Chen et al., 2011). Data regarding the Portuguese population shows that in 2012, 12.9% of the population aged between 20 and 79 years old had diabetes (Relatório Anual do Observatório Nacional da Diabetes, 2013). Indeed, the Portuguese population has one of the highest rates of prevalence among the European countries (Relatório Anual do Observatório Nacional da Diabetes, 2013). According to the International Diabetes Federation, in 2011 the healthcare costs with individuals with DM represented 11% of the total healthcare expenditures in the world, which illustrates the economic burden of this disorder (http://www.idf.org/diabetesatlas/5e/healthcare-expenditures).

Regarding the prevalence of the main types of DM, type 1 diabetes (T1D) accounts for 5-10%, while type 2 diabetes (T2D) accounts for the remaining 90-95% of the total cases of DM worldwide (Maahs et al., 2010; American Diabetes

Association, 2012). The most common type of DM in children and adolescents is T1D, although T2D is diagnosed in older people (Maahs et al., 2010).

T1D is subdivided in two categories: type 1a, which represents the vast majority of the cases and is originated by an autoimmune-mediated destruction of pancreatic beta cells, and type 1b, that represents the minority of cases and results from the idiopathic destruction or failure of pancreatic beta cells (Maahs et al., 2010). The combination of genetic risk factors and environmental triggers is involved in T1D development (Todd, 2010). So far, ten genes have been identified as being involved in T1D pathophysiology while viral infections also play a key role in disease onset (Todd, 2010). Nevertheless, the exact cause(s) of T1D is (are) still largely obscure.

In type 2 diabetes (T2D) insulin resistance and pancreatic beta-cell dysfunction are known to be the major pathophysiological factors driving this disorder (Maahs et al., 2010). Insulin resistance results from several factors including overweight and obesity, high calorie diets, sedentary habits, and genetics, among others (Chen et al., 2011).

Despite the disparities involving the etiology and the epidemiology of T1D and T2D, according to the World Health Organization (WHO), both types of diabetes share some clinical phenotypic features such as polyuria (excessive excretion of urine), polydipsia (extreme thirst), polyphagia (excessive hunger), vision changes and fatigue (http://www.who.int/mediacentre/factsheets/fs312/en/index.html accessed July, 2013). In the case of T2D the referred symptoms are often less marked. The American Diabetes Association (ADA) (American Diabetes Association, 2012), defined clear criteria for the diagnosis of diabetes: (1) glycated hemoglobin

(HbA1c) ≥ 6.5%; or (2) fasting plasma glucose ≥ 126 mg/dL (fasting is defined as no caloric intake for at least 8 h); or (3) 2 h plasma glucose ≥ 200 mg/dL during an oral glucose tolerance test (using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water); or (4) in a patient with classic symptoms of hyperglycemia or hyperglycemic crisis, a random plasma glucose ≥ 200 mg/dL.

Long-term complications associated with DM include retinopathy with potential loss of vision; nephropathy leading to renal failure; peripheral neuropathy with risk of foot ulcers, amputations, and Charcot joints; and autonomic neuropathy causing gastrointestinal, genitourinary, and cardiovascular symptoms and sexual dysfunction. DM also presents increased incidence of atherosclerotic cardiovascular, peripheral arterial, and cerebrovascular disease (American Diabetes Association, 2014). In sub-chapter 1.2 it will be discussed in further detail the relation between DM and the increased incidence of neurodegenerative diseases, particularly Alzheimer's disease (AD).

1.2. Diabetes increases the risk of cognitive decline and dementia: a focus on Alzheimer's disease

1.2.1. An overview about Alzheimer's disease

The classical histopathological hallmarks of AD are the senile plaques, extracellular deposits formed mainly of amyloid β (Aβ) protein, and the neurofibrillary tangles (NFTs), intracellular deposits composed mostly of

hyperphosphorylated tau protein (Fig. 1.1.) (Castellani et al., 2010; Querfurth and LaFerla, 2010).

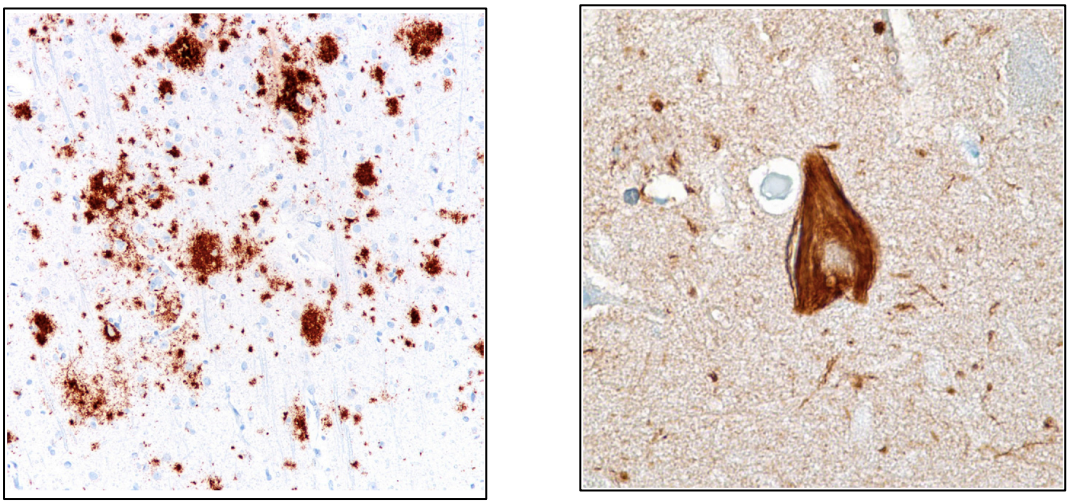


Figure 1.1. – Classical histopathological hallmarks of Alzheimer's disease. Left panel: Cerebral cortex immunohistochemistry of senile plaques. Right panel: Immunohistochemical stain of neurofibrillary tangles. Adapted from Castellani et al., 2010.

AD has either an age-associated, late onset sporadic form, which is the more common form of disease, or an early-onset familial form with a genetic origin involving mutations in the amyloid-β protein precursor (AβPP) and presenilin 1 and 2 (PS1 and PS2) genes (Querfurth and LaFerla, 2010). The etiopathogenesis of sporadic AD remains largely unclear and several competing hypotheses have been proposed. Whereas the hypothesis that still drives the investigation of most authors is the amyloid cascade hypothesis pointing the oligomeric Aβ as the most toxic entity and culprit of the disease (Haass and Selkoe, 2007; LaFerla et al., 2007), some authors implicate cerebrovascular damage as a potential cause of AD (Humpel and Marksteiner, 2005; Brenner, 2008). Others center their efforts on hyperphosphorylation of cytoskeletal proteins (Pei et al., 2008; Chung, 2009), oxidative stress (Smith et al., 2000),

abnormal cell cycle re-entry (Raina et al., 2000a; 2000b), and inflammation (Pasinetti, 1996). Mitochondrial dysfunction is also one of the earliest and most prominent features of AD and recent developments in the field support an involvement of mitochondrial-dependent mechanisms in the pathogenesis of AD (Swerdlow and Khan, 2004; 2009).

Clinically, AD is characterized by a decline in several cognitive domains such as memory, speech, personality and judgment, vision, association sensory-motor function, and culminates in the death of the individual typically within 3–9 years after diagnosis (Santos et al., 2011).

1.2.2. The interrelation between diabetes mellitus and Alzheimer's disease

The number of studies associating DM with the development of dementia and AD is much higher for T2D than for T1D. Even so, cognitive deficits, such as impaired learning, memory, problem solving, and mental flexibility have been recognized as being more common in T1D subjects than in the general population (Biessels et al., 2008; Ryan et al., 1985) suggesting a negative effect of hyperglycemia and/or hypoinsulinemia on central nervous system (CNS). Noteworthy, degeneration of cerebral cortex (Reske-Nielsen and Lundbaek, 1963), and neuronal loss (DeJong, 1977) are observed at autopsy and are more pronounced in patients suffering from T1D than in age-matched non-diabetic patients. In agreement, the streptozotocin (STZ)-induced animal model of T1D presented decreased exploratory activity, short- and long-term memory impairment and increased depression-like symptoms (Haider et al., 2013). Moreover, it has been demonstrated that the presence of learning deficits are

associated with increased glycogen synthase kinase-3 (GSK3) activity, increased tau protein phosphorylation and increased Aβ protein levels in the brain of a STZ-induced mouse model of T1D (Jolivalt et al., 2008). Similar changes were observed in a rat model of spontaneous T1D (Li et al., 2007). Increased tau protein phosphorylation, which was correlated with decreased activity of protein phosphatase 2A (PP2A), was observed in the brains of the non-obese diabetic (NOD) mice, a genetic model of spontaneous T1D (Papon et al 2013). Interestingly, the STZ-induced T1D in the AβPP transgenic mice exacerbated the accumulation of Aβ and phosphorylated tau protein, which was accompanied by an increase in the activity of GSK3β (Jolivalt et al., 2010). Insulin therapy partially prevented behavioral and biochemical changes observed in the STZ-induced mouse model of T1D (Jolivalt et al., 2008), suggesting that insulin deficiency plays a pathogenic role in the development of AD-like features.

Hoyer and Nitsch (1989) were the first to propose that defective insulin signaling, such as occurs in T2D, contributes to AD pathogenesis. Since then several epidemiological studies have associated diabetes and dementia. Indeed, an early prospective follow-up study positively associated T2D and dementia, particularly vascular dementia and AD (Ott et al., 1999). It has been estimated that T2D is associated with a 1.5-2.5-fold increased risk of dementia (Strachan et al., 2011). Moreover in a 9-years follow up study, T2D has been shown to accelerate the progression from mild cognitive impairment (MCI, a prodromal stage of AD) to dementia by 3.18 years (Xu et al., 2010). In 2005, Suzanne de la Monte and collaborators (2005) introduced the term “type 3 diabetes” in reference to AD. Indeed, AD present several pathogenic features (e.g. insulin

resistance, abnormal glucose metabolism, mitochondrial dysfunction) that overlap with T2D.

Insulin resistance can mechanistically explain, at least in part, the cognitive impairment that occurs in diabetes and AD (Sato et al., 2011). The mRNA levels of insulin, insulin-like growth factor (IGF) and their receptors and downstream signaling are reduced in postmortem AD brains compared with the controls (Rivera et al., 2005; Steen et al., 2005), these alterations being positively correlated with Braak staging (Rivera et al., 2005). Indeed, phosphorylation of insulin receptor substrate 1 (IRS1) at serine residue 616 or 636 is a recognized marker of insulin resistance and is elevated in AD brains (Morino et al., 2008; Fröjdö et al., 2009; Sun and Liu, 2009; Talbot et al., 2012). Interestingly, the same observations were made in the hippocampus and temporal cortex of a non-human primate model of AD, resulting from intracerebroventricular infusions of Aβ oligomers (Bomfim et al., 2012). Insulin signaling inactivates GSK3β, which is a serine/threonine kinase that is constitutively active (Cross et al., 1995). Therefore, it is not surprising that insulin resistance reduces signaling through phosphatidylinositol-3-kinase (PI3K)-Akt pathway, resulting in increased activation of GSK3β and hyperphosphorylation of tau protein (Schubert et al., 2003; 2004; Grunblatt et al., 2007). Likewise, overexpression of GSK3β in the CNS of rodents leads to tau protein hyperphosphorylation and cognitive deficits (Lucas et al., 2001). In contrast, inhibition of GSK3β decreases Aβ production and reduces hyperphosphorylated tau protein-associated neurodegeneration both in vivo and in vitro (Phiel et al., 2003; Noble et al., 2005; Qing et al., 2008; Ly et al., 2013). Strikingly, both diabetes (Kaidanovich and Eldar-Finkelman, 2002; Lee

and Kim, 2007) and AD (Balaraman et al., 2006) are characterized by a dysregulation of GSK3 activity.

Another molecular link between T2D and AD is the insulin-degrading enzyme (IDE). IDE is a metalloprotease with a molecular weight of 110 KDa, being ubiquitously expressed and predominantly localized, at the subcellular level, in the cytosol and peroxisomes (Qiu and Folstein, 2006). IDE primary function is to catabolize insulin, nevertheless it has also been found to degrade Aβ (Vekrellis et al., 2000; Farris et al., 2003). Indeed, due to its higher affinity to IDE, insulin competes with Aβ for the binding to the catalytic site of the enzyme. Therefore, if the insulin levels increase in the brain (i.e. brain hyperinsulinemia), it will affect the degradation of Aβ via IDE, which could cause Aβ accumulation potentiating the occurrence of neurodegenerative events (Qiu and Folstein, 2006). Indeed, a recent study suggested that IDE is the main peptidase involved in the degradation of soluble monomeric Aβ in AD brains (Stargardt et al., 2013). Notably, the degradation capacity by IDE was found to inversely correlate with the Braak stages of sporadic AD, diminishing already during the earliest Braak stages (Stargardt et al., 2013). These results suggest that a decreased IDE degrading capacity of Aβ occurs early in the progression of AD contributing to Aβ deposition.

Abnormal glucose metabolism underscores both T2D and AD, reinforcing the notion that AD may be a particular type of diabetes of the CNS. Brain region-specific alterations in glucose metabolism have been detected in the brains of individuals at risk of developing or suffering from AD (Sims-Robinson et al., 2010). In such cases, reductions in glucose metabolism have been observed in temporal and parietal brain regions (Small et al., 2000). Furthermore, reductions

in glucose metabolism have also been observed in the hippocampus of AD individuals (Garrido et al., 2002). Indeed, tricarboxylic acid (TCA) cycle enzymes such as α-ketoglutarate dehydrogenase, pyruvate dehydrogenase, and isocitrate dehydrogenase exhibit reduced activity in the brains of AD individuals (Ahmad, 2013). Also the animal model of sporadic AD characterized by central insulin resistance, generated by the intracerebroventricular injection of STZ, presents a reduction in the activity of α-ketoglutarate dehydrogenase and pyruvate dehydrogenase (Correia et al., 2013). These studies support the observation that ATP production from glucose is reduced by 50% in early AD (Ahmad, 2013).

Neurons are not capable of store glucose, thus there is the need of a constant supply of glucose through the blood-brain barrier (BBB) by glucose transporters (GLUTS). GLUTS isoforms 1, 3, 4 and 8 are abundant in the brain (Ahmad, 2013). Several studies have shown a decrease in the levels of GLUTS 1 and 3 in the brains of AD patients (Simpson et al., 1994; Harr et al 1995). The decrease in the levels of GLUTS correlates with tau protein hyperphosphorylation (Liu et al., 2008). In parallel, also in the brains of T2D patients a reduction in the levels of neuronal GLUT3 was observed in comparison to the control and AD brain (Liu et al., 2009a). Concomitantly, an increase in the levels of tau protein phosphorylation in the brains of T2D individuals was also observed, predisposing these subjects to the development of AD (Liu et al., 2009a).

As it will be further discussed in sub-chapter 1.4, mitochondria play a decisive role in cell life-death decisions since they regulate a number of processes that include energy production and homeostasis of important second messengers (reactive oxygen species - ROS and calcium - Ca^{2+}). Mitochondrial

dysfunction has been proposed as a pivotal link between diabetes and AD (Moreira et al., 2007). Neurons have limited glycolytic capacity, therefore highly depending on ATP synthesis by mitochondria to survive, rendering them particularly susceptible to disturbances in mitochondrial function (Han et al., 2011). Mitochondrial dysfunctions have been reported to occur in the brains of both T1D and T2D animal models. It has been demonstrated that brain mitochondria isolated from STZ diabetic rats have lower levels of coenzyme Q9 (CoQ9), which is suggestive of an impaired antioxidant system (Moreira et al., 2005). CoQ has been demonstrated to serve the dual functions of an electron carrier/proton translocator in the respiratory chain (Crane, 1989) and an antioxidant by directly scavenging radicals (Takayanagi et al., 1980) or indirectly by regenerating vitamin E (Stoyanovsky et al., 1995). Indeed, sensory neurons and peripheral nerves from animal models of both types of diabetes show increased ROS production, lipid peroxidation, protein nitrosylation, and decreased levels of glutathione (GSH) and ascorbate (Fernyhough et al., 2010). Moreover, it has been shown in brain mitochondria isolated from diabetic STZ rats a reduction in the activity of mitochondrial complexes III, IV and ATP synthase, culminating in decreased ATP synthesis, associated with an increase in oxidative and nitrosative stress (Mastrocola et al., 2005). An age-related decay of the respiratory chain efficiency and an uncoupling of oxidative phosphorylation system in brain mitochondria isolated from Goto-Kakizaki (GK) rats, an animal model of T2D, was also reported (Moreira et al., 2003). In addition, brain mitochondria isolated from 12- and 24-month-old male GK rats were more susceptible to the toxicity of Aβ peptide when compared to mitochondria isolated from age-matched control rats (Moreira et al., 2003). It was recently

showed that 11-month-old sucrose-induced T2D and AD mice present similar brain mitochondrial abnormalities (Carvalho et al., 2012), cognitive defects and vascular anomalies (Carvalho et al., 2013). Interestingly, sucrose-induced T2D mice presented a significant increase in Aβ levels in both cortex and hippocampus (Carvalho et al., 2012; 2013). These observations reinforce the idea that T2D and AD share common features.

Mitochondria have been implicated in the pathogenesis of AD as: 1) triggers of disease (Nunomura et al., 2001; Pratico et al., 2001; Swerdlow and Khan, 2004; Hauptmann et al., 2009; Swerdlow and Khan, 2009); 2) mediators and targets of the harmful effects of Aβ (LaFerla et al., 2007), causing mitochondrial dysfunction and increased ROS production; and 3) potential sites of Aβ production, since AβPP and an active γ-secretase enzymatic complex were found in mitochondrial membranes (Keil et al., 2004; Hansson et al., 2004).

In summary, DM has been pointed out as a risk factor for the development of AD. This notion is supported by the existence of several epidemiological and mechanistic studies that show a close interrelation between both disorders. Indeed, diabetes seems to precipitate the appearance of AD-like features, which may be partly explained by shared mechanisms such as insulin resistance, abnormal glucose metabolism and mitochondrial dysfunction.

1.3. An overview of the role of insulin in the brain and its neuroprotective effects

The classical view that the brain is insulin insensitive has been challenged by several lines of evidence, namely: 1) the presence of insulin and insulin

receptors (IR) in the brain and expression of insulin-sensitive GLUT-4 in neurons (El Messari et al., 2002; Sankar et al., 2002); and 2) the presence of proinsulin I and II mRNA showing that there is a local production of insulin (Devaskar et al., 1993; Schechter et al., 1996). Furthermore, a number of effects of insulin on the brain have been documented, including metabolic, neurotrophic, neuromodulatory, and neuroendocrine actions. The binding of insulin to IR leads to the phosphorylation of several tyrosine residues resulting in receptor autophosphorylation and phosphorylation of intracellular substrates, including IRS and the Src-homology-2-containing protein (Shc) (Fig. 1.2.) (Paz et al., 1996). The phosphorylation of these intracellular substrates initiates two main downstream signaling pathways, PI-3K and mitogen-activated protein kinase (MAPK) pathways (Fig. 1.2.) (Candeias et al., 2012). After PI-3K activation, downstream signaling proteins, such as the serine/threonine kinase protein kinase B (PKB)/Akt, are recruited to the plasma membrane, being then translocated to the cytosol and nucleus causing the phosphorylation of target proteins (e.g. GSK-3β) (Fig. 1.2.) (Lizcano and Alessi, 2002; Kim and Feldman, 2012). The insulin-mediated activation of Akt, protein kinase C, or c-AMP-dependent protein kinase triggers multiple effects via the inactivation of GSK-3β, including synthesis of proteins involved in neuronal glucose metabolism, antiapoptotic mechanisms, and antioxidant defense (Cross et al., 1995; van der Heide et al., 2006). It was also described that overexpression of a constitutively active GSK-3β promotes cell death, while its inhibition prevents apoptosis (Duarte et al., 2012). Other known molecules targeted by PI-3K/Akt signaling are FoxO3, nuclear factor-kB (NF-kB), and cAMP response element-binding protein (CREB). In fact, Akt may also phosphorylate and inhibit FoxO3, preventing the

disruption of mitochondrial membrane potential and cytochrome c release, thus promoting neuronal survival (van der Heide et al., 2006; Cole et al., 2007). NF-kB phosphorylation by Akt has been shown to protect against oxidative stress and apoptosis by increasing Cu/Zn superoxide dismutase (Cu/Zn SOD) mRNA and protein levels (Duarte et al., 2012; Cole et al., 2007). Moreover, Akt-mediated CREB phosphorylation has been involved in the stimulation of neuronal glucose metabolism and enhancement of mitochondrial membrane potential, ATP levels, nicotinamide adenine dinucleotide phosphate (NADPH) redox state, and hexokinase activity (Candeias et al., 2012). The activation of MAPK pathway seems to promote the expression of genes involved in cell and synapse growth as well as in cell repair and maintenance (Li and Holscher, 2007; Kim and Feldman, 2012). Interestingly, several studies suggest a crosstalk between PI-3K/Akt and MAPK pathways that, by converging at Bad phosphorylation, may play an antiapoptotic role (Fang et al., 1999). Thus, both PI-3K/Akt and MAPK pathways appear to underlie both neurotrophic and neuroprotective actions of insulin.

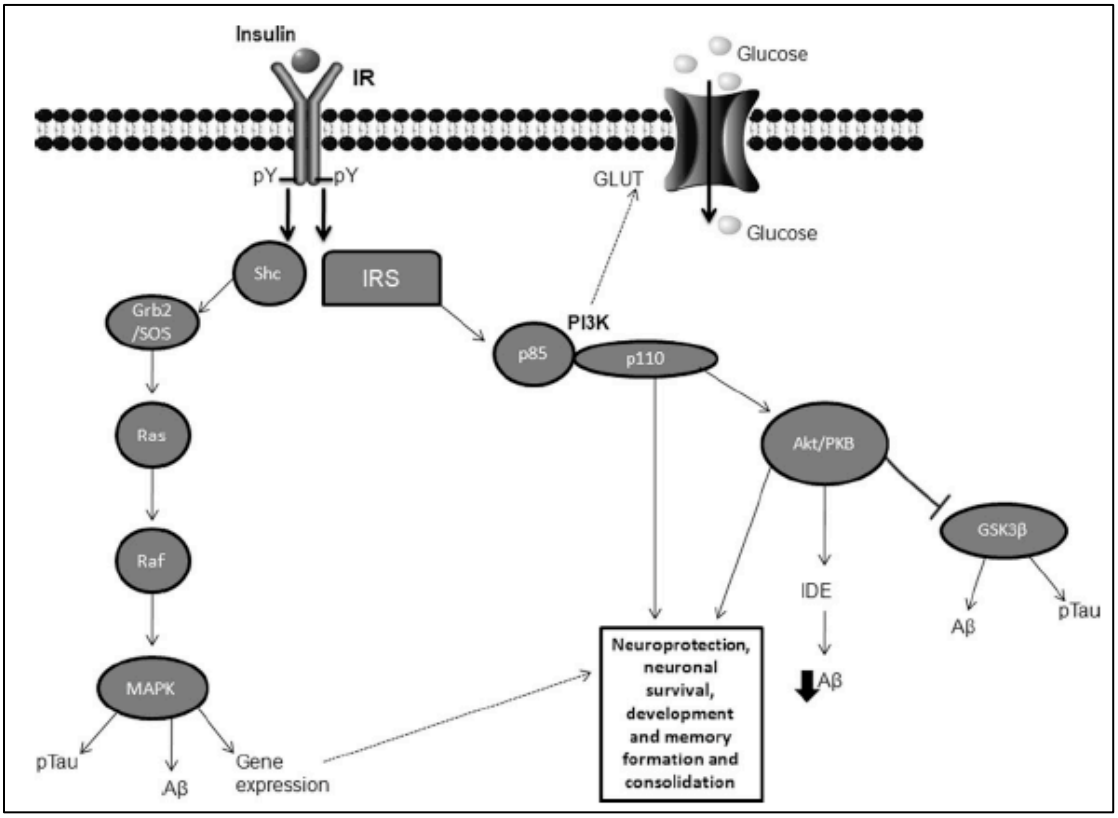


Figure 1.2. – Neuronal insulin signaling pathways. Binding of insulin to extracellular α-subunits of insulin receptors (IRs) induces a conformational change of these receptors, activating tyrosine kinase activity of the β-subunits and promoting receptor autophosphorylation (pY) at several tyrosine residues located inside the cell, thereby recruiting docking proteins (as Shc and IRS) to the cell membrane. Insulin receptor substrate (IRS) phosphorylation, namely at the Src homology 2 (SH2) domain of the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI-3K), stimulates the catalytic subunit p110, with subsequent recruitment of downstream signaling proteins (e.g., Akt). Once activated, Akt phosphorylates target proteins like the proapoptotic factors Bad and caspase-9, glycogen synthase 3-beta (GSK-3β), and insulin-degrading enzyme (IDE). Alternatively, phosphorylated Shc recruits the growth factor receptor binding protein (Grb2)/son of sevenless initiating downstream signals, namely from the Ras-Raf-mitogen-activated protein kinase (MAPK) cascade. Furthermore, Grb2 can be also recruited by IRS, promoting the crosstalk between PI3K/Akt and MAPK. Activation of these pathways may ultimately mediate several biological responses in the central nervous system such as apoptosis inhibition, regulation of gene transcription and glucose metabolism, modulation of neurotransmission, regulation of amyloid β protein (Aβ) clearance, and tau protein phosphorylation (pTau). GLUT, glucose transporters. Adapted from Candeias et al., 2012.

Previous studies showed that insulin improves cognitive performance in healthy and AD individuals, and in experimental animal models of insulin resistance (Kern et al., 2001; Reger et al., 2008; Cardoso et al., 2009). In rodents the intracerebroventricular administration of insulin improves memory for a passive avoidance task (Park et al., 2000). It was further suggested that intrahippocampal injections of insulin could have a dose-dependent effect on spatial learning and memory (Moosavi et al., 2006). Moreover, in humans, euglycemic intravenous infusions of insulin enhance verbal memory (Craft et al., 1996; Craft et al., 1999a; Craft et al., 1999b; Kern et al., 2001; Craft et al., 2003; Watson et al., 2003). However, peripheral insulin administration is not feasible for long-term treatment of memory deficits due to the increased risk of hypoglycemia. In fact, systemic administration of insulin is associated with memory deficits in animals, possibly due to hypoglycemia that occurs when exogenous insulin is not supplemented with glucose to maintain euglycemia (Izumi et al., 2003). Indeed, it has been demonstrated that both acute (Cardoso et al., 2010) and recurrent (Cardoso et al., 2012) insulin-induced hypoglycemia exacerbate diabetic brain mitochondrial dysfunction and oxidative imbalance, which may compromise the function, and integrity of brain cells. Nevertheless, Reger and collaborators (2006) suggested that intranasal administration of insulin provides an effective way to deliver this hormone into the brain, by passing the BBB and circumventing systemic side effects. Elevated insulin levels can be detected in cerebrospinal fluid within 10 minutes after intranasal insulin administration, but plasma insulin and glucose levels remain unchanged (Craft and Watson, 2004). Previous studies have demonstrated the beneficial effects of acute and long-term intranasal administration of regular human insulin (RH-I)

on declarative memory in humans (Craft and Watson, 2004; Benedict et al., 2004). Benedict and co-workers (2007) also reported that the beneficial effects of intranasal insulin treatment in memory deficits in humans can be enhanced by using insulin aspart (ASP-I). ASP-I enhanced efficiency when compared to RH-I can be explained by its reduced tendency to form hexamers facilitating its absorption and therefore its action (Benedict et al., 2007). Recently, in a randomized, double-blind, placebo-controlled trial with AD and MCI patients, intranasal delivery of insulin has been proven to have beneficial effects in cognition and in AD biochemical marker levels in the CSF (Craft et al., 2012). Regarding animal studies, intranasal insulin has also been demonstrated to ameliorate tau protein hyperphosphorylation in a rat model of T2D (Yang et al., 2013).

1.4. Mitochondria

Mitochondrial function is far from solely being energy production, due to the coupling of the electron transport chain (ETC) and the phosphorylative system. In fact, mitochondria are also involved in the production and buffering of second messengers and are the master regulators of the apoptotic cell death pathway. Mitochondrial physiology and function are highly dependent on mitochondrial dynamics and biogenesis.

1.4.1. Mitochondrial functions and oxidative stress

Mitochondria account for more than 90% of the cellular energetic production (Chance et al., 1979). Mitochondrial energy production assumes its maximum importance in the brain since neurons, which have a high energy demand, have a limited glycolytic capacity making them greatly dependent on aerobic oxidative phosphorylation (oxphos) (Moreira et al., 2010a). Mitochondria generate a proton gradient as electrons flow through the ETC, from donors with lower redox potentials to acceptors with higher redox potentials. During the flux of electrons across the mitochondrial respiratory chain, the mitochondrial complexes I, III, and IV pump protons across the inner mitochondrial membrane to the intermembrane space generating potential energy that drives the phosphorylation of ADP to ATP by the FoF1-ATP synthase (mitochondrial complex V) (Nelson and Cox, 2004; Scheffler, 2008).

Despite the high efficiency of mitochondrial respiration, 0.4-4.0% of all oxygen consumed is converted to superoxide anion ($O_2^{\bullet -}$), a short-lived free radical that results from the electron leak mostly in mitochondrial complexes I and III (Shigenaga et al., 1994; Fridovich, 1995; Droge, 2002; Evans et al., 2002; Carreras et al., 2004; Balaban et al., 2005). $O_2^{\bullet -}$ can be converted/dismutated to nonradical derivatives such as hydrogen peroxide (H_2O_2) either spontaneously or catalyzed by superoxide dismutase (SOD) (Weisiger and Fridovich, 1973a; Weisiger and Fridovich, 1973b; Turrens, 2003). H_2O_2 is relatively stable and membrane permeable. It diffuses within the cell and can be breakdown by cytosolic antioxidant systems such as catalase, glutathione peroxidase, and thioredoxin (TRX) peroxidase (Holmgren, 2000;Nordberg and Arner, 2001). If the excess of ROS is not properly neutralized, it can induce lipid peroxidation,

and protein and DNA oxidation processes. In the presence of nitric oxide ($NO^{\bullet}$), $O_2^{\bullet-}$ generates peroxynitrite ($ONOO^-$) that can react with proteins interfering with their function and/or can be decomposed in nitrogen dioxide radical ($NO^{\bullet}_2$), which further reacts with tyrosine residues, resulting in 3-nitrotyrosine formation (Hillet al., 2010).

As by-products of mitochondrial respiration, ROS assume a role as signaling messengers or harmful oxidizing agents depending on their rate of production, and the imbalance between ROS production and scavenging. ROS dual role reflects on the activation of cytoprotective pathways when low to moderate levels are present, while higher levels of ROS exacerbate oxidative stress and activate pro-death pathways.

The role of ROS as physiological signals is increasingly recognized, as for instance in chemotaxis, stem cell proliferation, neurogenesis and circadian rhythm (Dickinson et al., 2011). Correia et al. (2012c) recently demonstrated that moderate levels of mitochondrial ROS protected brain endothelial and neuron-like cells against glucotoxicity. Others have elegantly demonstrated that the responsiveness of the insulin signaling pathway is diametrically opposed depending on the levels of ROS, further identifying C-Jun N-terminal kinase (JNK) and phosphatases, such as protein tyrosine phosphatase (PTP) 1B, as candidate molecules responsible for the switch between the opposite effects (Iwakamiet al., 2011). Whereas low levels of ROS inhibit PTP1B activity, high levels of ROS stimulate JNK activity (Iwakami et al., 2011). Among the ROS effectors, PTP, TRX and peroxiredoxin (PRX) family proteins possess special domains/motifs where cysteine residues, which are easily modified by oxidation, directly sense and respond to fluctuations in ROS levels (Miki and Funato, 2012).

Oxidative stress can be concomitantly considered cause and consequence of mitochondrial dysfunction, as mitochondria are both generators and targets of ROS (Murphy, 2009). When ROS interact with mitochondrial DNA, they can lead to mutations, rearrangements, and transcriptional errors that impair important mitochondrial components leading to more oxidative stress and, eventually, cell death (Facecchia et al., 2011; Santos et al., 2013).

ROS also modulate mitochondrial Ca^{2+} homeostasis and apoptotic pathways by controlling the opening of permeability transition pore (PTP), which enables the release of some small pro-apoptotic proteins, such as cytochrome c and apoptosis-inducing factor (AIF) to the cytosol (Hengartner, 2000;Tayloret al., 2008). Although the exact nature of the PTP is uncertain, it is known that cyclophilin D (CypD), a protein with a regulatory role in the pore, senses the oxidative status (Wallace and Fan, 2009).

As buffers of cytoplasmic Ca^{2+}, mitochondria play a key role in normal neurotransmission, short- and long-term plasticity, excitotoxicity, and regulation of gene transcription, processes that are highly dependent on Ca^{2+} levels (Zimmermann, 1990; Rizzuto et al., 1999; Zucker, 1999; Rizzuto et al., 2000; Soderling, 2000; Sabatini et al., 2001; Wojda et al., 2008; Celsi et al., 2009). Ca^{2+} is internalized into mitochondria via the Ca^{2+} uniporter, a protein that is still to be fully identified and biochemically characterized. Nevertheless, a candidate protein, which was named MCU (from ‘‘mitochondrial Ca^{2+} uniporter’’), proved to be essential for high-capacity Ca^{2+} transport into mitochondria in a number of in vitro and in vivo experimental models (Baughman et al., 2011; De Stefani et al., 2011). On the other hand, Ca^{2+} release is mediated by Na^{+}/Ca^{2+} or H^{+}/Ca^{2+} exchangers (Wojda et al., 2008). It was shown that mitochondria are involved in

cells' Ca^{2+} buffering impairment, a situation that occurs in the aging brain and AD (Buchholz et al., 2007; Camandola and Mattson, 2011).

1.4.2. Mitochondrial fission/fusion

A group of GTPases mediates the processes of mitochondrial fission and fusion however, the mechanisms by which they govern those processes remain to be completely elucidated. Dynamin-like protein 1 (DLP1, also referred as DRP1) and Fis1 are fission-related proteins (Fig. 1.3.) (Su et al., 2010a; 2010b). DRP1 is a member of the conserved dynamin large GTPase superfamily that controls membrane fission, existing constitutively in a cytosolic pool and being recruited to the mitochondrial membrane where it is often detected as a pattern of punctated spots. The putative mechanistic action of DRP1 on mitochondrial membrane relies on the formation of a ring-like complex structure within the mitochondrial surface that constricts the organelle upon the hydrolysis of GTP, initiating fission (Smirnova et al., 2001). Fis1 is a mitochondrial outer membrane protein suggested to act as a receptor for DRP1 (James et al., 2003). However, this is controversial since two recent studies revealed that DRP1 interact with two factors present in the mitochondrial outer membrane, mitochondrial fission factor (MFF) (Otera et al., 2010) and mitochondrial elongation factor 1 (MIEF1) (Zhao et al., 2011). Most interestingly, while MFF/DRP1 interaction induces mitochondrial fission (Gandre-Babbe and van der Bliek, 2008; Otera et al., 2010), MIEF1/DLP1 interaction prevents mitochondrial fission due to the inhibition of DRP1 activity mediated by MIEF1 (Zhao et al., 2011).

Regarding the proteins involved in the process of mitochondrial fusion, three large GTPase proteins assume different functions and ultrastructural

locations. The fusion of the outer membrane is mediated by two mitofusins – Mfn1 and Mfn2 – that interact by their coiled-coil domains, forming homo- and hetero-oligomeric complexes, thus connecting the mitochondrial outer membranes of neighboring mitochondria (Fig. 1.3.) (Zuchner et al., 2004; Chen et al., 2005; Ishihara et al., 2006). However, the inner mitochondrial membrane also needs to be fused, and OPA1, being an inner membrane protein that faces the intermembrane space, is implicated in this event, requiring Mfn1, but not Mfn2, to mediate this process (Fig. 1.3.) (Cipolat et al., 2004; Chen et al., 2005).

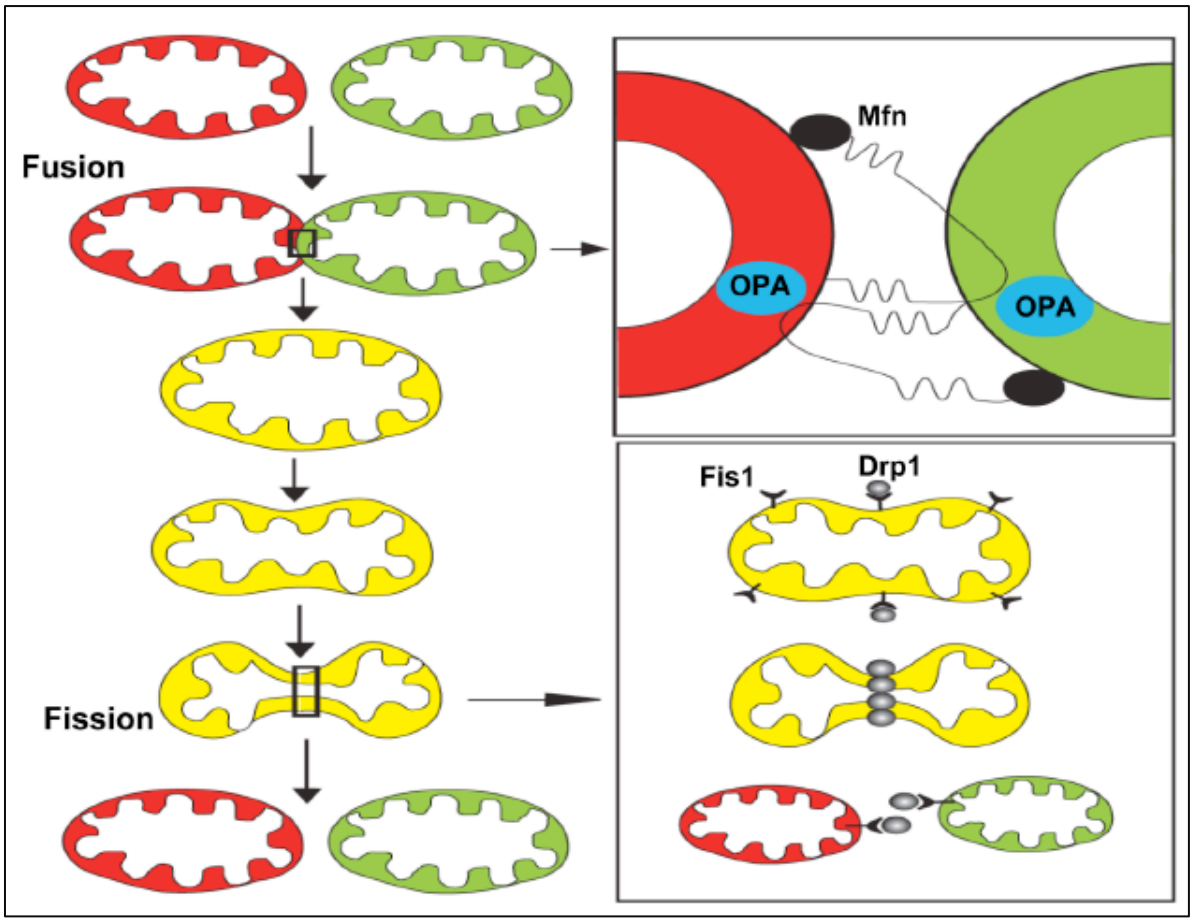


Figure 1.3. – Mechanisms of mitochondrial fusion and fission. Mitochondrial outer membrane fusion (upper part of the image) is mediated by homo- and hetero-oligomers formed between mitofusins 1 (Mfn1) and 2 (Mfn2). Fusion of the mitochondrial inner membrane (upper part of the image) is mediated by optic atrophy 1 (OPA1). Mitochondrial fission (lower part of the image) involves the recruitment of dynamin-related protein 1 (DRP1) to discrete foci in the

mitochondrial surface and also requires Fis1, a protein located in the outer mitochondrial membrane. Adapted from Mattson et al., 2008.

Mitochondria divide and fuse in response to several stimuli (Chan, 2006a; Knott et al., 2008) however, the precise mechanisms controlling these events are largely unclear. Some studies have examined post-translational modifications of mitochondrial dynamics-related proteins such as DRP1 and OPA1. DRP1 is known to undergo post-translational modifications such as phosphorylation (Chang and Blackstone, 2007; Cribbs and Strack, 2007;Taguchi et al., 2007), ubiquitinylation (Nakamura et al., 2006), s-nitrosylation (Cho et al., 2009), and sumoylation (Harder et al., 2004). Whereas phosphorylation at Ser616 (Chang and Blackstone, 2007; Cribbs and Strack, 2007; Taguchi et al., 2007), sumoylation (Harder et al., 2004), and s-nitrosylation are known to potentiate mitochondrial fission, ubiquitinylation (Nakamura et al., 2006) decreases the rate of mitochondrial fission.

For mitochondrial fusion to occur, the proteolytic cleavage of OPA1 into long and short isoforms is critical (Song et al., 2007). The machinery that cleaves OPA1 is not completely clear nevertheless, several proteases of the inner mitochondrial membrane have been associated with its processing (Cipolat et al., 2004;Ishihara et al., 2006; Griparic et al., 2007; Song et al., 2007; Ehses et al., 2009). However, low mitochondrial ATP levels, the dissipation of the membrane potential across the inner membrane ($\Delta\Psi_m$), or apoptotic stimuli (Griparic et al., 2007) induce OPA1 cleavage resulting in the loss of long isoforms and impairing mitochondrial fusion (Ishihara et al., 2006; Duvezin-Caubet et al., 2006; Baricault et al., 2007; Guillery et al., 2008).

Mitochondrial dynamics is critical for maintaining various mitochondrial functions: fusion-deficient cells demonstrate greatly reduced endogenous and uncoupled respiratory rates and demonstrate reversible interorganellar heterogeneity in $\Delta\Psi_m$ and inhibition of cell growth (Chen et al., 2003; Chen et al., 2005). Fission deficiency also causes a reduced rate of mitochondrial ATP synthesis due to a significant decrease in complex IV activity and an inefficient oxphos system (Benard et al., 2007). On the other hand it has been demonstrated that the maintenance of mitochondrial fusion was correlated with increased levels of dimerization and activity of ATP synthase, and ATP production (Gomes et al., 2011).

Excessive mitochondrial fission is also correlated with increased ROS production (Yu et al., 2006; Wang et al., 2008; Wang et al., 2009). Likewise, it has been reported that ROS are overproduced along with mitochondrial fragmentation when neuronal cells are exposed to hyperglycemic conditions (Yu et al., 2006). The genetic inhibition of mitochondrial fission by the dominant negative mutant form of DRP1 has been shown to prevent ROS production in hyperglycemic conditions, suggesting that mitochondrial fragmentation plays a critical role in ROS overproduction and oxidative imbalance (Yu et al., 2006). It has also been demonstrated that mitochondrial ETC complex inhibitors such as rotenone, 1-methyl-4-phenylpyridinium, and 3-nitropropionic acid cause fragmentation of the mitochondrial network along with increased ROS production (Benard et al., 2007; Liot et al., 2009; Wang et al., 2011). Antioxidants can only partially alleviate ETC complex inhibitor-induced mitochondrial fragmentation (Liot et al., 2009; Wang et al., 2011), while inhibition of mitochondrial fission significantly reduces ROS overproduction and block

mitochondrial/cellular dysfunction (Wang et al., 2011), further suggesting that mitochondrial morphological dynamics are essential for the maintenance of ROS balance.

A fragmented mitochondrial network is less efficient in mitochondrial Ca^{2+} uptake and intramitochondrial Ca^{2+} diffusion, and the formation of a mitochondrial network facilitates Ca^{2+} propagation within interconnected mitochondria suggesting that the balance of mitochondrial fission/fusion can significantly impact cellular Ca^{2+} ion homeostasis (Frieden et al., 2004; Szabadkai et al., 2004). Mitochondrial fragmentation is also an early event during apoptosis preceding cytochrome c release and caspases activation (Frank et al., 2001).

1.4.3. Mitochondrial biogenesis

Mitochondria cannot be made *de novo* (Arduíno et al., 2011). Several paradigmatic processes are involved in the formation of new mitochondria, called mitochondrial biogenesis. These include the synthesis, import, and incorporation of proteins and lipids to the existing mitochondrial reticulum, as well as the replication of the mtDNA (Arduíno et al., 2011). Indeed, not all the protein components involved in the assembly of these organelles are locally encoded and synthesized; thereby the coordination between the transcription of nuclear and mitochondrial genes is crucial. Mitochondrial genome only encodes 13 proteins, 22 transfer RNAs (tRNAs) and 2 ribossomal RNAs (rRNAs) required for the translation within the mitochondrial matrix (Taanman, 1999; Wallace, 2005). Thus, the vast majority of mitochondrial proteins are nuclear-encoded as is the case of the protein subunits that comprise the five inner membrane complexes of the ETC and oxphos system (Fig. 1.4.) (Scarpulla, 2008). The

involvement of two distinct genomes compels for an elaborate coordination of gene expression that involves a coordinate machinery of transcription factors. The machinery underlying mitochondrial biogenesis is mainly constituted by the nuclear respiratory factor 1 (NRF 1) and nuclear respiratory factor 2 (NRF 2), which control the nuclear genes that encode mitochondrial proteins, and mitochondrial transcription factor A (TFAM) that drives transcription and replication of mtDNA. The expression of NRF 1, NRF 2, and TFAM is regulated by the peroxisome proliferator activator receptor gamma-coactivator 1α (PGC-1α) (Correia et al., 2012b).

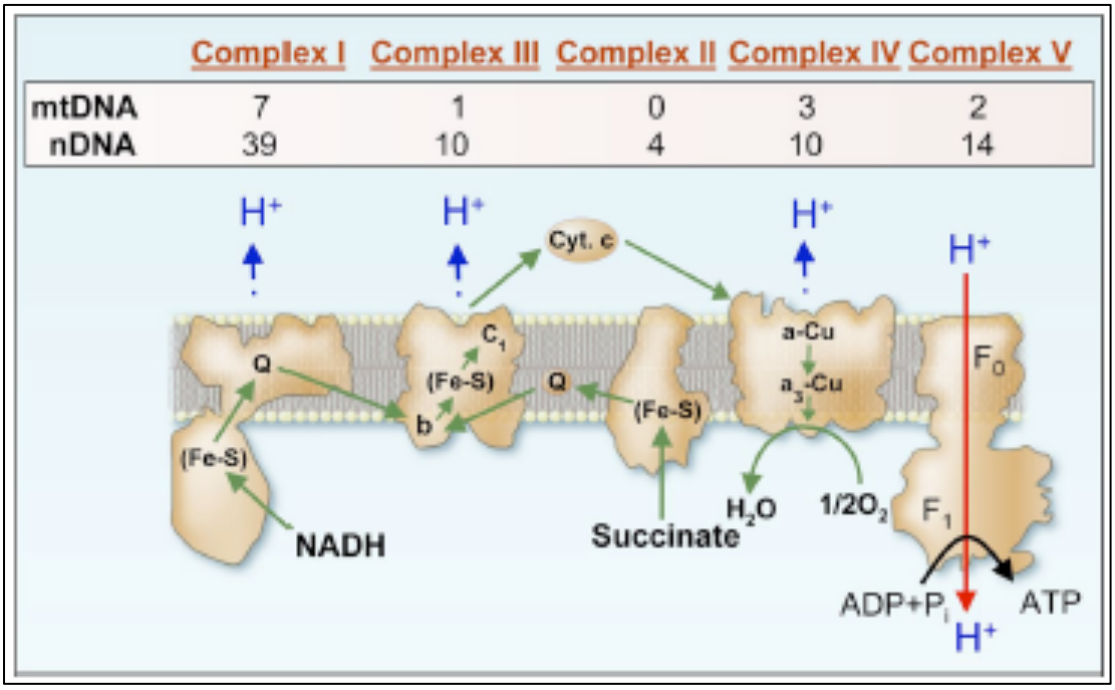


Figure 1.4. – Schematic representation of the five mitochondrial respiratory complexes in the inner mitochondrial membrane and the dissociable electron carriers cytochrome c (Cyt. c) and coenzyme Q (CoQ). Green arrows show the electron flow through the mitochondrial complexes. Blue arrows show the proton (H^+) pumping from the matrix to the intermembrane space. The red arrow shows the flow of protons through mitochondrial complex V (FoF1-ATP synthase) from the intermembrane space to the mitochondrial matrix with consequent production of adenosine triphosphate (ATP). It is indicated the number of subunits for each complex that are nuclear (nDNA) and mitochondrial (mtDNA) encoded. NADH: nicotinamide

adenine dinucleotide; ADP: adenosine diphosphate; Pi: inorganic phosphate. Adapted from Scarpulla, 2008.

1.5. Autophagy

Macroautophagy (hereafter referred to as autophagy) is an evolutionarily conserved housekeeping process within the cells, in which a double-membrane vesicle engulfs a portion of cytoplasmic content and ultimately fuses with a lysosome in order to expose its content to the action of hydrolases (Kundu and Thompson, 2008). Autophagy enables cells to get nutrients through the digestion of their own components and, at the same time, degrades misfolded proteins, damaged organelles and invading microorganisms (Mizushima et al., 2008). Since Christian De Duve, who coined the term autophagy in the CIBA Foundation Symposium on Lysosomes in 1963 (Klionsky, 2007), this process has received growing attention. Several breakthroughs emerged from the scientific literature, namely the elucidation of the molecular basis of autophagy and the knowledge that this process rather than being a random mechanism of degradation of cellular constituents, it degrades them selectively (Klionsky, 2007). Indeed, it was only in the early 1990s that genetic screenings performed in yeast mutants allowed the identification of several autophagy-related (Atg) genes essential in different stages of the assembly of the autophagic vacuoles (AVs) or autophagosomes (Tsukada and Ohsumi, 1993; Thumm et al., 1994).

The formation of the autophagic vesicle goes through a progressive process involving several steps such as regulation and nucleation, in which a signal triggers the formation of an isolation membrane that engulfs dysfunctional organelles and/or misfolded proteins in a first structure called phagophore.

When the membrane edges of the phagophore fuse the autophagosome is formed (Chu, 2006; Cherra and Chu, 2008). The newly formed autophagosome undergoes a maturation process, which follows in three stages, dissolution of the inner membrane, fusion with lysosomes and acidification, cargo degradation and release of macromolecules (Chu, 2006; Cherra and Chu, 2008).

Since autophagy is a tightly regulated process involving a complex cascade of signal transduction, it provides many possible targets for its modulation. The autophagic process is typically activated during nutrient deprivation (Fig. 1.5.), becoming the major source of several basic macromolecules. Several other natural and pharmacological modulators of autophagy are known such as glucagon, insulin, rapamycin and 3-methyladenine (Fig. 1.5.) (Cuervo, 2004).

The overall process is activated by a stress signal triggering the vesicle nucleation that is the first step on the autophagosome formation (Yue et al., 2009). At this stage two kinases assume importance, the serine/threonine protein kinase target of rapamycin (TOR) and the class III phosphatidylinositol 3-kinase (PI3K) complex (Fig. 1.5.). TOR is an evolutionarily conserved kinase involved in the initial steps of the signal transduction, acting upstream the Atg proteins. Autophagy is inhibited upon stimulation of TOR; conversely, the inhibition of TOR stimulates autophagy (Pattingre et al., 2008). When TOR is normally active, it phosphorylates Atg13 blocking its ability to associate with Atg1, the yeast homologue of the mammalian unc-51-like kinase 1 (ULK1) (Fig. 1.5.). Atg13 is a regulatory subunit of the Atg1 complex (Kamada et al., 2000). Atg1 and ULK1 are essential for autophagosome formation in Drosophila and mammalian cells, respectively (Scott et al., 2004; Young et al., 2006). It is known

that ULK1 acts downstream of TOR in mammalian cells (Chan et al., 2007), although little is known about this signaling pathway.

PI3K is a key regulator at the nucleation stage of the AVs formation. This enzymatic complex contains three highly conserved proteins, the protein kinase vacuolar protein sorting 15 (Vps15), the phosphatidylinositol 3-kinase Vps34 and Beclin 1/Atg6 (Fig. 1.5.) (Todde et al., 2009). The role of the PI3K enzymatic complex in the assembly of the AVs is not well understood. However, since PI3K is involved in phosphatidylinositol 3-phosphate (PI3P) formation, it presumably enables the recruitment of Atg proteins to the membrane because these proteins bind to this phospholipid (Xie and Klonsky, 2007; Todde et al., 2009). Beclin 1 is the mammalian homologue of Atg6 and is part of a multiprotein complex acting as a platform for the recruitment of activators or repressors of Beclin 1/Vps34-dependent autophagy (Pattingre et al., 2008).

As discussed above, the activity of the PI3K complex is required for the recruitment of Atg proteins. Indeed, those proteins are part of two evolutionarily conserved ubiquitin-like conjugation systems and are essential for the vesicle elongation and vesicle completion processes in both yeast and mammals (Kundu and Thompson, 2008). Atg5/Atg12 and Atg16 are one of the conjugation systems and the other is composed by lipidated LC3, the mammalian homologue of yeast Atg8. The first conjugation system requires the activity of the ubiquitin-like conjugating enzymes Atg7 and Atg10 to enable the covalent linking of Atg5 to Atg12. The second conjugation system requires the activity of the ubiquitin-like conjugating enzymes Atg7 and Atg3 followed by a LC3 modifying protease (Atg4) to convert LC3-I to LC3-II enabling its binding to phosphatidylethanolamine (PE) in the membrane (Fig. 1.5.) (Reggiori, 2006). LC3-PE associates with both

surfaces of the double membrane of the autophagosome, unlike the complexes of Atg5/Atg12 and Atg16 that are found only on the cytosolic surface. However, both complexes are critical for membrane expansion. Despite the relevance of the proteins Atg5 and Atg7 in the autophagic process, a recent work performed by Nishida and co-workers (2009) demonstrated the existence of an alternative process independent of Atg5/Atg7 in which the lipidation of LC3 to form LC3-II (a marker widely used as an indicator of autophagy) does not occur. Additionally, the same authors found that this alternative process of autophagy is regulated by several autophagic proteins including ULK1 and Beclin1 (Nishida et al., 2009).

The final step of the autophagic process involves the fusion of the autophagosome with the lysosome (autolysosome), exposing the content of the AVs to the action of hydrolases. Two families of proteins are critical for the fusion between the autophagosome and the lysosome; the soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) and Rab proteins (specifically Rab7) (Jager et al., 2004; Eskelinen, 2005).

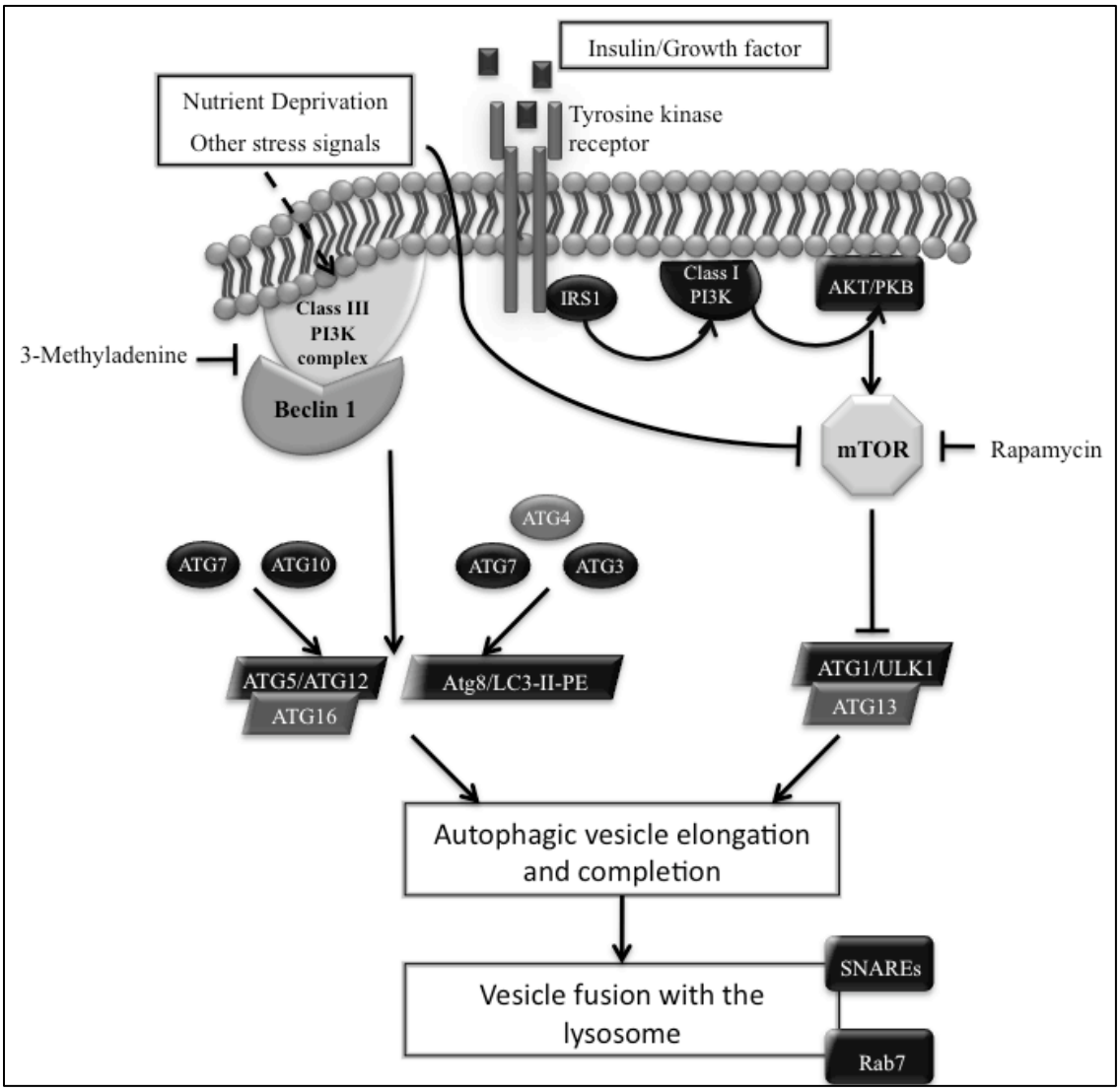


Figure 1.5. - Autophagy is triggered by a number of stress signals: nutrient deprivation, insulin and other growth factors. When the tyrosine kinase receptor is activated, insulin receptor substrate 1 (IRS1) is phosphorylated followed by the activation of a class I phosphatidylinositol 3-kinase (PI3K) that subsequently activates AKT/protein kinase B (PKB) and the mammalian target of rapamycin (TOR). The activation of TOR inhibits autophagy through the inhibition of a functional group of autophagy-related (Atg) proteins, phosphorylating Atg13 and disabling it to associate to Atg1, the yeast homologue of the mammalian ULK1. Other functional branch of autophagy proteins involves the activation of a class III PI3K, to generate phosphatidylinositol-3-phosphate (PI3P). The activation of this multiprotein enzyme depends on the formation of a complex with Beclin1 and leads to the activation of two functional Atg protein groups. The first group involves the conjugation of Atg12 to Atg5 and Atg16, with the help of the E1-like enzyme Atg7 and the E2-like enzyme Atg10. The second group involves the conjugation of LC3 (LC3 is

one of the mammalian homologues of Atg8) to phosphatidylethanolamine (PE) by the sequential action of the protease Atg4, the E1-like enzyme Atg7 and the E2-like enzyme Atg3. The final step involves the action of soluble NSF attachment protein receptors (SNAREs) and Rab proteins, particularly Rab7. Adapted from Santos et al., 2010b.

CHAPTER 2

Objectives

2.1. Objectives

Mitochondria are key organelles controlling a number of processes that ultimately determine cells' fate. Mitochondria were once seen has bean-shaped and isolated organelles however, currently, mitochondria are known to form a reticulum with the capability of fuse and divide and move within the cell. These events are crucial to maintain mitochondrial homeostatic processes such as energy production, generation of physiological levels of ROS and buffering of cytosolic Ca^{2+}. Furthermore, mitochondria have a life cycle controlled by mitochondrial biogenesis and mitophagy (the specific autophagic degradation of mitochondria).

Evidence shows that diabetes precipitates neurodegenerative events, being considered an important risk factor for vascular dementia and AD. Furthermore, it is known that mitochondrial alterations play a key role in diabetes as well as in several neurodegenerative diseases. Although it is known that mitochondrial dysfunction is involved in diabetes-associated complications, the full spectrum of mitochondrial alterations that occur in the diabetic brain, from early to later stages of the disease, remains unclear.

This work was mainly devoted to evaluate the alterations occurring in mitochondrial parameters considered essential for the maintenance of a healthy mitochondrial population in the brains of T2D and T1D rats. The reason for the use of two different animal models of diabetes relates to the fact that the two types of diabetes are etiologically different which may yield divergent observations in light of the parameters evaluated. To closely mimic humans suffering from T1D, who need exogenous insulin administration to survive, and

to evaluate the potential protective effects of insulin in the brain, we also performed experiments in T1D rats treated with insulin.

We started by evaluating brain mitochondrial function, fission/fusion and biogenesis and autophagy in 6-month-old male GK and Wistar control rats (Chapter 4). GK rats are a nonobese animal model of T2D mainly characterized by mild hyperglycemia and insulin resistance. At this age, GK rats are considered a model of prediabetes or early stage of T2D.

In Chapter 5, we performed experiments in male STZ-induced T1D rats (3 months of diabetes duration) treated or not with insulin during the last month. Vehicle-injected rats were used as control group. Several parameters were evaluated: mitochondrial function, fission/fusion and biogenesis, autophagy, tau protein phosphorylation state, cell death and synaptic integrity.

CHAPTER 3

Materials and Methods

3.1.Reagents

Streptozotocin (STZ), bacterial protease from Bacillus licheniformis - type VIII, bovine serum albumin (BSA), phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT) and Tween 20 were obtained from Sigma Aldrich (St. Louis, MO, USA). Insulin (Humulin NPH) was obtained from Eli Lilly and Company (USA). Digitonin was obtained from Calbiochem. Polyvinylidene difluoride (PVDF) Immobilon-P membranes were obtained from Millipore (Billerica, MA, USA). Enhanced chemifluorescence (ECF) reagent was obtained from Amersham Biosciences (Little Chalfont, UK). All the other chemicals used were of the highest grade of purity commercially available.

3.2. Animal models housing and treatment

3.2.1. Animal model of T2D

The GK rat is a nonobese, spontaneously T2D animal (Moreira et al., 2003) produced by selective breeding of glucose intolerant Wistar rats and first characterized by Goto and Kakizaki (Goto and Kakizaki, 1981). GK rats exhibit moderate but stable fasting hyperglycemia evident from 6 weeks of age (Ferreira et al., 1999). GK and respective Wistar control rats were obtained from our animal colony (Laboratory Research Center, Faculty of Medicine, University of Coimbra) and maintained under controlled light (12 h day/night cycle) and humidity with *ad libitum* access to water and powdered rodent chow. Following procedures approved by the Federation of European Laboratory Animal Science Associations (FELASA), fasted 6-month-old animals were sacrificed by

decapitation.

3.2.2. Animal model of T1D

2-month-old male Wistar rats purchased from Charles River were housed in our animal facility (Laboratory Research Center, Faculty of Medicine and Center for Neuroscience and Cell Biology, University of Coimbra) and maintained under controlled light (12 h day/night cycle) and humidity with *ad libitum* access to water and powdered rodent chow (except in the fasting period).

The STZ animal model of T1D is characterized by the ablation of insulin production due to the selective destruction of the pancreatic beta cells followed by STZ administration that results in severe hyperglycemia (Wei et al., 2003). Before STZ administration, rats were deprived of food overnight and randomly divided into two groups. One group received an intraperitoneal (i.p.) injection of STZ (50 mg/kg body weight) freshly dissolved in 100 mM citrate, pH 4.5. The volume administered was always 0.5 ml/200 g body weight. The control group received an i.p. injection with an equal volume of citrate (vehicle). In the following 24 h, animals were provided with free access to glycosylated serum in order to avoid hypoglycemia resulting from the massive destruction of pancreatic β-cells and consequent release of intracellular insulin associated with STZ treatment (Moreira et al., 2005). 3 days after STZ administration, the tail vein blood glucose levels were measured in all animals and those presenting levels above 250 mg/dl were considered diabetic. After 3 months of the induction of diabetes, and following procedures approved by FELASA, Wistar controls and STZ-induced diabetic rats were sacrificed by decapitation.

3.2.3. Insulin-treated T1D animals

Two months after the induction of diabetes with STZ, diabetic rats were randomly divided into two groups and one group of animals was subjected to daily subcutaneous (s.c.) injections of INS, in order to lower the systemic levels of glucose (dose adjusted to blood glucose levels as follows: if blood glucose levels ≤ 200 mg/dL, 2U INS were administered to rats; if blood glucose levels > 200 mg/dL an extra 2U INS per each 100 mg/dL blood glucose were given to rats), during one month. After insulin treatment period, rats were sacrificed by decapitation.

3.3. Measurement of biochemical parameters

Blood glucose was determined immediately after sacrifice by a glucose oxidase reaction, using a glucometer (Glucometer-Elite, Bayer). Hemoglobin A1C (HbA1c) levels were determined using Systems SYNCHRON CX 4 (Beckman). This system utilizes two cartridges, Hb and A1c to determine A1c concentration as a percentage of the total Hb. The hemoglobin was measured by a colorimetric method and the A1c concentration by a turbidimetric immunoinhibition method.

3.4. Measurement of brain insulin, glucose and pyruvate levels

Brain cortical tissues were homogenized in radioimmunoprecipitation assay (RIPA) buffer containing 0.1 M phenylmethylsulfonyl fluoride (PMSF), 0.2

M dithiothreitol (DTT), and protease and phosphatase inhibitors (commercial protease and phosphatase inhibitor cocktails from Roche Applied Science). The crude homogenate was incubated on ice for 15 minutes, frozen and defrozen 3 times to favor disruption, centrifuged at 14000 rpm (Eppendorf centrifuge 5415C) for 10 minutes, at 4°C, and the resulting supernatant collected and stored at -80°C.

INS quantification was performed using an ELISA kit (BertinPharma, France) according to manufacturers' instructions. The principle of the kit is based on the competition between unlabeled rat insulin and acetylcholinesterase (AChE) linked to rat insulin (tracer) for limited specific Guinea-Pig anti-rat insulin antiserum sites.

Glucose quantification was performed using a PicoProbe™ glucose fluorometric assay kit (BioVision, USA) according to manufacturers' instructions. The principle of the kit is based on the enzymatic oxidation of D-glucose to form a product which reacts with a colorless probe to generate fluorescence. The fluorescence generated is directly proportional to the amount of glucose present in the sample.

Pyruvate quantification was performed using a pyruvate colorimetric assay kit (BioVision, USA) according to manufacturers' instructions. The principle of the kit is based on the enzymatic oxidation of pyruvate by pyruvate oxidase to generate color upon reaction with a pyruvate probe. The color intensity is proportional to pyruvate content; therefore the pyruvate concentration can be accurately measured.

3.5. Preparation of mitochondrial fraction

Brain cortical mitochondria were isolated from rats by the method of Moreira et al. (2001), using 0.02% digitonin to allow the release of mitochondria from the synaptosomal fraction. Briefly, after animal decapitation, the cortex was immediately separated 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 5 mg of the bacterial protease (Sigma). Single brain homogenates were brought to 30 ml and then centrifuged at 2500 rpm (Sorvall Evolution RC Superspeed Refrigerated Centrifuge) for 5 minutes. The resulting supernatant was then centrifuged at 10000 rpm for 10 minutes. 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 for 10 minutes. The brown mitochondrial pellet without the synaptosomal layer was resuspended again in 10 ml of medium and centrifuged at 10000 rpm for 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 for 5 minutes. The final mitochondrial pellet was resuspended in the washing medium and the protein amount determined by the biuret method calibrated with bovine serum albumin (BSA)(Gornall et al., 1949).

3.6. Measurement of mitochondrial respiration

Oxygen consumption of 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 standard respiratory 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. State 3 of respiration (consumption of oxygen in the presence of substrate and ADP) was initiated with ADP (50 nmol/mg protein). States 3 and 4 (consumption of oxygen after ADP phosphorylation) of respiration, respiratory control ratio (RCR=state 3/state 4), and ADP/O index (a marker of the mitochondrial ability to couple oxygen consumption to ADP phosphorylation during state 3 of respiration) were determined according to Chance and Williams (Chance and Williams, 1956).

3.7. Measurement of mitochondrial transmembrane potential

The transmembrane potential ($\Delta\Psi_m$) 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 Linear 1200 recorder. The voltage response of the TPP^+ electrode to log[TPP^+] was linear with a slope of 59±1, which is 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 (100 mM sucrose, 100 mM KCl, 2 mM KH_2PO_4, 5 mM Hepes and 10 μM EGTA; pH 7.4) 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 Gunter, 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 (substrate of mitochondrial complex II) in the presence of 2 μM rotenone (inhibitor of mitochondrial complex I) in order to activate the mitochondrial electron transport chain. After a steady-state distribution of TPP^+ had been reached (ca. 1 min of recording), $\Delta\Psi_m$ fluctuations were recorded.

3.8. Protein extraction for Western blot analysis

Brain cortical tissue was homogenized in RIPA buffer containing 0.1 M PMSF, 0.2 M DTT, and protease and phosphatase inhibitors (commercial protease and phosphatase inhibitor cocktails from Roche Applied Science). The crude homogenate was incubated on ice for 15 minutes, frozen and defrozen 3 times to favor disruption, centrifuged at 14000 rpm (Eppendorf centrifuge 5415C) for 10 minutes, at 4°C, and the resulting supernatant collected and stored at -80°C. The amount of protein content in the samples was analyzed by the bicinchoninic acid (BCA) protein assay using the BCA kit (Pierce Thermo Fisher Scientific, Rockford, IL).

3.9. Western blot analysis

The samples (50-75 µg per lane) were resolved by electrophoresis in 8-15% sodium dodecyl sulfate (SDS)–polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes. Non-specific binding was blocked by gently agitating the membranes in 5% non-fat milk or 5% BSA for phosphoproteins and 0.05% Tween in Tris-buffered saline (TBS) for 1 hour at room temperature. The blots were subsequently incubated with specific primary antibodies, overnight at 4 °C, with gentle agitation. Blots were washed three times (3x15 minutes), with Tris buffer containing 0.05% Tween (TBS-T) and then were incubated with secondary antibodies for 1 hour 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).

In some cases, the same membrane was used to detect other proteins with very different molecular weights. In these situations, the membranes were gently washed with 40% methanol (30 minutes) and 3 times (15 minutes) with TBS-T and then incubated with the primary antibody. The following steps are identical to those described above.

Total and respective phosphorylated protein levels were examined in two distinct membranes. To preserve data accuracy the following procedures were adopted: 1) the same sample was used to analyze total and phosphorylated protein levels; 2) all samples had the same cycles of freezing and thawing (reduced to a minimum); 3) the two gels were prepared and ran at the same time; 4) after proteins have been transferred onto PVDF membranes and stained with specific antibodies, each band of interest were normalized with respect to the loading control (actin); 5) the ratio between phosphorylated and total protein levels was obtained by the formula (phosphorylated protein/actin membrane 1)/(total protein/actin membrane 2).

The antibodies used in our studies are listed in table 3.1.

Table 3.1. – List of primary and secondary antibodies used in the western blots (alphabetically ordered).

Antibody	Catalog No.	Supplier	Host Specie	Dilution
Anti-Beclin1	612113	BD Biosciences	Mouse	1:1000
Anti-DRP1	611113	BD Biosciences	Mouse	1:1000
Anti-ERK	9102	Cell Signaling	Rabbit	1:1000
Anti-Fis1	IMG-5113A	Imgenex	Rabbit	1:750
Anti-Goat IgG alkaline phosphatase conjugate	sc-2771	Santa Cruz Biotechnology	Rabbit	1:2500
Anti-GSK3β	sc-81462	Santa Cruz Biotechnology	Mouse	1:500
Anti-JNK	9258	Cell Signaling	Rabbit	1:1000
Anti-LC3	L7543	Sigma Aldrich	Rabbit	1:1000
Anti-Mfn1 (H-65)	sc-50330	Santa Cruz Biotechnology	Rabbit	1:1000
Anti-Mfn2	sc-100560	Santa Cruz Biotechnology	Mouse	1:1000
Anti-Mouse IgG alkaline phosphatase conjugate	NIF1316	Amersham Pharmacia Biotech	Goat	1:10000
Anti-mTOR	4517	Cell Signaling	Mouse	1:1000
Anti-ND1	sc-20493	Santa Cruz Biotechnology	Goat	1:500
Anti-NRF1	sc-33771	Santa Cruz Biotechnology	Rabbit	1:500
Anti-NRF2	ab31163	Abcam	Rabbit	1:500
Anti-OPA1	612607	BD Biosciences	Mouse	1:1000
Anti-p38 MAPK	9212	Cell Signaling	Rabbit	1:1000
Anti-p62	P0067	Sigma Aldrich	Rabbit	1:1000
Anti-PSD95	3450	Cell Signaling	Rabbit	1:1000
Anti-Pser2448 mTOR	2971	Cell Signaling	Rabbit	1:1000

Anti-Pser396 Tau	sc-101815	Santa Cruz Biotechnology	Rabbit	1:1000
Anti-Pser616 DRP1	4494	Cell Signaling	Rabbit	1:1000
Anti-Pser9 GSK3β	9336	Cell Signaling	Rabbit	1:1000
Anti-Pthr180/182 p38 MAPK	9216	Cell Signaling	Mouse	1:1000
Anti-Pthr181 Tau	sc-101816	Santa Cruz Biotechnology	Rabbit	1:250
Anti-Pthr183/tyr185 JNK	9255S	Cell Signaling	Mouse	1:2000
Anti-Pthr202/tyr204 ERK	4377	Cell Signaling	Rabbit	1:1000
Anti-Ptyr216 GSK3β	sc-135653	Santa Cruz Biotechnology	Rabbit	1:500
Anti-Rabbit IgG alkaline phosphatase conjugate	NIF1317	Amersham Pharmacia Biotech	Goat	1:10000
Anti-Synaptophysin	S5768	Sigma Aldrich	Mouse	1:20000
Anti-Tau (BT2)	MN1010	Thermo Scientific	Mouse	1:1000
Anti-TFAM	sc-23588	Santa Cruz Biotechnology	Goat	1:1000
Anti-α-Tubulin	2125	Cell Signaling	Rabbit	1:1000
Anti-β-Actin	A5441	Sigma Aldrich	Mouse	1:5000

3.10. Measurement of phosphatase 2A activity

Phosphatase 2A (PP2A) activity was measured using a PP2A DuoSet IC kit (R&D Systems) according to the manufacturer's instructions. Briefly, a portion of brain tissue was homogenized in ice cold lysis buffer (Cell Signaling) and 75 µg of protein were loaded onto 96-well plates coated with a capture antibody specific for PP2A (R&D Systems) for immunocapture at 4 °C for 3 h. After washing twice, synthetic phosphopeptide substrates (200 µM) were added for the dephosphorylation reaction catalyzed by PP2A. The level of free phosphate was determined by a sensitive dye-binding assay using malachite green and molybdic

acid according to the manufacturer's instructions followed by measurement of the absorbance at 620 nm.

3.11. Measurement of caspases 3 and 9 activation

Caspases 3 and 9 activation was measured using a colorimetric method. Brain cortical tissues were homogenized in cold RIPA buffer and frozen and defrozen three times. The lysates were centrifuged for 10 minutes at 14000 rpm (5417R, Eppendorf) at 4°C. The resulting supernatant was stored at −80 °C. Protein concentrations were measured by using the BCA protein assay kit (Pierce, Rockford, IL).

Samples (50 μg of protein) were incubated at 37 °C for 2 h in 25 mM Hepes, pH 7.5, containing 0.1% 3-[(3-cholamido-propyl) dimethylammonio]-1-propanesulfonate (CHAPS), 10% sucrose, 2 mM DTT, and40 μM Ac-DEVD-pNA for caspase 3 or 40μM Ac-LEHD-pNA for caspase 9. Caspase 3- and caspase 9-like activity was determined by measuring substrate cleavage at 405 nm in a microplate reader (SpectraMax Plus 384, Molecular Devices).

3.12.Total DNA extraction

Total DNA was extracted from brain cortical tissue using the TRIzol Reagent (Sigma-Aldrich) according to the manufacturer's instructions.

3.13. Determination of mtDNA Copy Number

Real-time polymerase chain reaction (qPCR) analysis was performed to determine mtDNA copy number in brain cortical samples, as described by Fuke and collaborators (2011), with slight modifications. Relative quantification of mtDNA levels was determined by the ratio of the mitochondrial ND1 (mt-Nd1) gene to the single-copy, nuclear-encoded beta-2-microglobulin (β2MG) gene. Reactions were carried out in an iQ5 system (Bio-Rad), and efficiency of the reactions was determined for the selected primers using serial dilutions of DNA samples. Primer concentration and annealing temperature were optimized, and the specificity of the amplicons was determined by melting curve analysis. The reactions mixture consisted of Maxima SYBR Green qPCR Master Mix (Fermentas), sense and antisense primers (see Table 3.2. for details), and 20 ng of DNA. Each reaction was run in triplicate to calculate relative mtDNA copy number. Control (Ct) values of all samples were within the linear range. Ct value differences were used to quantify mtDNA copy number relative to the beta-2-microglobulin gene with the following equation: Relative copy number = $2^{\Delta Ct}$, where ΔCt is $Ct_{\beta 2MG}$ -Ct_{ND1}.

Table 3.2. – Oligonucleotides and cycling conditions for qPCR amplification of ND1 and β-2-microglobulin.

Gene	Sequence (5'- 3')	AT (ºC)	Amplificon Size (bp)	C
ND1	Sense: GAG CCC TAC GAG CCG TTG CC Antisense: GCG AATG GTC CTG CGG CGT A	58	271	30
β2MG	Sense: GCG TGG GAG GAG CAT CAG GG Antisense: CTC ATC ACC ACC CCG GGG ACT	58	264	30

Abbreviations: AT - annealing temperature; C - Number of cycles of amplification

3.14. Statistical analysis

Data presented in Chapter 4 are presented as mean ± SEM of the indicated number of animals. Statistical significance was determined using the unpaired two-tailed student t-test.

Data presented in Chapter 5 and regarding the characterization of the experimental animal groups and mitochondrial function parameters are presented as mean ± SEM of the indicated number of animals and differences between groups were analyzed using one-way ANOVA followed by Tukey's post-hoc test. All other data presented in Chapter 5 are expressed as median ± interquartile range of the indicated number of animals and statistical significance was determined using the non-parametric test of Kruskal-Wallis followed by the posthoc Dunn's multiple comparison test.

CHAPTER 4

Mitochondrial quality control systems sustain brain mitochondrial bioenergetics in early stages of type 2 diabetes

4.1. Abstract

Mitochondria have a crucial role in the supply of energy to the brain. Mitochondrial alterations can lead to detrimental consequences on the function of brain cells and are thought to have a pivotal role in the pathogenesis of several neurological disorders. This study was aimed to evaluate mitochondrial function, fusion-fission and biogenesis and autophagy in brain cortex of 6-month-old Goto-Kakizaki (GK) rats, an animal model of non-obese type 2 diabetes (T2D). No statistically significant alterations were observed in mitochondrial respiratory chain and oxidative phosphorylation system. A significant decrease in the protein levels of optic atrophy 1 (OPA1), a protein that facilitates mitochondrial fusion, was observed in brain cortex of GK rats. Furthermore, a significant decrease in the protein levels of LC3-II and a significant increase in protein levels of mTOR phosphorylated at serine residue 2448 was observed in GK rats suggesting a suppression of autophagy in diabetic brain cortex. No significant alterations were observed in the parameters related to mitochondrial biogenesis. Altogether, these results demonstrate that during the early stages of T2D brain mitochondrial function is maintained in part due to a delicate balance between mitochondrial fusion-fission and biogenesis and autophagy. However, future studies are warranted to evaluate the role of mitochondrial quality control pathways in late stages of T2D.

4.2. Introduction

Diabetes mellitus has become a worldwide epidemic with a substantial social and economic burden (Lam and LeRoith, 2012). As a matter of fact the prevalence of this disorder is rising dramatically, being estimated that 370 million people worldwide will be suffering from diabetes in 2030 (Wild et al., 2004). Based on its etiology and clinical presentation, diabetes is mainly categorized in type 1 (T1D) or type 2 (T2D) diabetes (Umegaki, 2012). T2D is the most prevalent form of diabetes and is marked by chronic hyperglycemia as a consequence of insulin resistance and partial insulin deficiency (Umegaki, 2012). Amongst the wide range of chronic complications associated with diabetes, brain degenerative events, cognitive deterioration and dementia have assumed a pivotal importance in the last decades (J et al., 2009). Particularly, diabetes is considered a major risk factor for the development of Alzheimer's and Parkinson's diseases (Correia et al., 2012a; Santiago and Potashkin, 2013). While the mechanistic link between diabetes and neurodegeneration remains enigmatic, scientific advances revealed that mitochondria could be in the basis of this interrelation (Carvalho et al., 2012; Correia et al., 2012a; Santiago and Potashkin, 2013). In fact, mitochondria take center stage in the brain since neurons have a limited glycolytic capacity, making them highly dependent on aerobic oxidative phosphorylation to fulfill their energetic requirements (Santos et al., 2010a). Aside from their role in ATP generation, these organelles are also involved in the homeostasis of important cellular second messengers such as reactive oxygen species (ROS) and Ca^{2+} as well as in the control of apoptotic cell death (Santos et al., 2010a).

To ensure the maintenance of a healthy mitochondrial population, brain cells are equipped with mitochondrial quality control systems to regulate mitochondrial shape, function and mass. These mitochondrial quality control systems include, but are not restricted to mitochondrial fusion-fission, biogenesis and autophagy (Zhu et al., 2013). The frequent and balanced cycles of fusion and fission are crucial for neurons, given their unique architecture and special energy and Ca^{2+}-buffering requirements at the synapse (DuBoff et al., 2013). The core molecular machinery involved in mitochondrial fusion includes optic atrophy 1 (OPA1) and mitofusins 1 and 2 (Mfn1 and Mfn2, respectively). Whereas OPA1 can be found and is responsible for mitochondrial inner membrane fusion, Mfn1 and Mfn2 are found and are responsible for mitochondrial outer membrane fusion (Chan, 2006b). On the other hand, dynamin-related protein 1 (DRP1) and fission protein 1 (Fis1) are involved in mitochondrial fission (Chan, 2006b). Degradation of mitochondria through autophagy, usually termed as mitophagy, constitutes an important quality control process that degrades damaged/dysfunctional mitochondria (Twig et al., 2008a).

So far, the role of mitochondrial quality control mechanisms has not been investigated in the early stages of diabetic brain. Taking into account that the maintenance of a healthy mitochondrial pool is crucial for normal brain functioning, the present study was aimed to uncover the impact of moderate T2D on brain cortical mitochondrial function, fission-fusion and biogenesis and autophagy. For that, several parameters were determined in the brains of 6-month-old Goto-Kakizaki (GK) rats: respiration chain function, oxidative phosphorylation system, mitochondrial fusion-fission proteins levels,

mitochondrial mtDNA copy number, protein levels of mitochondrial biogenesis-related transcription factors and markers of autophagy.

4.3. Results

4.3.1. Characterization of the experimental animals

As shown in Table 4.1, GK rats presented a significant increase in HbA1c and postprandial glucose levels when compared to the respective control rats, which confirms the diabetic state of this animal model. Additionally, a marked reduction in body weight was also detected in GK rats (Table 4.1). No alterations in brain weight were observed (Table 4.1).

Table 4.1. – Characterization of the experimental animals.

	Body weight (g)	Brain weight (g)	Glycemia (mg/dL)	HbA1c (%)
Wistar	461.8 ± 39.46	2.07 ± 0.081	85.20 ± 6.41	3.6 ± 0.04
GK	357.4 ± 17.47 *	2.04 ± 0.192	208.20 ± 14.17 ***	5.9 ± 0.34 ***

Data are the mean ± SEM of 5 animals from each condition studied. Statistical significance: *p<0.05 when compared to the respective wistar control rats; ***p<0.001 when compared to the respective wistar control rats.

4.3.2. T2D does not affect mitochondrial bioenergetics in brain cortex

In a first approach to investigate the impact of T2D on mitochondrial bioenergetic function, mitochondrial respiration and oxidative phosphorylation

were evaluated in freshly isolated brain cortical mitochondria energized with succinate, a complex II substrate. Our results revealed that mitochondrial coupling and oxidative phosphorylation efficiency given by the RCR and ADP/O indexes, respectively, remained unaltered in brain cortical mitochondria from GK rats when compared to the respective control mitochondria (Table 4.2).

Mitochondrial membrane potential ($\Delta\Psi_m$) is generated through the respiratory chain pumping of protons from the mitochondrial matrix to the intermembrane space. The proton gradient originates an electrochemical potential (Δp) resulting in a pH (ΔpH) and a voltage gradient ($\Delta\Psi_m$) across the inner membrane. Lag phase represents the time necessary to mitochondria phosphorylate the exogenous ADP into ATP. As shown in Table 4.2, no statistical differences were found in the oxidative phosphorylation system parameters ($\Delta\Psi_m$, ADP-induced depolarization and lag phase) of brain cortical mitochondria from GK rats when compared to the respective control mitochondria.

Table 4.2. – Effect of type 2 diabetes (GK rats) in brain cortical mitochondrial respiratory and oxidative phosphorylation parameters.

		Wistar	GK
Respiratory parameters	RCR	3.53 ± 0.567	2.673 ± 0.162
	ADP/O (nmol ADP/nAtgO/min/mg)	1.52 ± 0.389	1.57 ± 0.350
Membrane potential and oxidative phosphorylation	$\Delta\Psi_m$ (-mV)	171.90 ± 4.654	174.20 ± 0.726
	ADP-induced depolarization (-mV)	18.59 ± 0.339	19.27 ± 2.690
	Lag phase (min)	1.57 ± 0.118	1.83 ± 0.300

Mitochondrial respiration and oxidative phosphorylation system parameters were evaluated in freshly isolated brain cortical mitochondria (0.5 mg) in 1 ml of the reaction medium supplemented with 3μM TPP^+ and energized with 5 mM succinate in the presence of 2 μM rotenone. Data are the mean ± SEM of 3-4 animals from each condition studied.

4.3.3. Mitochondrial fission prevails in the early stages of T2D

Maintenance of mitochondrial functionality in response to metabolic stress requires fission and fusion of these dynamic organelles. Therefore, we next analyzed by Western blot the levels of mitochondrial fusion (Mfn1, Mfn2 and

OPA1) and fission-related proteins (Fis1 and DRP1). Interestingly, a significant decrease in OPA1 protein levels was observed in the brain cortex of GK rats when compared with the respective control rats (Fig. 4.1C), whereas the levels of Mnf1 (Fig. 4.1A) and Mnf2 (Fig. 4.1B) proteins remained unchanged. Concerning the mitochondrial fission-related proteins, our results revealed that the protein levels of Fis1 (Fig.4.2A) and DRP1 phosphorylated at serine residue 616 (Fig. 4.2B) remained statistically unchanged in the brain cortices of GK rats when compared with the respective control rats.

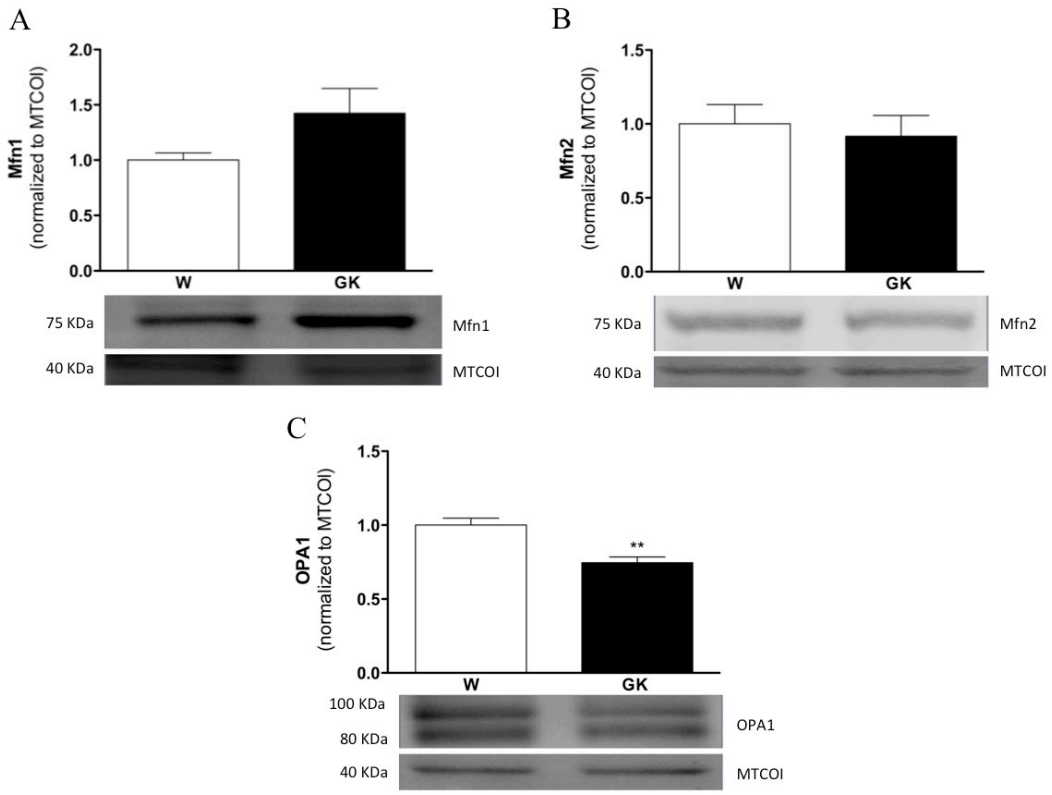


Figure 4.1. – Effect of T2D in the levels of mitochondrial fusion-related proteins: Mfn1 (A), Mfn2 (B) and OPA1 (C). Data are the mean ± SEM of 8 GK (and control) animals. Statistical significance: **p<0.01 when compared to the respective control rats.

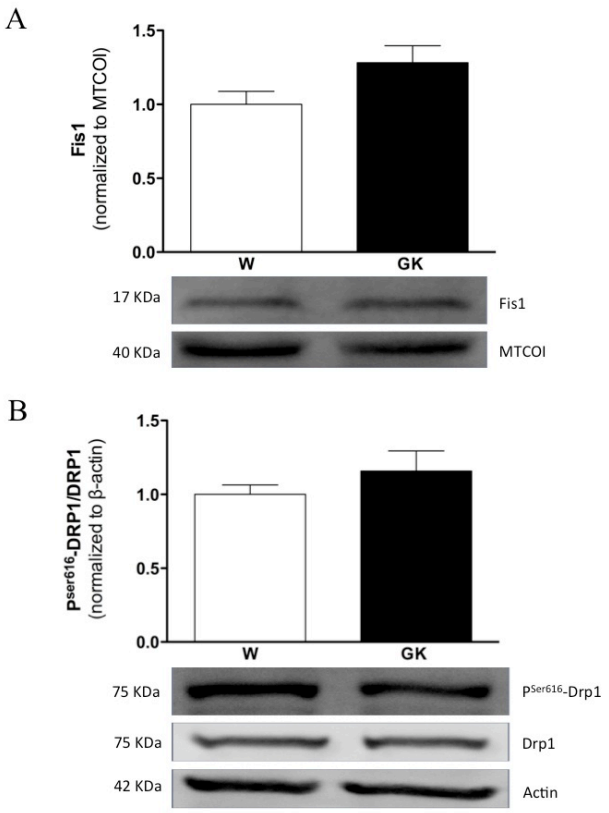


Figure 4.2. – Effect of T2D in the levels of mitochondrial fission-related proteins: Fis1 (A) and pser616-DRP1/DRP1 (B). Data are the mean ± SEM of 8 GK (and control) animals.

4.3.4. Autophagy is decreased in the early stages of T2D

Autophagy is an essential pro-survival response to stress conditions (e.g. nutrient limitation) that allows the maintenance of cellular homeostasis by promoting the timely turnover of long-lived proteins and organelles, including mitochondria. However, under certain circumstances, autophagy can also trigger a cell death program (Santos et al., 2010b). Given this duality of autophagy and in an effort to dissect how autophagy behaves under diabetic conditions, the next step of this study was to determine the levels of some key proteins (mTOR,

Beclin 1, p62 and LC3) associated to the autophagic process. Briefly, mTOR and Beclin 1 exert opposite effects on the regulation of autophagy. While mTOR acts a potent repressor, Beclin 1 functions as an inducer of the autophagic pathway. The autophagic substrate p62 binds directly to LC3, which is known to be a classic marker of autophagy and a participant in autophagosomal membrane elongation (Santos et al., 2010b). A significant decrease in LC3-II protein levels (Fig. 4.3C) was detected in the brain cortex of GK rats when compared to the respective control rats. Accordingly, GK rats exhibited a significant increase in the active form of mTOR ($p^{ser2448}$mTOR/mTOR) (Fig. 4.3A). Meanwhile, beclin 1 (Fig. 4.3B), and p62 (Fig. 4.3D) proteins levels remained unchanged in the brain cortex of GK rats. Overall, these findings suggest that in early stages of T2D autophagy is decreased in the brain cortex.

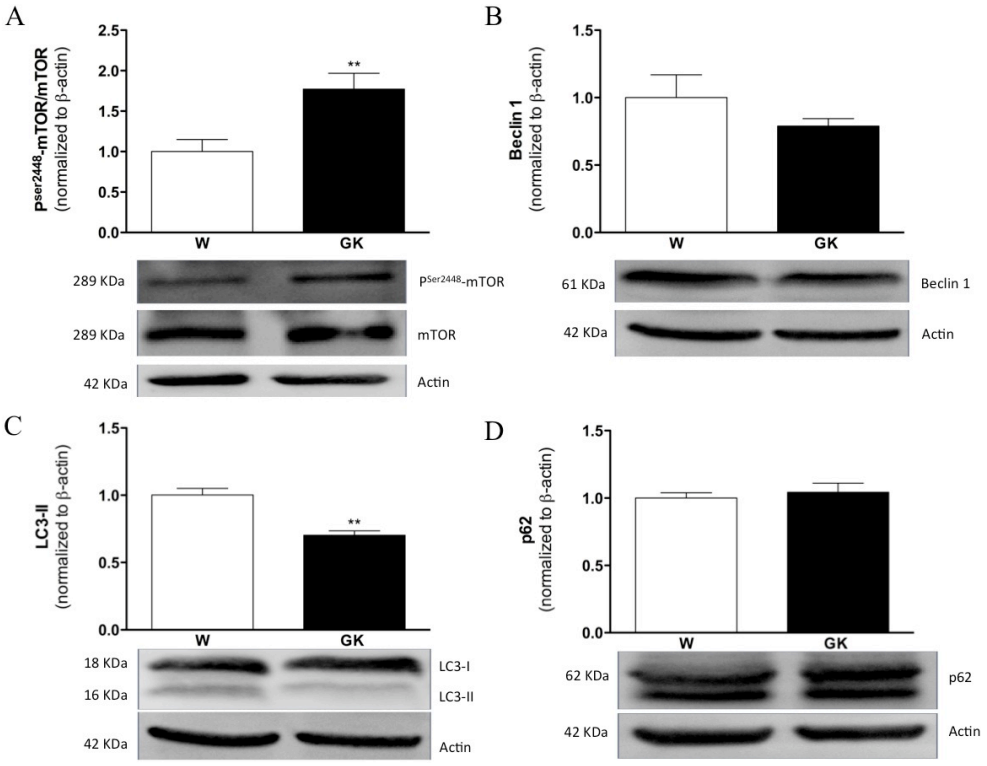


Figure 4.3. – Effect of T2D in the levels of autophagy-related proteins: $p^{ser2448}$-mTOR/mTOR (A), Beclin1 (B), LC3-II (C), p62 (D). Data are the mean ± SEM of 8

GK (and control) animals. Statistical significance: **p<0.01 when compared to the respective control rats.

4.3.5. Mitochondrial biogenesis is maintained in early stages of T2D

Preservation of normal mitochondrial function is known to depend, in part, on mitochondrial biogenesis. In this context, real-time PCR was used to quantify the copy number ratio of mtDNA to nuclear DNA (nDNA) in brain cortex samples. No significant alterations were observed in mtDNA copy number (Fig. 4.4A). Also the protein levels of a ND1, a mitochondrial subunit coded by mtDNA, (Fig. 4.4B), and nuclear respiratory factor 1 (NRF1) and mitochondrial transcription factor A (TFAM), two transcription factors involved in mitochondrial biogenesis, remained statistically unchanged in the brains of GK rats when compared to the controls (Fig. 4.4C and 4.4D).

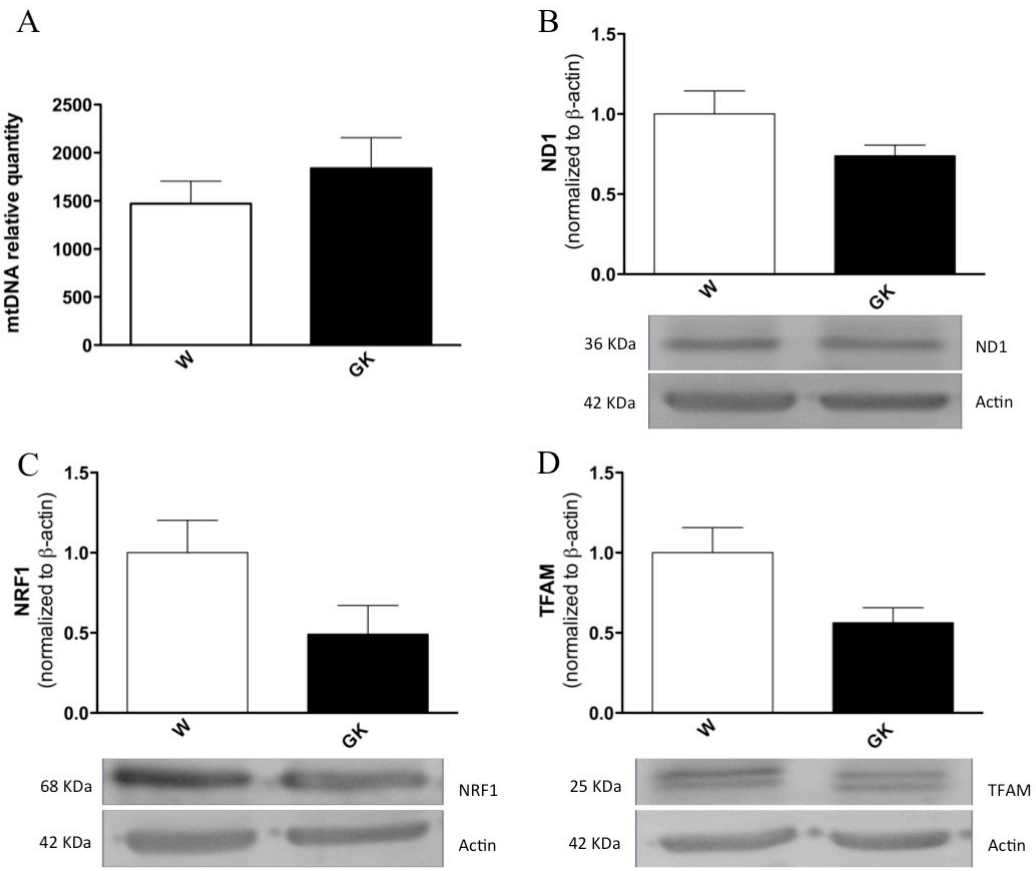


Figure 4.4. – Effect of T2D in (A) mtDNA copy number, and protein levels of (B) ND1, a mitochondrial subunit coded by mtDNA, and (C) NRF1, and (D) TFAM, two transcription factors involved in mitochondrial biogenesis. Data are the mean ± SEM of 5 animals.

4.4. Discussion

Although the molecular basis underlying cognitive deterioration and neurodegeneration in diabetes mellitus remains elusive, it has been attributed a central role to mitochondrial dysfunction (Correia et al., 2012a). Due to the characteristics of GK rats (non-obese, mild hyperglycemia and insulin resistance) the present study extends our knowledge on the role of quality control systems in the maintenance of mitochondrial bioenergetics in the early stages of T2D. Particularly, this study revealed that in the early stages of T2D: 1) mitochondrial fission prevails; 2) mitochondrial biogenesis is maintained; and 3) autophagy decreases. Collectively, these alterations seem to constitute an adaptive metabolic strategy to preserve mitochondrial bioenergetic function and restrict brain cells damage.

Within the brain, mitochondria regulate synaptic transmission, neurotransmitter recycling, dendritic and axonal transport, ion channels, and ion pump activity, which are processes with high energetic requirements (Kann and Kovacs, 2007). In this sense, mitochondrial bioenergetic failure has been pinpointed as a mechanistic event underlying brain malfunction and neurodegeneration in diabetes mellitus (Correia et al., 2012a). Using isolated brain cortical mitochondria from 6-month-old GK rats, this study revealed that

neither respiratory chain function nor oxidative phosphorylation efficiency was compromised in early stages of T2D-like pathology (Table 4.2). An age-related impairment of the respiratory chain and an uncoupling of oxidative phosphorylation were documented in brain mitochondria isolated from 12- and 24-month GK rats (Moreira et al., 2003). So, it is tempting to speculate that in young T2D animals compensatory mechanisms exist involving mitochondrial quality control systems. Integration of mitochondrial fusion-fission, biogenesis and autophagy forms a surveillance mechanism to maintain a healthy mitochondrial population (Twig et al., 2008a; Westermann, 2010). Mitochondria form a dynamic, interconnected network within a cell, and frequent cycles of fusion and fission are crucial for mitochondrial respiration, mitochondrial trafficking and distribution, formation and function of synapses and dendritic spines, and mitochondrial quality control, particularly in post-mitotic cells such as neurons (Detmer and Chan, 2007; Chen and Chan, 2009). A significant decrease in OPA1 protein levels was observed in the brain cortex of GK rats (Fig. 4.1C). This observation points for a remodeling of brain mitochondrial network in T2D, where the balance of mitochondrial fusion-fission events seems to be shifted towards fission. The first evidence of an altered mitochondrial structure in “diabetic neurons” was provided by Schmeichel et al. (2003). Subsequent studies revealed that dorsal root ganglia (DRG) neurons from a T1D animal model or DGR neurons exposed to high glucose levels exhibit smaller mitochondria as a consequence of an excessive mitochondrial fragmentation/fission (Leinninger et al., 2006; Edwards et al., 2010; Vincent et al., 2010). Remarkably, Leinninger and collaborators (2006) sophisticatedly proposed that early mitochondrial fission promoted by the up-regulation of

DRP1 represents a protective or metabolic fission in order to cope with hyperglycemia. However, in a later stage excessive mitochondrial fission is associated with the activation of Bim and Bax, culminating in apoptosis (Leinninger et al., 2006). More recently, it was also found an increase in the levels of mitochondrial fission protein DRP1 in cultured DRG neurons made insulin resistant by chronic exposure to hyperinsulinemic conditions (Kim et al., 2011). Under hyperglycemic conditions, mitochondrial fission is also increased in pancreatic-β cells, hepatocytes, skeletal muscle cells, and endothelial cells, promoting the disruption of mitochondrial network and accumulation of small mitochondria (Yoon et al., 2011).

Mitochondrial fission is involved in mitochondrial motility and efficient dissemination of energy across cell structure – metabolic fission (Cagalinec et al., 2013). Distressing fission or fusion mechanisms either by inhibiting expression of the fission protein DRP1 or by overexpressing the fusion protein Mfn1 has been shown to prevent mitochondria from distributing to synapses due to defective mitochondrial trafficking, leading to the loss of mitochondria from dendritic spines and, consequently, to a reduction of synapse formation (Santel et al., 2003; Tondera et al., 2005; Verstreken et al., 2005; Hoppins et al., 2007). Cardoso and collaborators (Cardoso et al., 2012) have previously reported that synaptic integrity, given by the protein levels of synaptophysin, is preserved in the brain cortex of STZ-treated rats, a model of T1D. The maintenance of synaptic integrity was also documented in animal models of T2D (Belanger et al., 2004). Thereby, we hypothesize that mitochondrial fission could somehow facilitate the recruitment and transport of mitochondria to critical subcellular compartments with high energy demand, such as synaptic terminals, where these organelles

remain stationary and preserve synaptic and neuronal function and integrity, in part by supplying ATP.

Mitochondrial fission together with autophagy form a surveillance mechanism to maintain a pool of healthy mitochondria by isolating and targeting damaged organelles for removal (Liesa and Shirihai, 2013). Autophagy assumes particular importance in post-mitotic cells, such as neurons, since it constitutes a way of "cleaning" cells from metabolic debris (Terman et al., 2010). During the last decade, autophagy has received increasing attention due to its involvement in various aspects of cell physiology, especially cell survival and maintenance of energy homeostasis under conditions of nutrient or energy deprivation-starvation (Mizushima, 2005). However, autophagy can also trigger cell death, underscoring its nature as a double-edged sword that could be either protective or injurious depending on the cellular environment, the nature and intensity of the stimulus, and the levels of autophagy (Kang and Avery, 2008). Our study revealed that autophagy is decreased in GK rats. Particularly, it was observed a decrease in LC3-II protein levels (Fig. 4.3C) and an increase in the protein levels of mTOR phosphorylated at serine residue 2448 (Fig. 4.3A). GK rats exhibit an insulin resistant state that in an initial phase is accompanied by hyperinsulinemia resulting from a compensatory increase in insulin secretion in response to increased insulin demand (King, 2012). Since insulin activates the class I phosphatidylinositol 3-kinase (PtdIns3K) and mTOR not only to stimulate glucose uptake and protein synthesis but also to inhibit autophagy, this could be a possible explanation for the diminished autophagy observed in the brain cortex of GK rats (Fig. 4.3) (Meijer and Codogno, 2006). It has been documented that diminished autophagy is an adaptive response that limits cardiac dysfunction in

T1D (Xu et al., 2013). Inhibition of autophagy is a beneficial adaptive response that protects cardiomyocytes against high glucose toxicity (Kobayashi et al., 2012). In accordance with our data, these authors found that increased mTOR signaling underlies the inhibition of autophagy (Kobayashi et al., 2012).

Our observations also revealed no significant alterations in mtDNA copy number and protein levels of ND1 and NRF1 and TFAM (Fig. 4.4). These results supports the notion that mitochondrial biogenesis is spared in the early stages of T2D. According to our hypothesis, it was previously demonstrated that mitochondrial biogenesis and function are negatively affected by hyperglycemia in a time-dependent manner (Palmeira et al., 2007).

Taken together, our results suggest that mitochondrial fusion-fission and biogenesis and autophagy act as "mitocheckpoints" to sustain brain mitochondrial bioenergetics during the early stages of T2D in order to maintain brain cells health. However, it is of utmost importance to evaluate how these mechanisms interact during the progression of T2D. Within this scenario, future studies involving the genetic manipulation of key proteins involved in mitochondrial quality control will be crucial to gain further insights on the role of these mechanisms during the development of T2D.

CHAPTER 5

Insulin therapy modulates mitochondrial dynamics and biogenesis, autophagy and tau protein phosphorylation in the brain of type 1 diabetic rats

Based in: Santos RX, Correia SC, Alves MG, Oliveira PF, Cardoso S, Carvalho C, Duarte AI, Santos MS, Moreira PI. Insulin therapy modulates mitochondrial dynamics and biogenesis, autophagy and tau protein phosphorylation in the brain of type 1 diabetic rats. Biochim Biophys Acta. 2014, 1842 (7): 1154-1166.

5.1. Abstract

The main purpose of this study was to examine whether streptozotocin (STZ)-induced type 1 diabetes (T1D) and insulin (INS) treatment affect mitochondrial function, fission/fusion and biogenesis, autophagy and tau protein phosphorylation in cerebral cortex from diabetic rats treated or not with INS. No significant alterations were observed in mitochondrial function as well as pyruvate levels, despite the significant increase in glucose levels observed in INS-treated diabetic rats. A significant increase in DRP1 protein phosphorylated at Ser616 residue was observed in the brain cortex of STZ rats. Also an increase in NRF2 protein levels and in the number of copies of mtDNA were observed in STZ diabetic rats, these alterations being normalized by INS. A slight decrease in LC3-II levels was observed in INS-treated rats when compared to STZ diabetic animals. An increase in tau protein phosphorylation at Ser396 residue was observed in STZ diabetic rats while INS treatment partially reversed that effect. Accordingly, a modest reduction in the activation of GSK3β and a significant increase in the activity of phosphatase 2A were found in INS-treated rats when compared to STZ diabetic animals. No significant alterations were observed in caspases 9 and 3 activity and synaptophysin and PSD95 levels. Altogether our results show that mitochondrial alterations induced by T1D seem to involve compensation mechanisms since no significant changes in mitochondrial function and synaptic integrity were observed in diabetic animals. In addition, INS treatment is able to normalize the alterations induced by T1D supporting the importance of INS signaling in the brain.

5.2. Introduction

Type 1 diabetes (T1D) is a metabolic disease that originates from the autoimmune destruction of β-cells due to lymphocytic infiltration of pancreatic islets, resulting in the permanent dependency of patients on exogenous insulin (INS) to survive (Gan et al., 2012).

Cognitive deficits, such as impaired learning, memory, problem solving, and mental flexibility have been recognized as being more common in T1D subjects than in the general population (Biessels et al., 2008; Ryan et al., 1985). It has also been demonstrated that T1D exacerbates tau protein hyperphosphorylation and amyloid beta (Aβ) formation contributing to the deposition of neurofibrillary tangles and Aβ plaques, the two major pathological hallmarks of Alzheimer's disease (AD), in the amyloid precursor protein (APP) transgenic mice (Jolivalt et al., 2010).

INS has been proven to exert a role in synaptic plasticity and memory consolidation through the modulation of the activity of excitatory and inhibitory receptors such as those for glutamate and GABA, and by triggering signal transduction cascades leading to the alteration of gene expression (Cardoso et al., 2009; Zhao et al., 2004). Furthermore, INS and insulin-like growth factors (IGFs) have been shown to protect neurons against Aβ toxicity (Dore et al., 1997; Takadera et al., 1993). Likewise, insulin reduced tau protein phosphorylation and promoted its binding to microtubules, the effects of insulin being mediated through the inhibition of glycogen synthase kinase-3β (GSK-3β) via the phosphoinositide 3-kinase (PI3-K)/Akt signaling pathway (Hong and Lee, 1997).

Mitochondria account for more than 90% of the cellular energy production (Chance et al., 1979). This bioenergetic production assumes its maximum importance in the brain since neurons have a high energy demand and a limited glycolytic capacity, making them highly dependent on aerobic oxidative phosphorylation (Moreira et al., 2010a).

Mitochondrial network is maintained through the fine balance between mitochondrial fission and fusion. Mitochondrial fission is governed by dynamin-like protein 1 (DRP1), a large cytosolic GTPase that is recruited to the mitochondrial membrane upon a fission-like stimuli, and by Fis1, a small mitochondrial molecule located in the outer membrane (Santos et al., 2010a). In turn, mitochondrial fusion is directed by three large GTPases, Mitofusin 1 (Mfn1) and Mitofusin 2 (Mfn2), both located in the mitochondrial outer membrane, and optic atrophy 1 (OPA1) protein, located in the inner mitochondrial membrane (Santos et al., 2010a).

Mitochondrial biogenesis results from an intricate crosstalk between both nuclear and mitochondrial genomes. The molecular machinery underlying mitochondrial biogenesis is constituted by the nuclear respiratory factor 1 (NRF 1) and nuclear respiratory factor 2 (NRF 2), which control the nuclear genes that encode mitochondrial proteins, and mitochondrial transcription factor A (TFAM) that drives transcription and replication of mitochondrial (mt) DNA (Correia et al., 2012b; Virbasius and Scarpulla, 1994). The expression of NRF1, NRF2, and TFAM is regulated by the peroxisome proliferator activator receptor gamma-coactivator 1 α (PGC-1 α) (Wu et al., 1999).

Autophagy is an evolutionarily conserved housekeeping process that enables cells to get nutrients through the digestion of their own components and,

at the same time, degrades misfolded proteins and aggregates, damaged organelles and invading microorganisms (Santos et al., 2010b). Autophagy is a tightly regulated process in a multistep manner. To the level of vesicle nucleation/initiation two main proteins are involved: the mammalian target of rapamycin (mTOR), which is an autophagic repressor; and Beclin1, which is an autophagic inducer (Moreira et al., 2010b). Regarding membrane elongation, cytosolic LC3-I is transformed to a membrane-bound form, LC3-II (Moreira et al., 2010b). In the autophagic degradation of ubiquitinated protein aggregates in mammalian cells, LC3 interacts with p62, which is an ubiquitin-binding protein therefore being considered an autophagic substrate (Moreira et al., 2010b).

The main aim of this study was the evaluation of the effects of streptozotocin (STZ)-induced T1D and INS treatment on brain cortical mitochondria, autophagy and tau protein phosphorylation. We evaluated several mitochondrial parameters: respiration [respiratory control ratio (RCR), and ADP/O index], phosphorylation system [transmembrane potential ($\Delta\Psi m$), ADP-induced depolarization, repolarization lag phase], fission/fusion protein levels (DRP1, Fis 1 and OPA1, MFN1, MFN2, respectively), biogenesis (NRF1, NRF2, TFAM and the number of copies of mtDNA). Autophagy (mTOR, Beclin1, LC3 and p62 protein levels), the activity of several kinases and phosphatase 2A that modulate tau protein phosphorylation, activity of caspases 3 and 9 and protein levels of synaptophysin and PSD95 were also evaluated.

5.3. Results

5.3.1. Characterization of the experimental animal models

STZ animals present a significant reduction in their body weight, and a significant increase in glycemia and glycated hemoglobin (HbA1c), when compared to Wistar (W) control rats (Table 5.1), confirming their diabetic state. Compared to untreated STZ diabetic rats, diabetic rats treated with INS present a statistically significant increase in body weight and a significant reduction in glycemia and HbA1c levels, which demonstrates the effectiveness of INS treatment in the amelioration of the diabetic phenotype. Additionally, a decrease in INS brain cortical levels is observed in STZ animals, an effect that is partially reversed by INS treatment (Fig. 5.1A). INS-treated rats show an increase in the levels of brain glucose (Fig. 5.1B), although no statistically significant alterations are observed in pyruvate levels (Fig. 5.1C).

Table 5.1. – Characterization of the experimental animal models

	Body weight (gr)	Brain weight (gr)	Glycemia (mg/dL)	HbA1c (%)
Wistar	457.2 ± 24.70	2.29 ± 0.07	92.60 ± 2.27	3.53 ± 0.03
STZ	261.2 ± 10.72 ***	2.16 ± 0.10	502.5 ± 29.90 ***	9.66 ± 0.53 ***
INS	332.2 ± 7.27 *** #	2.05 ± 0.10	347.2 ± 36.61 *** ###	6.15 ± 0.27 *** ###

Data are the mean ± SEM of 6 animals from each condition studied. Statistical significance: ***$p<0.001$ when compared to the respective wistar control rats; #$p<0.05$, ##$p<0.001$ and ###$p<0.001$ when compared to the respective STZ diabetic rats.

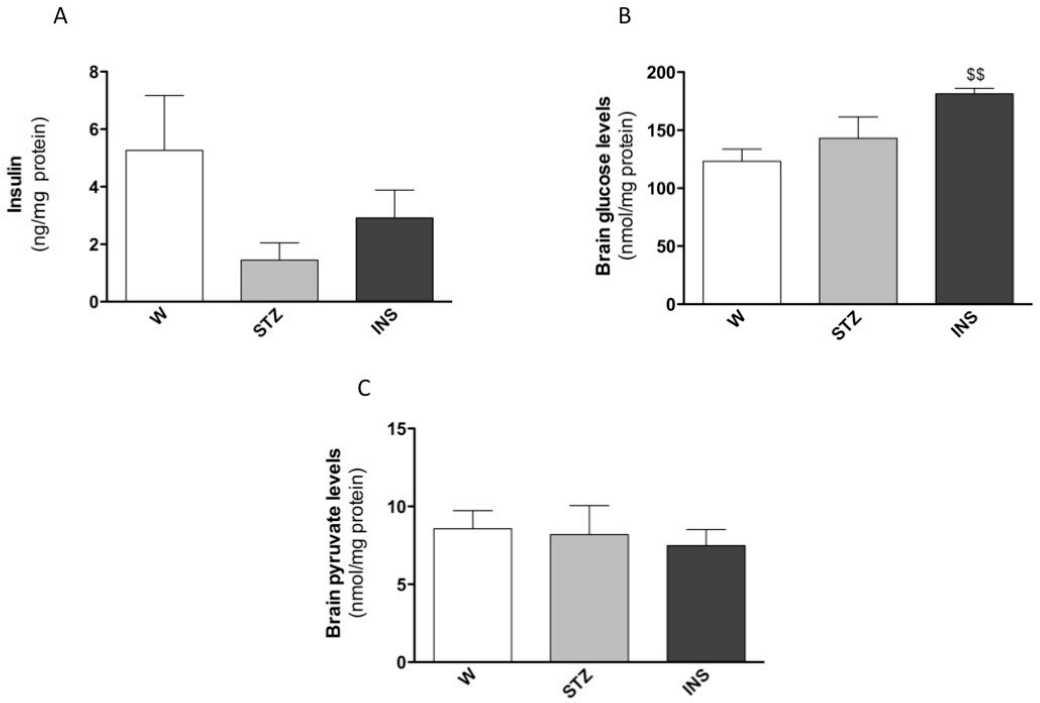


Figure 5.1. – Effects of T1D (STZ-induced diabetes) and insulin (INS) treatment in brain cortical insulin (A), glucose (B) and pyruvate (C) levels. Data are the mean ± SEM of 5-6 animals from each condition studied. Statistical significance: $^{\$\$}p<0.01$ when compared to the respective wistar (W) control animals.

5.3.2. Neither T1D nor insulin treatment affect mitochondrial function

RCR is a measure of the coupling between substrate oxidation and phosphorylation and is a good indicator of mitochondrial integrity. ADP/O index shows the efficiency of the mitochondrial phosphorylative system (Silva and Oliveira, 2012). Both mitochondrial indexes present no significant differences between the experimental groups (Table 5.2). Also FCCP and oligomycin respiratory states show no significant differences between the experimental groups (Table 5.2).

Mitochondrial membrane potential ($\Delta\Psi_m$) is generated through the respiratory chain pumping of protons from the mitochondrial matrix to the intermembrane space. The proton gradient originates an electrochemical potential (Δp) resulting in a pH (ΔpH) and a voltage gradient ($\Delta\Psi_m$) across the inner membrane. No statistically significant differences are observed in $\Delta\Psi_m$ and ADP-induced depolarization between the three experimental groups of animals (Table 5.2). Lag phase is the time necessary for mitochondria to phosphorylate the added ADP into ATP. Again, no statistically significant differences are observed between the experimental groups (Table 5.2).

Table 5.2. – Effects of T1D (STZ-induced diabetes) and insulin (INS) treatment in brain cortical mitochondrial respiration and oxidative phosphorylation system

		Wistar	STZ	INS
Respiratory parameters	**RCR**	4.65 ± 0.314	4.50 ± 0.157	4.51 ± 0.129
	ADP/O (nmol ADP/nAtgO/min/mg)	2.10 ± 0.299	2.10 ± 0.280	2.23 ± 0.201
	FCCP-stimulated respiration (nAtgO/min/mg)	97.39 ± 6.080	106.4 ± 23.64	100.1 ± 15.31
	Oligomycin-inhibited respiration (nAtgO/min/mg)	25.09 ± 4.070	29.12 ± 5.656	24.18 ± 3.451
Membrane potential and oxidative phosphorylation	**$\Delta\Psi_m$** (-mV)	185.4 ± 0.73	185.2 ± 2.73	184.8 ± 1.53
	ADP-induced depolarization (-mV)	19.79 ± 1.51	20.21 ± 2.99	17.34 ± 0.80
	Lag phase (min)	0.97 ± 0.083	0.92 ± 0.053	0.88 ± 0.071

Data are the mean ± SEM of 6 animals from each condition studied.

5.3.3. INS reverses mitochondrial fission promoted by T1D

Regarding mitochondrial fusion, no significant alterations are observed in Mfn1 (Fig. 5.2A), Mfn2 (Fig. 5.2B) and OPA1 (Fig. 5.2C) protein levels. The mitochondrial fission-related protein Fis1 is statistically unaltered between the experimental groups (Fig. 5.3A) however, the active form of the fission protein DRP1 (P^{Ser616}-DRP1) is significantly increased in diabetic animals when

compared to control animals, this effect being reversed by INS treatment (Fig. 5.3B).

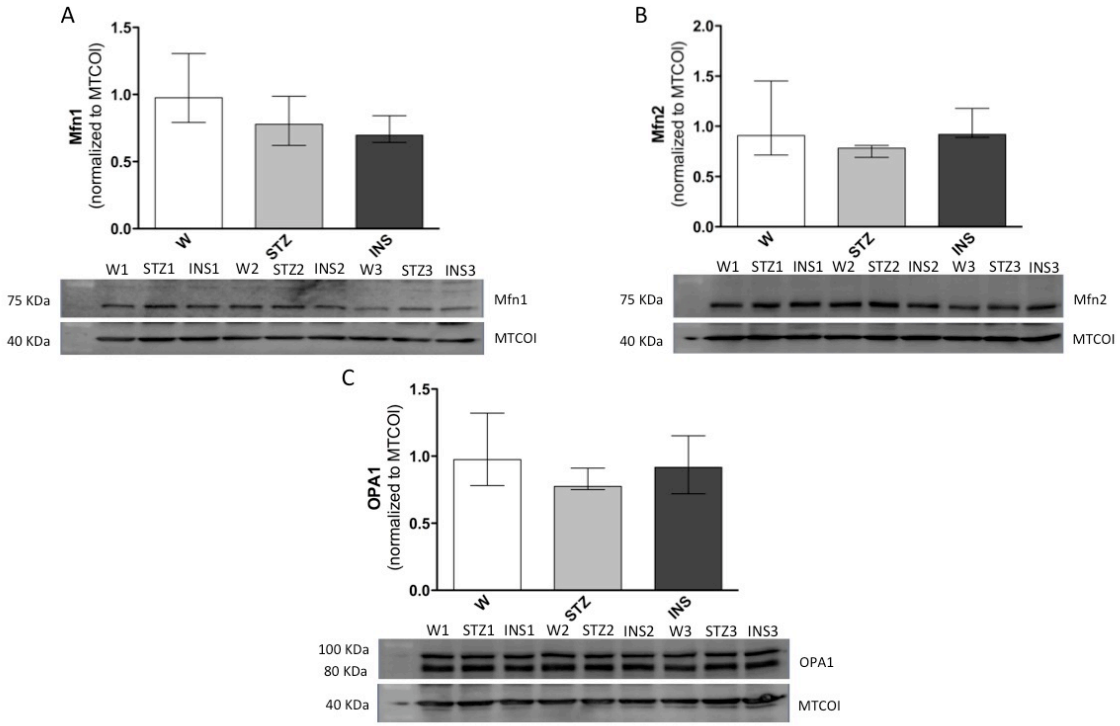


Figure 5.2. - Effects of T1D (STZ-induced diabetes) and insulin (INS) treatment in the levels of mitochondrial fusion-related proteins: Mfn1 (A), Mfn2 (B) and OPA1 (C). Data are the median ± interquartile range of 6 animals from each condition studied.

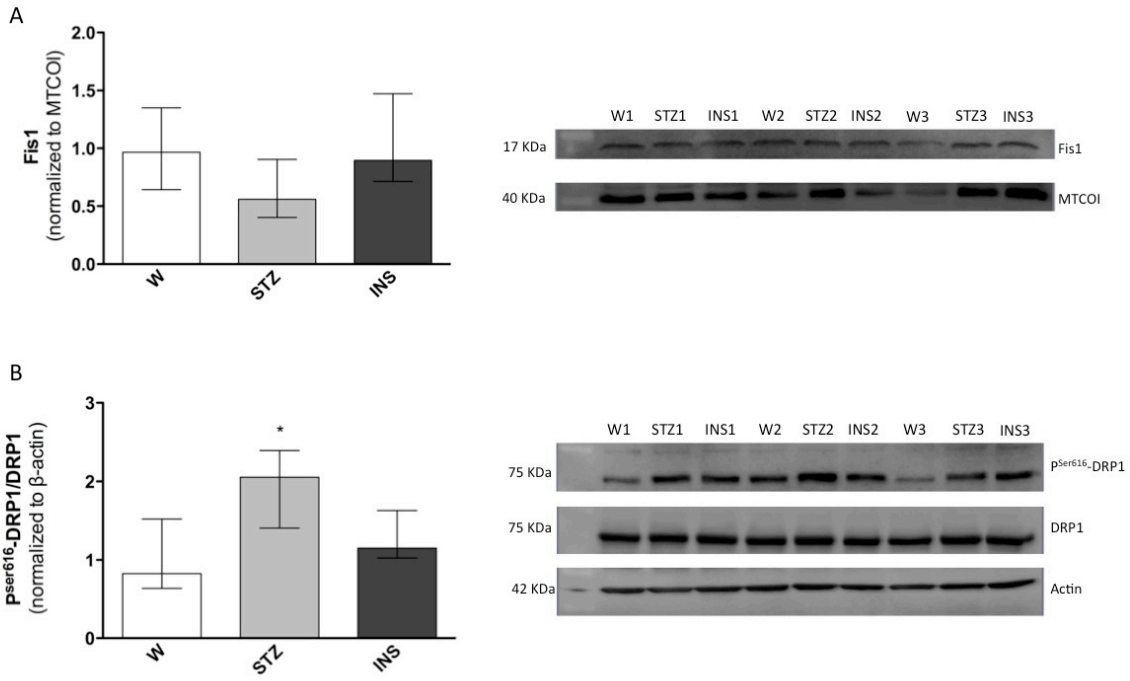


Figure 5.3. – Effects of T1D (STZ-induced diabetes) and insulin (INS) treatment in the levels of mitochondrial fission-related proteins: Fis1 (A) and P^{ser616}-DRP1/DRP1 (B). Data are the median ± interquartile range of 6 animals from each condition studied. Statistical significance: *$p<0.05$ when compared to the respective wistar (W) control animals.

5.3.4. Insulin treatment reverses T1D-induced mitochondrial biogenesis alterations

Several transcription factors have been enrolled in the transcription of genes that are crucial to mitochondria. To ascertain the role of T1D in brain cortical mitochondrial biogenesis the protein levels of NRF1 (Fig. 5.4A), NRF2 (Fig. 5.4B) and TFAM (Fig. 5.4C) were evaluated. STZ-induced T1D induces a significant increase in the levels of NRF2 (Fig. 5.4B) and a slight increase in the levels of TFAM (Fig. 5.4C), these effects being reversed by INS treatment (Fig.

5.4A-C). Accordingly, a significant increase in the relative number of mtDNA copies is observed in STZ diabetic animals (Fig. 5.5). Again, INS treatment tends to normalize the increase in the number of mtDNA copies induced by STZ-induced T1D (Fig. 5.5).

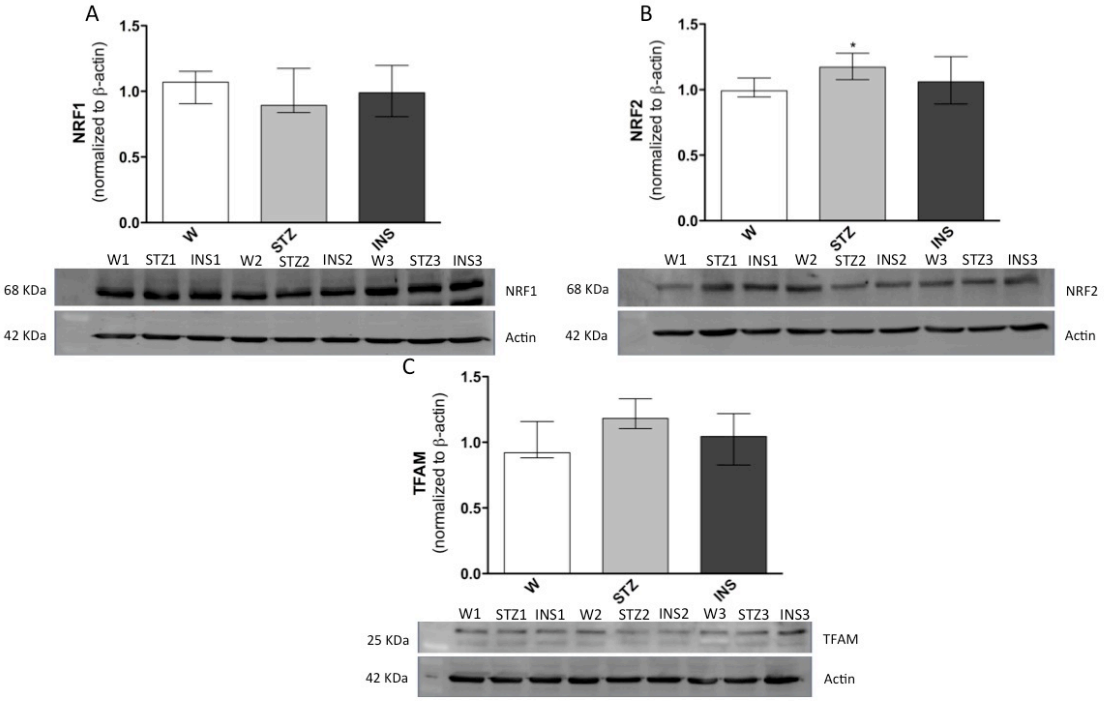


Figure 5.4. – Effects of T1D (STZ-induced diabetes) and insulin (INS) treatment in the levels of transcription factors related with mitochondrial biogenesis: NRF1 (A); NRF2 (B) and TFAM (C). Data are the median ± interquartile range of 6 animals from each condition studied. Statistical significance: *p<0.05 when compared to the respective wistar (W) control animals.

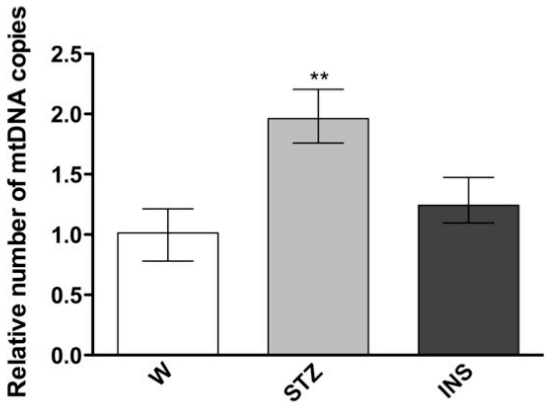


Figure 5.5. – Effects of T1D (STZ-induced diabetes) and insulin (INS) treatment in the number of copies of mitochondrial DNA (mtDNA). Data are the median ± interquartile range of 5 animals from each condition studied. Statistical significance: **p<0.01 when compared to the respective wistar (W) control animals.

5.3.5. Insulin therapy modulates autophagy

mTOR is a recognized repressor of the autophagic pathway. As shown, the active form of mTOR ($P^{Ser2448}$-mTOR) (Fig. 5.6A) is not significantly altered in the three experimental groups. The same occurs with the autophagic inducer Beclin1 (Fig. 5.6B) and the autophagic substrate p62 (Fig. 5.6D). However, the levels of LC3-II, a gold-standard marker of autophagic vesicle elongation, are tendentiously decreased by INS treatment (Fig. 5.6C).

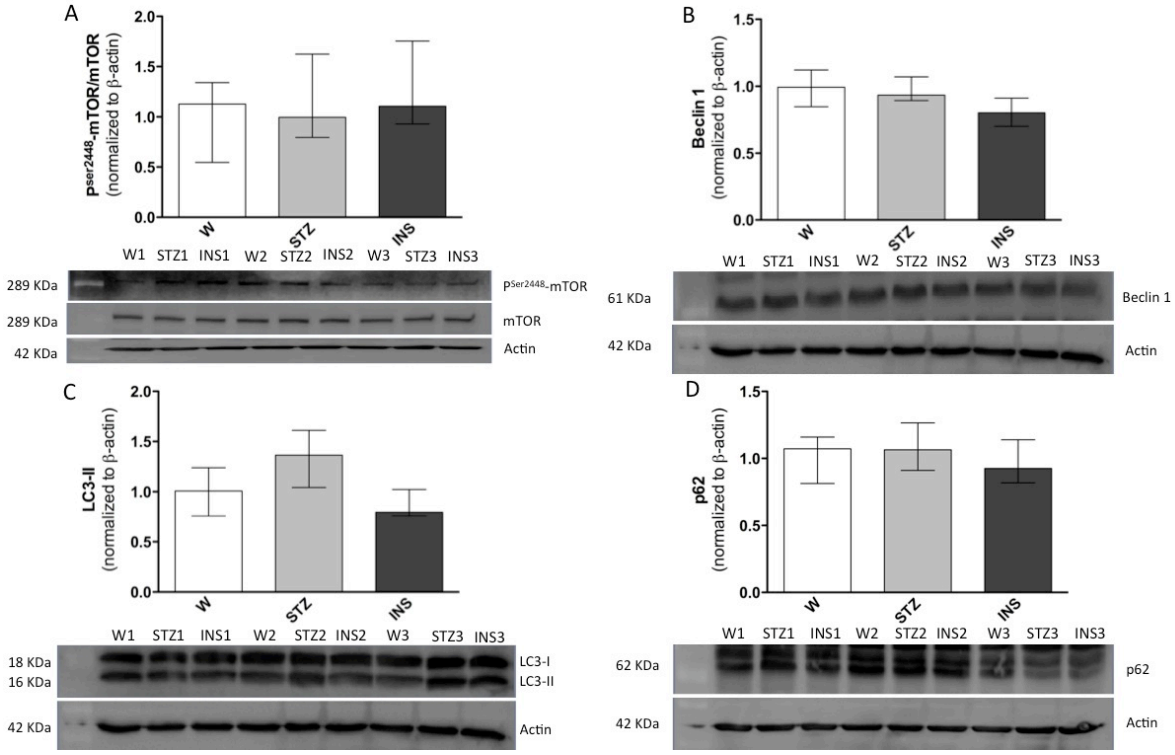


Figure 5.6. - Effects of T1D (STZ-induced diabetes) and insulin (INS) treatment in the levels of autophagy-related proteins: $P^{ser2448}$-mTOR/mTOR (A), Beclin1 (B), LC3-II (C), p62 (D). Data are the median ± interquartile range of 6 animals from each condition studied.

5.3.6. INS partially reverses tau protein phosphorylation promoted by T1D by the increase in PP2A activity

To investigate whether T1D and INS treatment affect tau protein phosphorylation, we evaluated the levels of tau protein phosphorylated at residues Ser396 and Thr181. T1D increases the protein levels of P^{Ser396}-Tau and decreases the protein levels of P^{Thr181}-Tau, while INS partially reverses these effects (Fig. 5.7). The protein levels of some kinases involved in tau protein phosphorylation were also assessed. T1D does not significantly alter the levels of

the active form of GSK3β (P^{Tyr216}-GSK3β) (Fig. 5.8A and 5.8B), however INS treatment tends to reduces the active form of this enzyme when compared to diabetic animals. A slight decrease in the levels of the inactive form of GSK3β (P^{Ser9}-GSK3β) is also observed in INS-treated rats (Fig. 5.8A and 5.8B). No significant alterations are observed in other kinases (JNK, ERK and p38 MAPK) known to modulate tau protein phosphorylation (Fig. 5.8A and 5.8B). Although not statistically significant, T1D decreases the activity of PP2A (Fig. 5.9) while INS treatment significantly increases the activity of this phosphatase when compared to diabetic animals (Fig. 5.9).

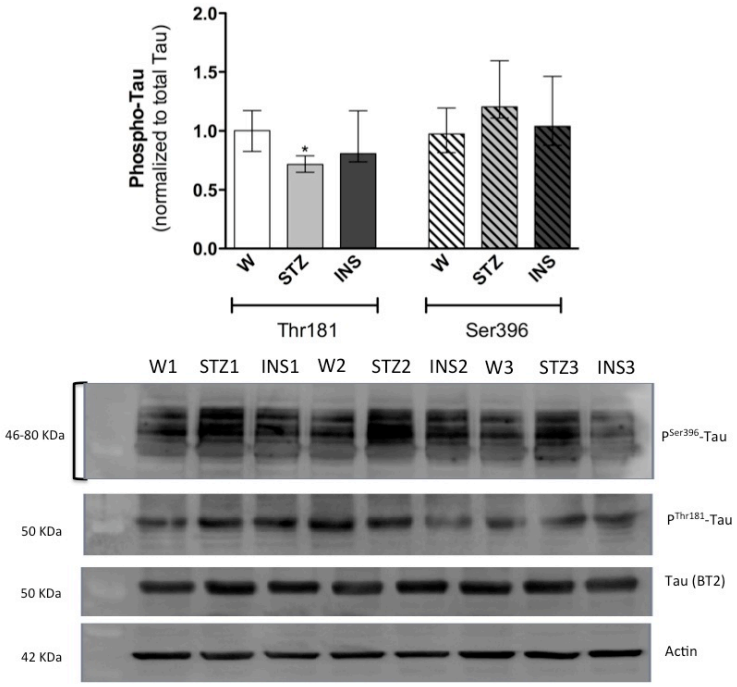
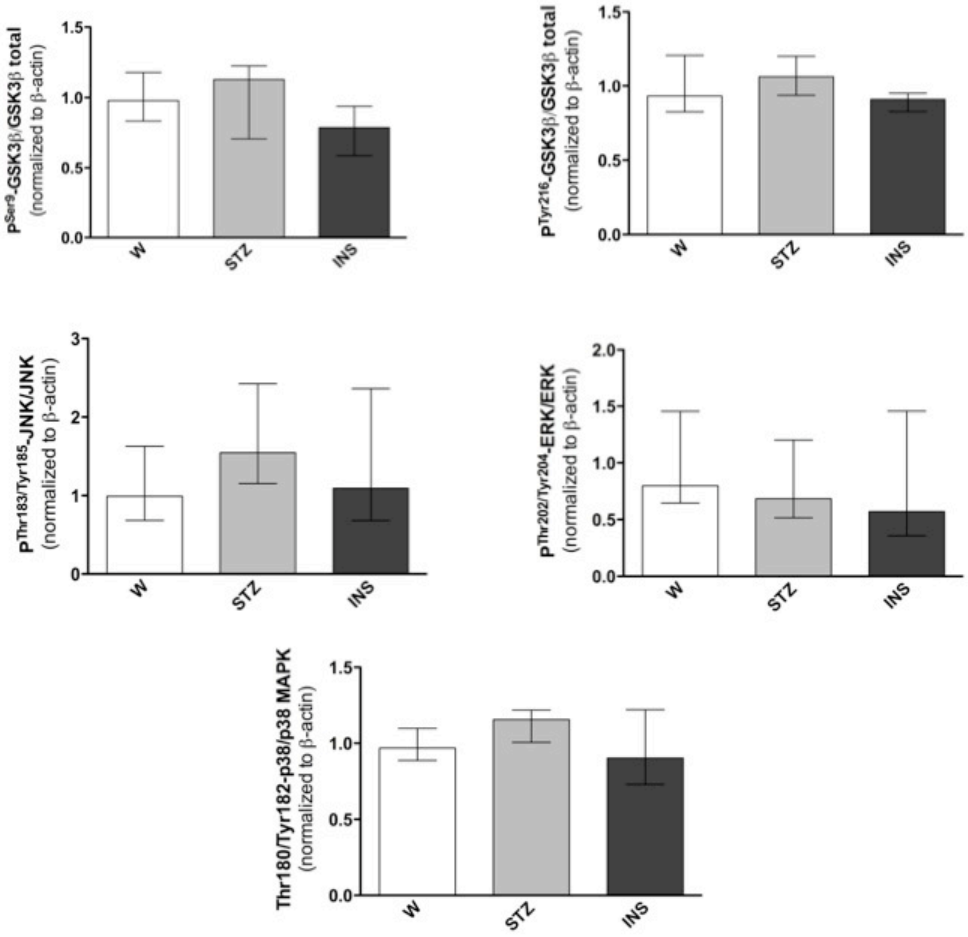


Figure 5.7. – Effects of T1D (STZ-induced diabetes) and insulin (INS) treatment on the ratio between phosphorylated (Thr181 and Ser396 residues) tau and total tau protein levels. Data are the median ± interquartile range of 6 animals from each condition studied. Statistical significance: *p<0.05 when compared to the respective Wistar (W) control animals.

A


P^Ser9-GSK3β/GSK3β total (normalized to β-actin)
P^Tyr216-GSK3β/GSK3β total (normalized to β-actin)
P^Thr183/Tyr185-JNK/JNK (normalized to β-actin)
P^Thr202/Tyr204-ERK/ERK (normalized to β-actin)
Thr180/Tyr182-p38/p38 MAPK (normalized to β-actin)
W
STZ
INS

B

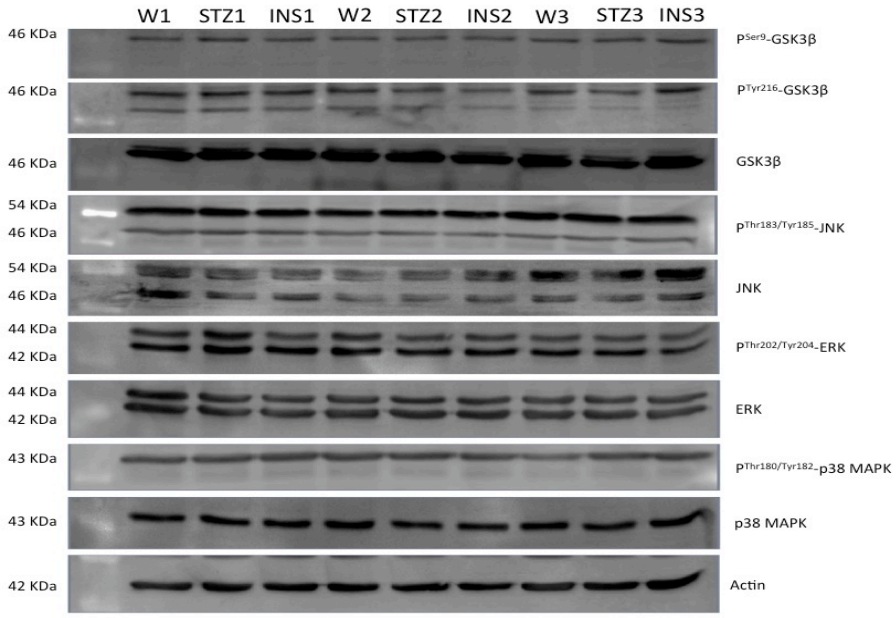

W1 STZ1 INS1 W2 STZ2 INS2 W3 STZ3 INS3
46 KDa
P^Ser9-GSK3β
P^Tyr216-GSK3β
GSK3β
54 KDa
46 KDa
P^Thr183/Tyr185-JNK
JNK
44 KDa
42 KDa
P^Thr202/Tyr204-ERK
ERK
43 KDa
P^Thr180/Tyr182-p38 MAPK
p38 MAPK
42 KDa
Actin

Figure 5.8. – Effects of T1D (STZ-induced diabetes) and insulin (INS) treatment in the phosphorylation of several kinases: GSK3β, JNK, ERK and p38 MAPK (A and B). Data are the median ± interquartile range of 6 animals from each condition studied.

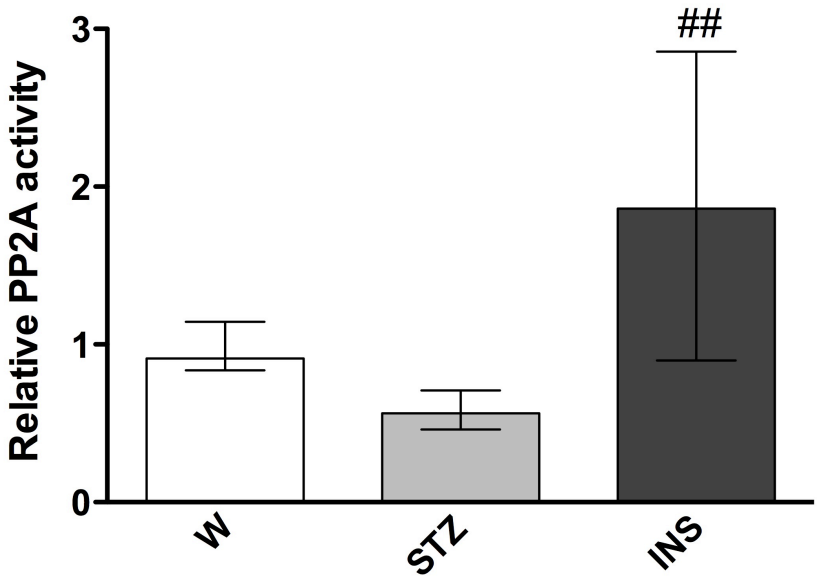


Figure 5.9. – Effects of T1D (STZ-induced diabetes) and insulin (INS) treatment in the activity of protein phosphatase 2A (PP2A). Data are the median ± interquartile range of 6 animals from each condition studied. Statistical significance: ##p<0.01 when compared to the respective STZ diabetic animals.

5.3.7. Neither T1D nor insulin treatment alter brain cells survival and synaptic integrity

The initiator caspase 9 is responsible for the mitochondrial-dependent apoptotic cell death. Caspase 3 is an effector caspase involved in both extrinsic and intrinsic (i.e. mitochondrial pathway) apoptosis pathways. Both STZ-induced

diabetes and INS treatment do not alter caspases 9- and 3-like activities (Fig. 5.10A and 5.10B, respectively), which is indicative of no effects on apoptotic cell death. Likewise, synaptophysin and PSD95 levels are not altered in the three experimental groups (Fig. 5.11A and 5.11B, respectively), which suggest that there is no significant loss of synaptic integrity.

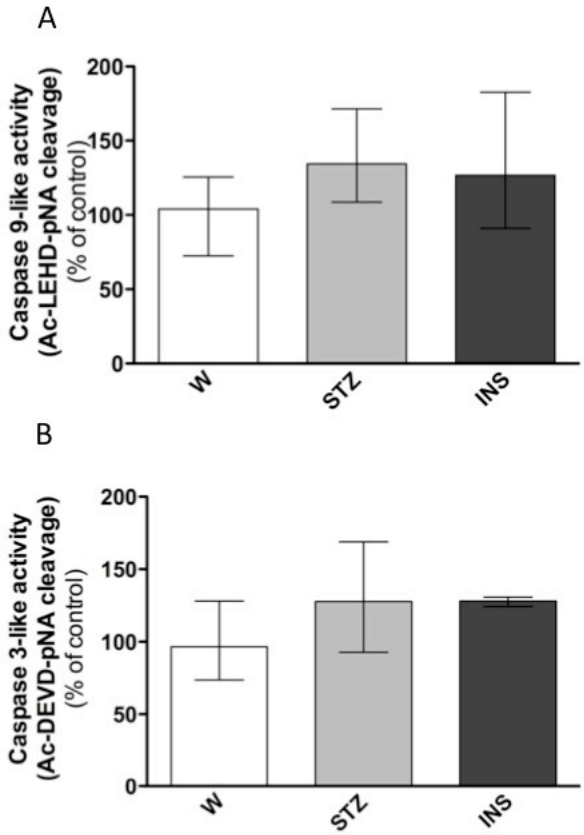


Figure 5.10. – Effects of T1D (STZ-induced diabetes) and insulin (INS) treatment in the caspase 9- (A) and caspase 3-like (B) activities. Data are the median ± interquartile range of 5 animals from each condition studied.

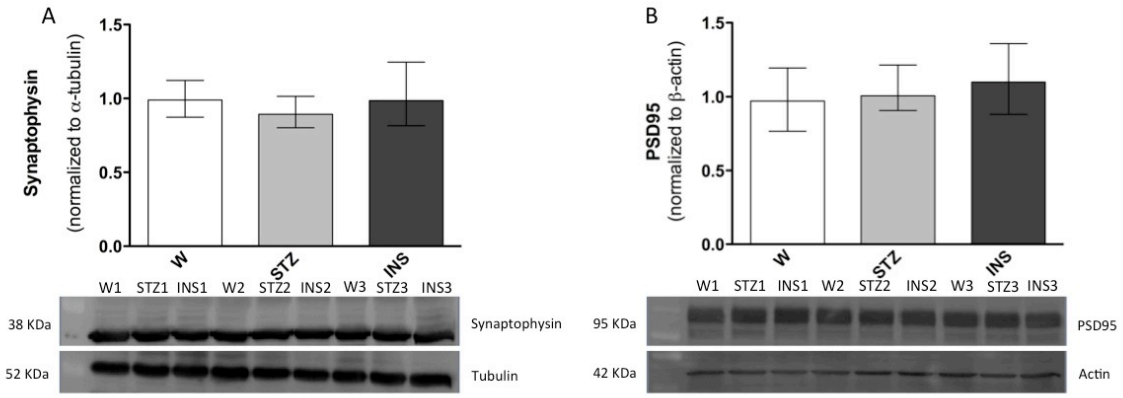


Figure 5.11. – Effects of T1D (STZ-induced diabetes) and insulin (INS) treatment in the protein levels of synaptophysin (A) and PSD95 (B). Data are the median ± interquartile range of 6 animals from each condition studied.

5.4. Discussion

T1D patients critically depend on lifelong INS treatment to survive. Indeed, T1D leads to several long-term complications due to the extremely high levels of blood glucose, which is expected to be controlled by INS therapy (Reichard et al., 1993; Brussee et al., 2004; Francis et al., 2009).

STZ administration significantly increased glycemia and HbA1c levels and avoided body weight gain (Table 5.1). The daily s.c. administration of INS ameliorated the levels of blood glucose and HbA1c. Strikingly, INS treatment also induced a significant body weight gain when compared to diabetic animals (Table 5.1). The phenotypic effects induced by STZ injection are comparable to those described by others in STZ-induced diabetic animals (Moreira et al., 2005; Cardoso et al., 2010, 2012) and T1D patients (Cooke and Plotnick, 2008).

Nevertheless, the levels of glucose (Fig. 5.1B) and pyruvate (Fig. 5.1C) in cerebral cortex of diabetic rats remained statistically unchanged suggesting that glucose uptake and its metabolism by glycolysis are unaffected in this animal model of T1D. It was previously reported that the function of glucose transporter 1 (GLUT 1), the main glucose transporter found in the blood-brain barrier (BBB), is not altered in cerebral microvessels of diabetic rats (Shah et al., 2012). In addition, it was reported an increase in glucose use throughout the brain 1 week after STZ administration in rats, while normal rates were found 4 weeks after diabetes induction (Mans et al., 1988). The systemic characterization of the INS-treated animals (Table 5.1) and the levels of INS found in the brains of these animals (Fig. 5.1A) show the effectiveness of the INS treatment. The increase in glucose levels in INS-treated diabetic rats is not related with alterations in glucose utilization since the levels of pyruvate (Fig. 5.1C) and mitochondrial function (Table 5.2) remained statistically unaltered. The increase in glucose levels in INS-treated diabetic brains may represent a stimulatory action of INS in promoting glucose uptake into brain.

INS treatment partially reversed the increase in the ratio of tau phosphorylated at Ser396 to total tau promoted by STZ-induced diabetes (Fig. 5.7). Our results converge with other studies showing an increased phosphorylation of tau protein in the brains of STZ diabetic animals (Planel et al., 2007; Qu et al., 2011). In vitro, pseudophosphorylation of Ser396 and Ser404 generates tau that is more fibrillogenic (Abraha et al., 2000). Mutational studies with human tau also showed that phosphorylation of tau at Ser396 is primarily responsible for the functional loss of tau-mediated tubulin polymerization (Evans et al., 2000). Interestingly, the ratio of tau phosphorylated at Thr181 to

total tau is decreased in the brain cortices of STZ diabetic animals (Fig. 5.7). A protective role for tau phosphorylation could be suggested when that modification takes place at Thr181 (Simón et al., 2012), which could facilitate its binding to exosomes and the release of tau excess (Johnson et al., 1989). Under these circumstances, the decrease in the ratio of tau phosphorylated at Thr181 to total tau observed in the brains of STZ-induced diabetic rats may indicate an increased probability for an over accumulation and deposition of tau.

Several mechanisms modulate tau protein phosphorylation levels, such as the decrease in the activity of PP2A, as observed in the brains of STZ animals and spontaneous model of T1D, the nonobese diabetic (NOD) mouse (Planel et al., 2007; Qu et al., 2011; Papon et al., 2013). Accordingly, we observed a non-statistically significant decrease of PP2A activity in the cerebral cortex of STZ diabetic rats (Fig. 5.9). It was also reported that the active form of p38-MAPK was increased in neurons from the olfactory bulb of STZ diabetic rats, which was accompanied by an increase in tau protein phosphorylation (Sharma et al., 2010). Moreover, Clodfelder-Miller et al. (2006) reported that STZ diabetic animals present an increase in p38 MAPK and JNK active forms while ERK remains unchanged. However, in our study, no significant alterations were observed in the active forms of p38-MAPK and JNK (Fig. 5.8A and 5.8B).

The decrease in the levels of phosphorylated tau protein promoted by INS treatment can be explained, at least in part, by the slight decrease in the levels of the active form of the Ser/Thr kinase GSK3β (P^{Tyr216}-GSK3β) (Fig. 5.8A and 5.8B). Duarte et al. (2008) showed that insulin decreased P^{Tyr216}-GSK3β phosphorylation in vitro. Additionally, INS treatment significantly increased the activity of PP2A (Fig. 5.9), contributing to the normalization of the levels of

phosphorylated tau protein. Ho and collaborators (Ho et al., 2004) demonstrated that insulin and IGF-1 reduce tau protein phosphorylation via the PI3-K pathway promoting its binding to microtubules. It was also shown that intranasal administration of insulin ameliorated tau protein phosphorylation in type 2 diabetic mice (Yang et al., 2013a).

Also, the slight decrease in LC3-II levels observed in INS-treated animals (Fig. 5.6C), which suggest a decrease in autophagy, can be correlated with the decrease in tau protein phosphorylation via stimulation of tau proteolysis by activation of calpains (Zhang et al., 2009). Indeed, Zhang et al (2009) demonstrated that inhibition of autophagy with 3-methyladenine (3-MA) elicited an increased calpain activity and, concomitantly, a reduction in the levels of phosphorylated tau protein. The decrease in LC3-II levels in INS-treated animals may result from the stimulation of mTOR activity by INS (Scott et al., 1998). However, unexpectedly, the levels of both $P^{ser2448}$-mTOR (Fig. 5.6A), an active form of the autophagy repressor, and Beclin1 (Fig. 5.6B), an autophagy inducer, remained unaltered. Nonetheless, it is noteworthy that mTOR phosphorylation does not always correlate with its activity (Caccamo et al., 2010). It has been also reported that the activation of mTOR may occur when one of the three residues (T2446, S2448 and S2481) is phosphorylated (Copp et al., 2009). Additionally, p62, an autophagic substrate, also remained unchanged in both experimental groups when compared to the control group (Fig. 5.6D). This result regarding the levels of p62 is quite surprising taking into account the levels of LC3-II (Fig. 5.6). Indeed, it is expectable that when autophagy is inhibited, as suggested by the decrease in LC3-II in INS-treated animals (Fig. 5.6C), the levels of p62 increase (Klionsky et al., 2012). However, p62 is also involved in proteasomal degradation

(Seibenhener et al., 2004), which indicate that the relation between autophagy and p62 levels is not always straightforward.

It has been described that tau protein hyperphosphorylation causes mitochondrial dynamics abnormalities such as changes in mitochondrial distribution (Kopeinika et al., 2011), and mitochondrial enlargement (DuBoff et al., 2012), which is putatively due to an abnormal interaction between the mitochondrial fission protein DRP1 and hyperphosphorylated tau protein (Manczak and Reddy, 2012). In our model of T1D, the expression pattern of the proteins involved in mitochondrial fusion and fission is more compatible with enhanced mitochondrial fragmentation due to increased phosphorylation of DRP1 at Ser616 (Fig. 5.3B). In agreement with our observations, increased mitochondrial fission associated to increased protein levels of Fis1 and DRP1 was observed in venous endothelial cells from patients with diabetes (Shenouda et al., 2011). INS treatment reversed the increase in P^{ser616}-DRP1 (Fig. 5.3B) promoted by STZ-induced T1D. Also the observation that OPA1 protein levels remained unaltered (Fig. 5.2C) in both STZ- and INS-treated animals is consistent with the unaltered activity of caspases 9 and 3 (Fig. 5.10). Only 10–15% of cytochrome c is found free in the intermembrane space, while the major fraction can be found in the cristae (Bernardi and Azzone, 1981; Scorrano et al., 2002). OPA1 complexes are involved in cristae remodeling, and the assembly and disassembly of such complexes regulates cytochrome c release from inside the cristae, and therefore caspase-dependent apoptotic cell death (Yamaguchi and Perkins, 2009; Scarpulla, 2006).

There is scarce evidence about the role of hyperglycemia in mitochondrial biogenesis, particularly in the central nervous system. A nuclear-mitochondrial

crosstalk regulates mitochondria biogenesis via PGC1α-NRF1/2-TFAM (Scarpulla, 2006, 2008). Indeed, Edwards et al. (2010) demonstrated in a mouse model of type 2 diabetes (T2D) with well-established diabetic neuropathy, that neurons from the dorsal root ganglia (DRG) presented greater mitochondrial biogenesis, when compared with non-diabetic mice. Also in vitro experiments demonstrated that cultured DRG exposed to hyperglycemic conditions exhibited increased mitochondrial biogenesis (Edwards et al., 2010; Vincent et al., 2010). The authors proposed that the increase in mitochondrial biogenesis is occurring in an attempt to overcome the metabolic load induced by the hyperglycemic status as well as to compensate for the enhanced mitochondrial fragmentation (Edwards et al., 2010; Vincent et al., 2010). Accordingly, we also observed an increase in mitochondrial biogenesis through the increase in the levels of NRF2 (Fig. 5.4B) and TFAM levels (Fig. 5.4C) and mtDNA copy number (Fig. 5.5) in T1D animals, those effects being normalized by INS treatment. Studies performed in primary cultures of mice hepatocytes revealed that prolonged exposure to INS decreased the levels of NRF1, TFAM and the cellular mitochondrial content (Liu et al., 2009). The alterations in mitochondrial biogenesis occurring in the cerebral cortex of T1D animals may represent a compensation mechanism against mitochondrial fragmentation, since there is no alteration of mitochondrial function (Table 5.2) and no activation of the apoptotic cascade (Fig. 5.10A and 5.10B), which protect synapse integrity, as demonstrated by the maintenance of synaptophysin and PSD95 levels (Fig. 5.11), two proteins crucial for neurotransmission and synaptic plasticity (Valtorta et al., 2004). Accordingly, it has been recently demonstrated by our group, that mitochondria isolated from

the cortex of T1D animals did not show significant alterations neither in the respiratory chain nor in the phosphorylation system (Cardoso et al., 2012).

Altogether our results support the idea that T1D modulates brain cortical mitochondria through possible compensatory mechanisms avoiding the decline of mitochondrial function, which is crucial for brain cells integrity and survival. Nevertheless, STZ-induced diabetes increases the abnormal phosphorylation of tau protein that in short- or medium-term may predispose to neurodegenerative events. Our study also shows beneficial effects of INS therapy that is able to normalize or attenuate brain cortical alterations promoted by STZ-induced T1D. However, longer-term studies must be done to elucidate the impact of T1D in brain biochemistry, structure and function. It would be also interesting to study the effectiveness of INS therapy after a longer period of time.

CHAPTER 6

General Conclusion

6.1. General Conclusion

Overall the results presented in this thesis demonstrate for the first time that in the brain cortices of T2D (Chapter 4) and T1D (Chapter 5) rats, at the studied stages of disease, mitochondrial dynamics is shifted towards mitochondrial fission, which may alter mitochondrial morphology. An alteration of mitochondrial morphology towards smaller mitochondria can have several implications, on the one side damaged mitochondrial components are possibly being segregated to smaller mitochondria that are subsequently degraded by mitophagy in order to maintain a healthy mitochondrial pool, and on the other side increased mitochondrial fission can impact mitochondrial motility. Considering our results about autophagy, a decrease in T2D and insulin-treated T1D rats, we suspect that mitochondrial fission is aimed to facilitate mitochondrial motility. Taking into consideration that neurons are polarized cells that have a complex and unique morphology, with long processes (axons and dendrites) extending far from the cell body, mitochondrial motility has a critical importance.

Curiously, we found that the alterations in mitochondrial fission/fusion are different in both types of diabetes, despite both pointing towards increased mitochondrial fragmentation. Although the reason for this difference is yet to be explored it most likely resides in the different etiologies of the metabolic disorders.

A significant increase in mitochondrial biogenesis was also found in T1D brains. An increase in mitochondrial number may be advantageous in organs

with high energy demand such as the brain rendering it more resistant against deleterious events such as promoted by diabetes.

What is most interesting is that despite the alterations in mitochondrial dynamics and/or biogenesis, brain mitochondrial function is sustained in both diabetic animal models. These observations suggest that the diabetic brain, at initial and mild stages of the disease, triggers adaptive mechanisms in an attempt to avoid structural and functional alterations. This hypothesis is supported by our observations concerning the maintenance of synaptic integrity and lack of apoptotic signs in T1D.

We also found that insulin reverses T1D-induced brain changes supporting the notion that insulin is an effective agent for the management of diabetes and associated complications. These observations also suggest that the amelioration of insulin signaling in the brain may prevent cognitive decline and dementia.

Future studies should focus on the evaluation of mitochondrial dynamics and autophagy in the brains of aged diabetic rats or subjected to a longer duration of diabetes since it is known that mitochondrial dysfunction is associated with long-term diabetic complications. As previously discussed, diabetes is a main risk factor for AD, an idea supported by the increase in tau protein phosphorylation found in the brains of T1D rats.

In addition, the genetic manipulation of mitochondrial biogenesis-related proteins in the brains of diabetic animals would certainly provide further insights into the role of this process during disease development. Also *in vitro* studies may help elucidate the mechanistic basis underlying mitochondrial dynamics, biogenesis and autophagy in hyperglycemic and/or insulin resistance conditions since it is easier to manipulate key individual molecules.

Since tau protein is involved in microtubules stabilization and an increase in tau protein phosphorylation may negatively impact this function, it would also be interesting to correlate tau pathology (e.g. excessive phosphorylation) with mitochondrial motility and function.

The clarification of the role of the mitochondrial processes discussed above in the brains of T2D and T1D subjects will spur the search of effective therapies against diabetes-associated neurodegeneration.

CHAPTER 7

References

7.1. References

Abraha A, Ghoshal N, Gamblin TC, Cryns V, Berry RW, Kuret J, Binder LI (2000) C-terminal inhibition of tau assembly in vitro and in Alzheimer's disease. J Cell Sci 113 Pt 21:3737-3745.

Ahmad W (2013) Overlapped metabolic and therapeutic links between Alzheimer and diabetes. Mol Neurobiol 47:399-424.

American Diabetes A (2012) Diagnosis and classification of diabetes mellitus. Diabetes Care 35 Suppl 1:S64-71.

American Diabetes A (2014) Diagnosis and classification of diabetes mellitus. Diabetes Care 37 Suppl 1:S81-90.

Balaban RS, Nemoto S, Finkel T (2005) Mitochondria, oxidants, and aging. Cell 120:483-495.

Balaraman Y, Limaye AR, Levey AI, Srinivasan S (2006) Glycogen synthase kinase 3beta and Alzheimer's disease: pathophysiological and therapeutic significance. Cell Mol Life Sci 63:1226-1235.

Baricault L, Segui B, Guegand L, Olichon A, Valette A, Larminat F, Lenaers G (2007) OPA1 cleavage depends on decreased mitochondrial ATP level and bivalent metals. Exp Cell Res 313:3800-3808.

Baughman JM, Perocchi F, Girgis HS, Plovanich M, Belcher-Timme CA, Sancak Y, Bao XR, Strittmatter L, Goldberger O, Bogorad RL, Koteliansky V, Mootha VK (2011) Integrative genomics identifies MCU as an essential component of the mitochondrial calcium uniporter. Nature 476:341-345.

Beique JC, Andrade R (2003) PSD-95 regulates synaptic transmission and plasticity in rat cerebral cortex. J Physiol 546:859-867.

Belanger A, Lavoie N, Trudeau F, Massicotte G, Gagnon S (2004) Preserved LTP and water maze learning in hyperglycaemic-hyperinsulinemic ZDF rats. Physiol Behav 83:483-494.

Benard G, Bellance N, James D, Parrone P, Fernandez H, Letellier T, Rossignol R (2007) Mitochondrial bioenergetics and structural network organization. J Cell Sci 120:838-848.

Benedict C, Hallschmid M, Hatke A, Schultes B, Fehm HL, Born J, Kern W (2004) Intranasal insulin improves memory in humans. Psychoneuroendocrinology 29:1326-1334.

Benedict C, Hallschmid M, Schmitz K, Schultes B, Ratter F, Fehm HL, Born J, Kern W (2007) Intranasal insulin improves memory in humans: superiority of insulin aspart. Neuropsychopharmacology 32:239-243.

Bernardi P, Azzone GF (1981) Cytochrome c as an electron shuttle between the outer and inner mitochondrial membranes. J Biol Chem 256:7187-7192.

Biessels GJ, Deary IJ, Ryan CM (2008) Cognition and diabetes: a lifespan perspective. Lancet Neurol 7:184-190.

Bomfim TR, Forny-Germano L, Sathler LB, Brito-Moreira J, Houzel JC, Decker H, Silverman MA, Kazi H, Melo HM, McClean PL, Holscher C, Arnold SE, Talbot K, Klein WL, Munoz DP, Ferreira ST, De Felice FG (2012) An anti-diabetes agent protects the mouse brain from defective insulin signaling caused by Alzheimer's disease- associated Abeta oligomers. J Clin Invest 122:1339-1353.

Brenner SR (2008) Neurovascular unit dysfunction: a vascular component of Alzheimer disease? Neurology 70:243-244.

Brussee V, Cunningham FA, Zochodne DW (2004) Direct insulin signaling of neurons reverses diabetic neuropathy. Diabetes 53:1824-1830.

Buchholz JN, Behringer EJ, Pottorf WJ, Pearce WJ, Vanterpool CK (2007) Age-dependent changes in Ca2+ homeostasis in peripheral neurones: implications for changes in function. Aging Cell 6:285-296.

Caccamo A, Majumder S, Richardson A, Strong R, Oddo S (2010) Molecular interplay between mammalian target of rapamycin (mTOR), amyloid-beta, and Tau: effects on cognitive impairments. J Biol Chem 285:13107-13120.

Cagalinec M, Safiulina D, Liiv M, Liiv J, Choubey V, Wareski P, Veksler V, Kaasik A (2013) Principles of the mitochondrial fusion and fission cycle in neurons. J Cell Sci 126:2187-2197.

Camandola S, Mattson MP (2011) Aberrant subcellular neuronal calcium regulation in aging and Alzheimer's disease. Biochim Biophys Acta 1813:965-973.

Candeias E, Duarte AI, Carvalho C, Correia SC, Cardoso S, Santos RX, Placido AI, Perry G, Moreira PI (2012) The impairment of insulin signaling in Alzheimer's disease. IUBMB Life 64:951-957.

Cardoso S, Correia S, Santos RX, Carvalho C, Santos MS, Oliveira CR, Perry G, Smith MA, Zhu X, Moreira PI (2009) Insulin is a two-edged knife on the brain. J Alzheimers Dis 18:483-507.

Cardoso S, Santos MS, Seica R, Moreira PI (2010) Cortical and hippocampal mitochondria bioenergetics and oxidative status during hyperglycemia and/or insulin-induced hypoglycemia. Biochim Biophys Acta 1802:942-951.

Cardoso S, Santos RX, Correia SC, Carvalho C, Santos MS, Baldeiras I, Oliveira CR, Moreira PI (2012) Insulin-induced recurrent hypoglycemia exacerbates diabetic brain mitochondrial dysfunction and oxidative imbalance. Neurobiol Dis 49C:1-12.

Carreras MC, Franco MC, Peralta JG, Poderoso JJ (2004) Nitric oxide, complex I, and the modulation of mitochondrial reactive species in biology and disease. Mol Aspects Med 25:125-139.

Carvalho C, Cardoso S, Correia SC, Santos RX, Santos MS, Baldeiras I, Oliveira CR, Moreira PI (2012) Metabolic alterations induced by sucrose intake and Alzheimer's disease promote similar brain mitochondrial abnormalities. Diabetes 61:1234-1242.

Carvalho C, Machado N, Mota PC, Correia SC, Cardoso S, Santos RX, Santos MS, Oliveira CR, Moreira PI (2013) Type 2 diabetic and Alzheimer's disease mice present similar behavioral, cognitive, and vascular anomalies. J Alzheimers Dis 35:623-635.

Castellani RJ, Rolston RK, Smith MA (2010) Alzheimer disease. Dis Mon 56:484-546.

Celsi F, Pizzo P, Brini M, Leo S, Fotino C, Pinton P, Rizzuto R (2009) Mitochondria, calcium and cell death: a deadly triad in neurodegeneration. Biochim Biophys Acta 1787:335-344.

Chan DC (2006a) Mitochondria: dynamic organelles in disease, aging, and development. Cell 125:1241-1252.

Chan DC (2006b) Mitochondrial fusion and fission in mammals. Annu Rev Cell Dev Biol 22:79-99.

Chan EY, Kir S, Tooze SA (2007) siRNA screening of the kinome identifies ULK1 as a multidomain modulator of autophagy. J Biol Chem 282:25464-25474.

Chance B, Sies H, Boveris A (1979) Hydroperoxide metabolism in mammalian organs. Physiol Rev 59:527-605.

Chance B, Williams GR (1956) The respiratory chain and oxidative phosphorylation. Adv Enzymol Relat Subj Biochem 17:65-134.

Chang CR, Blackstone C (2007) Cyclic AMP-dependent protein kinase phosphorylation of Drp1 regulates its GTPase activity and mitochondrial morphology. J Biol Chem 282:21583-21587.

Chen H, Chan DC (2009) Mitochondrial dynamics--fusion, fission, movement, and mitophagy--in neurodegenerative diseases. Hum Mol Genet 18:R169-176.

Chen H, Chomyn A, Chan DC (2005) Disruption of fusion results in mitochondrial heterogeneity and dysfunction. J Biol Chem 280:26185-26192.

Chen H, Detmer SA, Ewald AJ, Griffin EE, Fraser SE, Chan DC (2003) Mitofusins Mfn1 and Mfn2 coordinately regulate mitochondrial fusion and are essential for embryonic development. J Cell Biol 160:189-200.

Chen L, Magliano DJ, Zimmet PZ (2012) The worldwide epidemiology of type 2 diabetes mellitus--present and future perspectives. Nat Rev Endocrinol 8:228-236.

Cherra SJ, 3rd, Chu CT (2008) Autophagy in neuroprotection and neurodegeneration: A question of balance. Future Neurol 3:309-323.

Cho DH, Nakamura T, Fang J, Cieplak P, Godzik A, Gu Z, Lipton SA (2009) S-nitrosylation of Drp1 mediates beta-amyloid-related mitochondrial fission and neuronal injury. Science 324:102-105.

Chu CT (2006) Autophagic stress in neuronal injury and disease. J Neuropathol Exp Neurol 65:423-432.

Chung SH (2009) Aberrant phosphorylation in the pathogenesis of Alzheimer's disease. BMB Rep 42:467-474.

Cipolat S, Martins de Brito O, Dal Zilio B, Scorrano L (2004) OPA1 requires mitofusin 1 to promote mitochondrial fusion. Proc Natl Acad Sci U S A 101:15927-15932.

Cole AR, Astell A, Green C, Sutherland C (2007) Molecular connexions between dementia and diabetes. Neurosci Biobehav Rev 31:1046-1063.

Cooke DW, Plotnick L (2008) Type 1 diabetes mellitus in pediatrics. Pediatr Rev 29:374-384; quiz 385.

Copp J, Manning G, Hunter T (2009) TORC-specific phosphorylation of mammalian target of rapamycin (mTOR): phospho-Ser2481 is a marker for intact mTOR signaling complex 2. Cancer Res 69:1821-1827.

Correia SC, Santos RX, Cardoso S, Carvalho C, Candeias E, Duarte AI, Placido AI, Santos MS, Moreira PI (2012b) Alzheimer disease as a vascular disorder: where do mitochondria fit? Exp Gerontol 47:878-886.

Correia SC, Santos RX, Cardoso SM, Santos MS, Oliveira CR, Moreira PI (2012c) Cyanide preconditioning protects brain endothelial and NT2 neuron-like cells against glucotoxicity: role of mitochondrial reactive oxygen species and HIF-1alpha. Neurobiol Dis 45:206-218.

Correia SC, Santos RX, Carvalho C, Cardoso S, Candeias E, Santos MS, Oliveira CR, Moreira PI (2012a) Insulin signaling, glucose metabolism and mitochondria: major players in Alzheimer's disease and diabetes interrelation. Brain Res 1441:64-78.

Correia SC, Santos RX, Santos MS, Casadesus G, Lamanna JC, Perry G, Smith MA, Moreira PI (2013) Mitochondrial abnormalities in a streptozotocin-induced rat model of sporadic Alzheimer's disease. Curr Alzheimer Res 10:406-419.

Craft S, Asthana S, Cook DG, Baker LD, Cherrier M, Purganan K, Wait C, Petrova A, Latendresse S, Watson GS, Newcomer JW, Schellenberg GD, Krohn AJ (2003) Insulin dose-response effects on memory and plasma amyloid precursor protein in Alzheimer's disease: interactions with apolipoprotein E genotype. Psychoneuroendocrinology 28:809-822.

Craft S, Asthana S, Newcomer JW, Wilkinson CW, Matos IT, Baker LD, Cherrier M, Lofgreen C, Latendresse S, Petrova A, Plymate S, Raskind M, Grimwood K, Veith RC (1999a) Enhancement of memory in Alzheimer disease with insulin and somatostatin, but not glucose. Arch Gen Psychiatry 56:1135-1140.

Craft S, Asthana S, Schellenberg G, Cherrier M, Baker LD, Newcomer J, Plymate S, Latendresse S, Petrova A, Raskind M, Peskind E, Lofgreen C, Grimwood K (1999b) Insulin metabolism in Alzheimer's disease differs according to apolipoprotein E genotype and gender. Neuroendocrinology 70:146-152.

Craft S, Baker LD, Montine TJ, Minoshima S, Watson GS, Claxton A, Arbuckle M, Callaghan M, Tsai E, Plymate SR, Green PS, Leverenz J, Cross D, Gerton B (2012) Intranasal insulin therapy for Alzheimer disease and amnestic mild cognitive impairment: a pilot clinical trial. Arch Neurol 69:29-38.

Craft S, Newcomer J, Kanne S, Dagogo-Jack S, Cryer P, Sheline Y, Luby J, Dagogo-Jack A, Alderson A (1996) Memory improvement following induced hyperinsulinemia in Alzheimer's disease. Neurobiol Aging 17:123-130.

Craft S, Watson GS (2004) Insulin and neurodegenerative disease: shared and specific mechanisms. Lancet Neurol 3:169-178.

Crane FL (1989) Remarks on the discovery of coenzyme Q. Biochim Biophys Acta 1000:359-361.

Cribbs JT, Strack S (2007) Reversible phosphorylation of Drp1 by cyclic AMP-dependent protein kinase and calcineurin regulates mitochondrial fission and cell death. EMBO Rep 8:939-944.

Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA (1995) Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature 378:785-789.

Cuervo AM (2004) Autophagy: many paths to the same end. Mol Cell Biochem 263:55-72.

De Stefani D, Raffaello A, Teardo E, Szabo I, Rizzuto R (2011) A forty-kilodalton protein of the inner membrane is the mitochondrial calcium uniporter. Nature 476:336-340.

DeJong RN (1977) CNS manifestations of diabetes mellitus. Postgrad Med 61:101-107.

Detmer SA, Chan DC (2007) Functions and dysfunctions of mitochondrial dynamics. Nat Rev Mol Cell Biol 8:870-879.

Devaskar SU, Singh BS, Carnaghi LR, Rajakumar PA, Giddings SJ (1993) Insulin II gene expression in rat central nervous system. Regul Pept 48:55-63.

Dickinson BC, Chang CJ (2011) Chemistry and biology of reactive oxygen species in signaling or stress responses. Nat Chem Biol 7:504-511.

Dore S, Kar S, Quirion R (1997) Insulin-like growth factor I protects and rescues hippocampal neurons against beta-amyloid- and human amylin-induced toxicity. Proc Natl Acad Sci U S A 94:4772-4777.

Droge W (2002) Free radicals in the physiological control of cell function. Physiol Rev 82:47-95.

Duarte AI, Moreira PI, Oliveira CR (2012) Insulin in central nervous system: more than just a peripheral hormone. J Aging Res 2012:384017.

DuBoff B, Feany M, Gotz J (2013) Why size matters - balancing mitochondrial dynamics in Alzheimer's disease. Trends Neurosci 36:325-335.

DuBoff B, Gotz J, Feany MB (2012) Tau promotes neurodegeneration via DRP1 mislocalization in vivo. Neuron 75:618-632.

Duvezin-Caubet S, Jagasia R, Wagener J, Hofmann S, Trifunovic A, Hansson A, Chomyn A, Bauer MF, Attardi G, Larsson NG, Neupert W, Reichert AS (2006) Proteolytic processing of OPA1 links mitochondrial dysfunction to alterations in mitochondrial morphology. J Biol Chem 281:37972-37979.

Edwards JL, Quattrini A, Lentz SI, Figueroa-Romero C, Cerri F, Backus C, Hong Y, Feldman EL (2010) Diabetes regulates mitochondrial biogenesis and fission in mouse neurons. Diabetologia 53:160-169.

Ehses S, Raschke I, Mancuso G, Bernacchia A, Geimer S, Tondera D, Martinou JC, Westermann B, Rugarli EI, Langer T (2009) Regulation of OPA1 processing and mitochondrial fusion by m-AAA protease isoenzymes and OMA1. J Cell Biol 187:1023-1036.

El Messari S, Ait-Ikhlef A, Ambroise DH, Penicaud L, Arluison M (2002) Expression of insulin-responsive glucose transporter GLUT4 mRNA in the

rat brain and spinal cord: an in situ hybridization study. J Chem Neuroanat 24:225-242.

Eskelinen EL (2005) Maturation of autophagic vacuoles in Mammalian cells. Autophagy 1:1-10.

Estabrook RE (1967) Mitochondrial respiratory control and the polarographic measurement of ADP/O ratios. Methods Enzymol 10:41-47.

Evans DB, Rank KB, Bhattacharya K, Thomsen DR, Gurney ME, Sharma SK (2000) Tau phosphorylation at serine 396 and serine 404 by human recombinant tau protein kinase II inhibits tau's ability to promote microtubule assembly. J Biol Chem 275:24977-24983.

Evans JL, Goldfine ID, Maddux BA, Grodsky GM (2002) Oxidative stress and stress-activated signaling pathways: a unifying hypothesis of type 2 diabetes. Endocr Rev 23:599-622.

Facecchia K, Fochesato LA, Ray SD, Stohs SJ, Pandey S (2011) Oxidative toxicity in neurodegenerative diseases: role of mitochondrial dysfunction and therapeutic strategies. J Toxicol 2011:683728.

Fang X, Yu S, Eder A, Mao M, Bast RC, Jr., Boyd D, Mills GB (1999) Regulation of BAD phosphorylation at serine 112 by the Ras-mitogen-activated protein kinase pathway. Oncogene 18:6635-6640.

Farris W, Mansourian S, Chang Y, Lindsley L, Eckman EA, Frosch MP, Eckman CB, Tanzi RE, Selkoe DJ, Guenette S (2003) Insulin-degrading enzyme regulates the levels of insulin, amyloid beta-protein, and the beta-amyloid precursor protein intracellular domain in vivo. Proc Natl Acad Sci U S A 100:4162-4167.

Fernyhough P, Roy Chowdhury SK, Schmidt RE (2010) Mitochondrial stress and the pathogenesis of diabetic neuropathy. Expert Rev Endocrinol Metab 5:39-49.

Ferreira FM, Palmeira CM, Seica R, Santos MS (1999) Alterations of liver mitochondrial bioenergetics in diabetic Goto-Kakizaki rats. Metabolism 48:1115-1119.

Francis G, Martinez J, Liu W, Nguyen T, Ayer A, Fine J, Zochodne D, Hanson LR, Frey WH, 2nd, Toth C (2009) Intranasal insulin ameliorates experimental diabetic neuropathy. Diabetes 58:934-945.

Frank S, Gaume B, Bergmann-Leitner ES, Leitner WW, Robert EG, Catez F, Smith CL, Youle RJ (2001) The role of dynamin-related protein 1, a mediator of mitochondrial fission, in apoptosis. Dev Cell 1:515-525.

Fridovich I (1995) Superoxide radical and superoxide dismutases. Annu Rev Biochem 64:97-112.

Frieden M, James D, Castelbou C, Danckaert A, Martinou JC, Demaurex N (2004) Ca(2+) homeostasis during mitochondrial fragmentation and perinuclear clustering induced by hFis1. J Biol Chem 279:22704-22714.

Frojdo S, Vidal H, Pirola L (2009) Alterations of insulin signaling in type 2 diabetes: a review of the current evidence from humans. Biochim Biophys Acta 1792:83-92.

Fuke S, Kubota-Sakashita M, Kasahara T, Shigeyoshi Y, Kato T (2011) Regional variation in mitochondrial DNA copy number in mouse brain. Biochim Biophys Acta 1807:270-274.

Gan MJ, Albanese-O'Neill A, Haller MJ (2012) Type 1 diabetes: current concepts in epidemiology, pathophysiology, clinical care, and research. Curr Probl Pediatr Adolesc Health Care 42:269-291.

Gandre-Babbe S, van der Bliek AM (2008) The novel tail-anchored membrane protein Mff controls mitochondrial and peroxisomal fission in mammalian cells. Mol Biol Cell 19:2402-2412.

Garrido GE, Furuie SS, Buchpiguel CA, Bottino CM, Almeida OP, Cid CG, Camargo CH, Castro CC, Glabus MF, Busatto GF (2002) Relation between medial temporal atrophy and functional brain activity during memory processing in Alzheimer's disease: a combined MRI and SPECT study. J Neurol Neurosurg Psychiatry 73:508-516.

Gomes LC, Di Benedetto G, Scorrano L (2011) During autophagy mitochondria elongate, are spared from degradation and sustain cell viability. Nat Cell Biol 13:589-598.

Gornall AG, Bardawill CJ, David MM (1949) Determination of serum proteins by means of the biuret reaction. J Biol Chem 177:751-766.

Goto Y, Kakizaki M (1981) The spontaneous diabetic rat a model of non-insulin dependent diabetes mellitus. Proc Jpn Acad 57:381-384.

Griparic L, Kanazawa T, van der Bliek AM (2007) Regulation of the mitochondrial dynamin-like protein Opa1 by proteolytic cleavage. J Cell Biol 178:757-764.

Grunblatt E, Salkovic-Petrisic M, Osmanovic J, Riederer P, Hoyer S (2007) Brain insulin system dysfunction in streptozotocin intracerebroventricularly treated rats generates hyperphosphorylated tau protein. J Neurochem 101:757-770.

Guillery O, Malka F, Landes T, Guillou E, Blackstone C, Lombes A, Belenguer P, Arnoult D, Rojo M (2008) Metalloprotease-mediated OPA1 processing is modulated by the mitochondrial membrane potential. Biol Cell 100:315-325.

Haass C, Selkoe DJ (2007) Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer's amyloid beta-peptide. Nat Rev Mol Cell Biol 8:101-112.

Haider S, Ahmed S, Tabassum S, Memon Z, Ikram M, Haleem DJ (2013) Streptozotocin-induced insulin deficiency leads to development of behavioral deficits in rats. Acta Neurol Belg 113:35-41.

Han XJ, Tomizawa K, Fujimura A, Ohmori I, Nishiki T, Matsushita M, Matsui H (2011) Regulation of mitochondrial dynamics and neurodegenerative diseases. Acta Med Okayama 65:1-10.

Hansson CA, Frykman S, Farmery MR, Tjernberg LO, Nilsberth C, Pursglove SE, Ito A, Winblad B, Cowburn RF, Thyberg J, Ankarcrona M (2004) Nicastrin, presenilin, APH-1, and PEN-2 form active gamma-secretase complexes in mitochondria. J Biol Chem 279:51654-51660.

Harder Z, Zunino R, McBride H (2004) Sumo1 conjugates mitochondrial substrates and participates in mitochondrial fission. Curr Biol 14:340-345.

Harr SD, Simonian NA, Hyman BT (1995) Functional alterations in Alzheimer's disease: decreased glucose transporter 3 immunoreactivity in the perforant pathway terminal zone. J Neuropathol Exp Neurol 54:38-41.

Hauptmann S, Scherping I, Drose S, Brandt U, Schulz KL, Jendrach M, Leuner K, Eckert A, Muller WE (2009) Mitochondrial dysfunction: an early event in

Alzheimer pathology accumulates with age in AD transgenic mice. Neurobiol Aging 30:1574-1586.

Hengartner MO (2000) The biochemistry of apoptosis. Nature 407:770-776.

Hill BG, Dranka BP, Bailey SM, Lancaster JR, Jr., Darley-Usmar VM (2010) What part of NO don't you understand? Some answers to the cardinal questions in nitric oxide biology. J Biol Chem 285:19699-19704.

Ho L, Qin W, Pompl PN, Xiang Z, Wang J, Zhao Z, Peng Y, Cambareri G, Rocher A, Mobbs CV, Hof PR, Pasinetti GM (2004) Diet-induced insulin resistance promotes amyloidosis in a transgenic mouse model of Alzheimer's disease. FASEB J 18:902-904.

Holmgren A (2000) Antioxidant function of thioredoxin and glutaredoxin systems. Antioxid Redox Signal 2:811-820.

Hong M, Lee VM (1997) Insulin and insulin-like growth factor-1 regulate tau phosphorylation in cultured human neurons. J Biol Chem 272:19547-19553.

Hoppins S, Lackner L, Nunnari J (2007) The machines that divide and fuse mitochondria. Annu Rev Biochem 76:751-780.

Hoyer S, Nitsch R (1989) Cerebral excess release of neurotransmitter amino acids subsequent to reduced cerebral glucose metabolism in early-onset dementia of Alzheimer type. J Neural Transm 75:227-232.

http://www.idf.org/diabetesatlas/5e/healthcare-expenditures accessed July, 2013.

http://www.who.int/mediacentre/factsheets/fs312/en/index.html accessed July, 2013.

Humpel C, Marksteiner J (2005) Cerebrovascular damage as a cause for Alzheimer's disease. Curr Neurovasc Res 2:341-347.

Ishihara N, Fujita Y, Oka T, Mihara K (2006) Regulation of mitochondrial morphology through proteolytic cleavage of OPA1. EMBO J 25:2966-2977.

Iwakami S, Misu H, Takeda T, Sugimori M, Matsugo S, Kaneko S, Takamura T (2011) Concentration-dependent dual effects of hydrogen peroxide on insulin signal transduction in H4IIEC hepatocytes. PLoS One 6:e27401.

Izumi Y, Yamada KA, Matsukawa M, Zorumski CF (2003) Effects of insulin on long-term potentiation in hippocampal slices from diabetic rats. Diabetologia 46:1007-1012.

J SR-F, Sa-Roriz TM, Rosset I, Camozzato AL, Santos AC, Chaves ML, Moriguti JC, Roriz-Cruz M (2009) (Pre)diabetes, brain aging, and cognition. Biochim Biophys Acta 1792:432-443.

Jager S, Bucci C, Tanida I, Ueno T, Kominami E, Saftig P, Eskelinen EL (2004) Role for Rab7 in maturation of late autophagic vacuoles. J Cell Sci 117:4837-4848.

James DI, Parone PA, Mattenberger Y, Martinou JC (2003) hFis1, a novel component of the mammalian mitochondrial fission machinery. J Biol Chem 278:36373-36379.

Jensen BD, Gunter TR (1984) The use of tertaphenylphosphonium (TPP+) to measure membrane potentials in mitochondria: membrane binding and respiratory effects. Biophys J 45:92.

Johnson GV, Jope RS, Binder LI (1989) Proteolysis of tau by calpain. Biochem Biophys Res Commun 163:1505-1511.

Jolivalt CG, Hurford R, Lee CA, Dumaop W, Rockenstein E, Masliah E (2010) Type 1 diabetes exaggerates features of Alzheimer's disease in APP transgenic mice. Exp Neurol 223:422-431.

Jolivalt CG, Lee CA, Beiswenger KK, Smith JL, Orlov M, Torrance MA, Masliah E (2008) Defective insulin signaling pathway and increased glycogen synthase kinase-3 activity in the brain of diabetic mice: parallels with Alzheimer's disease and correction by insulin. J Neurosci Res 86:3265-3274.

Kaidanovich O, Eldar-Finkelman H (2002) The role of glycogen synthase kinase-3 in insulin resistance and type 2 diabetes. Expert Opin Ther Targets 6:555-561.

Kamada Y, Funakoshi T, Shintani T, Nagano K, Ohsumi M, Ohsumi Y (2000) Tor-mediated induction of autophagy via an Apg1 protein kinase complex. J Cell Biol 150:1507-1513.

Kamo N, Muratsugu M, Hongoh R, Kobatake Y (1979) Membrane potential of mitochondria measured with an electrode sensitive to tetraphenyl phosphonium and relationship between proton electrochemical potential and phosphorylation potential in steady state. J Membr Biol 49:105-121.

Kang C, Avery L (2008) To be or not to be, the level of autophagy is the question: dual roles of autophagy in the survival response to starvation. Autophagy 4:82-84.

Kann O, Kovacs R (2007) Mitochondria and neuronal activity. Am J Physiol Cell Physiol 292:C641-657.

Keil U, Bonert A, Marques CA, Scherping I, Weyermann J, Strosznajder JB, Muller-Spahn F, Haass C, Czech C, Pradier L, Muller WE, Eckert A (2004) Amyloid

beta-induced changes in nitric oxide production and mitochondrial activity lead to apoptosis. J Biol Chem 279:50310-50320.

Kern W, Peters A, Fruehwald-Schultes B, Deininger E, Born J, Fehm HL (2001) Improving influence of insulin on cognitive functions in humans. Neuroendocrinology 74:270-280.

Kim B, Feldman EL (2012) Insulin resistance in the nervous system. Trends Endocrinol Metab 23:133-141.

Kim B, McLean LL, Philip SS, Feldman EL (2011) Hyperinsulinemia induces insulin resistance in dorsal root ganglion neurons. Endocrinology 152:3638-3647.

King AJ (2012) The use of animal models in diabetes research. Br J Pharmacol 166:877-894.

Klionsky DJ (2007) Autophagy: from phenomenology to molecular understanding in less than a decade. Nat Rev Mol Cell Biol 8:931-937.

Klionsky DJ, Abdalla FC, Abeliovich H, Abraham RT, Acevedo-Arozena A, Adeli K, Agholme L, Agnello M, Agostinis P, Aguirre-Ghiso JA, Ahn HJ, Ait-Mohamed O, Ait-Si-Ali S, Akematsu T, Akira S, Al-Younes HM, Al-Zeer MA, Albert ML, Albin RL, Alegre-Abarrategui J, Aleo MF, Alirezaei M, Almasan A, Almonte-Becerril M, Amano A, Amaravadi R, Amarnath S, Amer AO, Andrieu-Abadie N, Anantharam V, Ann DK, Anoopkumar-Dukie S, Aoki H, Apostolova N, Arancia G, Aris JP, Asanuma K, Asare NY, Ashida H, Askanas V, Askew DS, Auberger P, Baba M, Backues SK, Baehrecke EH, Bahr BA, Bai XY, Bailly Y, Baiocchi R, Baldini G, Balduini W, Ballabio A, Bamber BA, Bampton ET, Banhegyi G, Bartholomew CR, Bassham DC, Bast RC, Jr., Batoko H, Bay BH, Beau I, Bechet DM, Begley TJ, Behl C, Behrends C, Bekri

S, Bellaire B, Bendall LJ, Benetti L, Berliocchi L, Bernardi H, Bernassola F, Besteiro S, Bhatia-Kissova I, Bi X, Biard-Piechaczyk M, Blum JS, Boise LH, Bonaldo P, Boone DL, Bornhauser BC, Bortoluci KR, Bossis I, Bost F, Bourquin JP, Boya P, Boyer-Guittaut M, Bozhkov PV, Brady NR, Brancolini C, Brech A, Brenman JE, Brennand A, Bresnick EH, Brest P, Bridges D, Bristol ML, Brookes PS, Brown EJ, Brumell JH, Brunetti-Pierri N, Brunk UT, Bulman DE, Bultman SJ, Bultynck G, Burbulla LF, Bursch W, Butchar JP, Buzgariu W, Bydlowski SP, Cadwell K, Cahova M, Cai D, Cai J, Cai Q, Calabretta B, Calvo-Garrido J, Camougrand N, Campanella M, Campos-Salinas J, Candi E, Cao L, Caplan AB, Carding SR, Cardoso SM, Carew JS, Carlin CR, Carmignac V, Carneiro LA, Carra S, Caruso RA, Casari G, Casas C, Castino R, Cebollero E, Cecconi F, Celli J, Chaachouay H, Chae HJ, Chai CY, Chan DC, Chan EY, Chang RC, Che CM, Chen CC, Chen GC, Chen GQ, Chen M, Chen Q, Chen SS, Chen W, Chen X, Chen YG, Chen Y, Chen YJ, Chen Z, Cheng A, Cheng CH, Cheng Y, Cheong H, Cheong JH, Cherry S, Chess-Williams R, Cheung ZH, Chevet E, Chiang HL, Chiarelli R, Chiba T, Chin LS, Chiou SH, Chisari FV, Cho CH, Cho DH, Choi AM, Choi D, Choi KS, Choi ME, Chouaib S, Choubey D, Choubey V, Chu CT, Chuang TH, Chueh SH, Chun T, Chwae YJ, Chye ML, Ciarcia R, Ciriolo MR, Clague MJ, Clark RS, Clarke PG, Clarke R, Codogno P, Coller HA, Colombo MI, Comincini S, Condello M, Condorelli F, Cookson MR, Coombs GH, Coppens I, Corbalan R, Cossart P, Costelli P, Costes S, Coto-Montes A, Couve E, Coxon FP, Cregg JM, Crespo JL, Cronje MJ, Cuervo AM, Cullen JJ, Czaja MJ, D'Amelio M, Darfeuille-Michaud A, Davids LM, Davies FE, De Felici M, de Groot JF, de Haan CA, De Martino L, De Milito A, De Tata V, Debnath J, Degterev A, Dehay B, Delbridge LM,

Demarchi F, Deng YZ, Dengjel J, Dent P, Denton D, Deretic V, Desai SD, Devenish RJ, Di Gioacchino M, Di Paolo G, Di Pietro C, Diaz-Araya G, Diaz-Laviada I, Diaz-Meco MT, Diaz-Nido J, Dikic I, Dinesh-Kumar SP, Ding WX, Distelhorst CW, Diwan A, Djavaheri-Mergny M, Dokudovskaya S, Dong Z, Dorsey FC, Dosenko V, Dowling JJ, Doxsey S, Dreux M, Drew ME, Duan Q, Duchosal MA, Duff K, Dugail I, Durbeej M, Duszenko M, Edelstein CL, Edinger AL, Egea G, Eichinger L, Eissa NT, Ekmekcioglu S, El-Deiry WS, Elazar Z, Elgendy M, Ellerby LM, Eng KE, Engelbrecht AM, Engelender S, Erenpreisa J, Escalante R, Esclatine A, Eskelinen EL, Espert L, Espina V, Fan H, Fan J, Fan QW, Fan Z, Fang S, Fang Y, Fanto M, Fanzani A, Farkas T, Farre JC, Faure M, Fechheimer M, Feng CG, Feng J, Feng Q, Feng Y, Fesus L, Feuer R, Figueiredo-Pereira ME, Fimia GM, Fingar DC, Finkbeiner S, Finkel T, Finley KD, Fiorito F, Fisher EA, Fisher PB, Flajolet M, Florez-McClure ML, Florio S, Fon EA, Fornai F, Fortunato F, Fotedar R, Fowler DH, Fox HS, Franco R, Frankel LB, Fransen M, Fuentes JM, Fueyo J, Fujii J, Fujisaki K, Fujita E, Fukuda M, Furukawa RH, Gaestel M, Gailly P, Gajewska M, Galliot B, Galy V, Ganesh S, Ganetzky B, Ganley IG, Gao FB, Gao GF, Gao J, Garcia L, Garcia-Manero G, Garcia-Marcos M, Garmyn M, Gartel AL, Gatti E, Gautel M, Gawriluk TR, Gegg ME, Geng J, Germain M, Gestwicki JE, Gewirtz DA, Ghavami S, Ghosh P, Giammarioli AM, Giatromanolaki AN, Gibson SB, Gilkerson RW, Ginger ML, Ginsberg HN, Golab J, Goligorsky MS, Golstein P, Gomez-Manzano C, Goncu E, Gongora C, Gonzalez CD, Gonzalez R, Gonzalez-Estevez C, Gonzalez-Polo RA, Gonzalez-Rey E, Gorbunov NV, Gorski S, Goruppi S, Gottlieb RA, Gozuacik D, Granato GE, Grant GD, Green KN, Gregorc A, Gros F, Grose C, Grunt TW, Gual P, Guan JL, Guan KL,

Guichard SM, Gukovskaya AS, Gukovsky I, Gunst J, Gustafsson AB, Halayko AJ, Hale AN, Halonen SK, Hamasaki M, Han F, Han T, Hancock MK, Hansen M, Harada H, Harada M, Hardt SE, Harper JW, Harris AL, Harris J, Harris SD, Hashimoto M, Haspel JA, Hayashi S, Hazelhurst LA, He C, He YW, Hebert MJ, Heidenreich KA, Helfrich MH, Helgason GV, Henske EP, Herman B, Herman PK, Hetz C, Hilfiker S, Hill JA, Hocking LJ, Hofman P, Hofmann TG, Hohfeld J, Holyoake TL, Hong MH, Hood DA, Hotamisligil GS, Houwerzijl EJ, Hoyer-Hansen M, Hu B, Hu CA, Hu HM, Hua Y, Huang C, Huang J, Huang S, Huang WP, Huber TB, Huh WK, Hung TH, Hupp TR, Hur GM, Hurley JB, Hussain SN, Hussey PJ, Hwang JJ, Hwang S, Ichihara A, Ilkhanizadeh S, Inoki K, Into T, Iovane V, Iovanna JL, Ip NY, Isaka Y, Ishida H, Isidoro C, Isobe K, Iwasaki A, Izquierdo M, Izumi Y, Jaakkola PM, Jaattela M, Jackson GR, Jackson WT, Janji B, Jendrach M, Jeon JH, Jeung EB, Jiang H, Jiang JX, Jiang M, Jiang Q, Jiang X, Jimenez A, Jin M, Jin S, Joe CO, Johansen T, Johnson DE, Johnson GV, Jones NL, Joseph B, Joseph SK, Joubert AM, Juhasz G, Juillerat-Jeanneret L, Jung CH, Jung YK, Kaarniranta K, Kaasik A, Kabuta T, Kadowaki M, Kagedal K, Kamada Y, Kaminskyy VO, Kampinga HH, Kanamori H, Kang C, Kang KB, Kang KI, Kang R, Kang YA, Kanki T, Kanneganti TD, Kanno H, Kanthasamy AG, Kanthasamy A, Karantza V, Kaushal GP, Kaushik S, Kawazoe Y, Ke PY, Kehrl JH, Kelekar A, Kerkhoff C, Kessel DH, Khalil H, Kiel JA, Kiger AA, Kihara A, Kim DR, Kim DH, Kim EK, Kim HR, Kim JS, Kim JH, Kim JC, Kim JK, Kim PK, Kim SW, Kim YS, Kim Y, Kimchi A, Kimmelman AC, King JS, Kinsella TJ, Kirkin V, Kirshenbaum LA, Kitamoto K, Kitazato K, Klein L, Klimecki WT, Klucken J, Knecht E, Ko BC, Koch JC, Koga H, Koh JY, Koh YH, Koike M, Komatsu M,

Kominami E, Kong HJ, Kong WJ, Korolchuk VI, Kotake Y, Koukourakis MI, Kouri Flores JB, Kovacs AL, Kraft C, Krainc D, Kramer H, Kretz-Remy C, Krichevsky AM, Kroemer G, Kruger R, Krut O, Ktistakis NT, Kuan CY, Kucharczyk R, Kumar A, Kumar R, Kumar S, Kundu M, Kung HJ, Kurz T, Kwon HJ, La Spada AR, Lafont F, Lamark T, Landry J, Lane JD, Lapaquette P, Laporte JF, Laszlo L, Lavandero S, Lavoie JN, Layfield R, Lazo PA, Le W, Le Cam L, Ledbetter DJ, Lee AJ, Lee BW, Lee GM, Lee J, Lee JH, Lee M, Lee MS, Lee SH, Leeuwenburgh C, Legembre P, Legouis R, Lehmann M, Lei HY, Lei QY, Leib DA, Leiro J, Lemasters JJ, Lemoine A, Lesniak MS, Lev D, Levenson VV, Levine B, Levy E, Li F, Li JL, Li L, Li S, Li W, Li XJ, Li YB, Li YP, Liang C, Liang Q, Liao YF, Liberski PP, Lieberman A, Lim HJ, Lim KL, Lim K, Lin CF, Lin FC, Lin J, Lin JD, Lin K, Lin WW, Lin WC, Lin YL, Linden R, Lingor P, Lippincott-Schwartz J, Lisanti MP, Liton PB, Liu B, Liu CF, Liu K, Liu L, Liu QA, Liu W, Liu YC, Liu Y, Lockshin RA, Lok CN, Lonial S, Loos B, Lopez-Berestein G, Lopez-Otin C, Lossi L, Lotze MT, Low P, Lu B, Lu Z, Luciano F, Lukacs NW, Lund AH, Lynch-Day MA, Ma Y, Macian F, MacKeigan JP, Macleod KF, Madeo F, Maiuri L, Maiuri MC, Malagoli D, Malicdan MC, Malorni W, Man N, Mandelkow EM, Manon S, Manov I, Mao K, Mao X, Mao Z, Marambaud P, Marazziti D, Marcel YL, Marchbank K, Marchetti P, Marciniak SJ, Marcondes M, Mardi M, Marfe G, Marino G, Markaki M, Marten MR, Martin SJ, Martinand-Mari C, Martinet W, Martinez-Vicente M, Masini M, Matarrese P, Matsuo S, Matteoni R, Mayer A, Mazure NM, McConkey DJ, McConnell MJ, McDermott C, McDonald C, McInerney GM, McKenna SL, McLaughlin B, McLean PJ, McMaster CR, McQuibban GA, Meijer AJ, Meisler MH, Melendez A, Melia TJ, Melino G,

Mena MA, Menendez JA, Menna-Barreto RF, Menon MB, Menzies FM, Mercer CA, Merighi A, Merry DE, Meschini S, Meyer CG, Meyer TF, Miao CY, Miao JY, Michels PA, Michiels C, Mijaljica D, Milojkovic A, Minucci S, Miracco C, Miranti CK, Mitroulis I, Miyazawa K, Mizushima N, Mograbi B, Mohseni S, Molero X, Mollereau B, Mollinedo F, Momoi T, Monastyrska I, Monick MM, Monteiro MJ, Moore MN, Mora R, Moreau K, Moreira PI, Moriyasu Y, Moscat J, Mostowy S, Mottram JC, Motyl T, Moussa CE, Muller S, Munger K, Munz C, Murphy LO, Murphy ME, Musaro A, Mysorekar I, Nagata E, Nagata K, Nahimana A, Nair U, Nakagawa T, Nakahira K, Nakano H, Nakatogawa H, Nanjundan M, Naqvi NI, Narendra DP, Narita M, Navarro M, Nawrocki ST, Nazarko TY, Nemchenko A, Netea MG, Neufeld TP, Ney PA, Nezis IP, Nguyen HP, Nie D, Nishino I, Nislow C, Nixon RA, Noda T, Noegel AA, Nogalska A, Noguchi S, Notterpek L, Novak I, Nozaki T, Nukina N, Nurnberger T, Nyfeler B, Obara K, Oberley TD, Oddo S, Ogawa M, Ohashi T, Okamoto K, Oleinick NL, Oliver FJ, Olsen LJ, Olsson S, Opota O, Osborne TF, Ostrander GK, Otsu K, Ou JH, Ouimet M, Overholtzer M, Ozpolat B, Paganetti P, Pagnini U, Pallet N, Palmer GE, Palumbo C, Pan T, Panaretakis T, Pandey UB, Papackova Z, Papassideri I, Paris I, Park J, Park OK, Parys JB, Parzych KR, Patschan S, Patterson C, Pattingre S, Pawelek JM, Peng J, Perlmutter DH, Perrotta I, Perry G, Pervaiz S, Peter M, Peters GJ, Petersen M, Petrovski G, Phang JM, Piacentini M, Pierre P, Pierrefite-Carle V, Pierron G, Pinkas-Kramarski R, Piras A, Piri N, Platanias LC, Poggeler S, Poirot M, Poletti A, Pous C, Pozuelo-Rubio M, Praetorius-Ibba M, Prasad A, Prescott M, Priault M, Produit-Zengaffinen N, Progulske-Fox A, Proikas-Cezanne T, Przedborski S, Przyklenk K, Puertollano R, Puyal J, Qian SB, Qin

L, Qin ZH, Quaggin SE, Raben N, Rabinowich H, Rabkin SW, Rahman I, Rami A, Ramm G, Randall G, Randow F, Rao VA, Rathmell JC, Ravikumar B, Ray SK, Reed BH, Reed JC, Reggiori F, Regnier-Vigouroux A, Reichert AS, Reiners JJ, Jr., Reiter RJ, Ren J, Revuelta JL, Rhodes CJ, Ritis K, Rizzo E, Robbins J, Roberge M, Roca H, Roccheri MC, Rocchi S, Rodemann HP, Rodriguez de Cordoba S, Rohrer B, Roninson IB, Rosen K, Rost-Roszkowska MM, Rouis M, Rouschop KM, Rovetta F, Rubin BP, Rubinsztein DC, Ruckdeschel K, Rucker EB, 3rd, Rudich A, Rudolf E, Ruiz-Opazo N, Russo R, Rusten TE, Ryan KM, Ryter SW, Sabatini DM, Sadoshima J, Saha T, Saitoh T, Sakagami H, Sakai Y, Salekdeh GH, Salomoni P, Salvaterra PM, Salvesen G, Salvioli R, Sanchez AM, Sanchez-Alcazar JA, Sanchez-Prieto R, Sandri M, Sankar U, Sansanwal P, Santambrogio L, Saran S, Sarkar S, Sarwal M, Sasakawa C, Sasnauskiene A, Sass M, Sato K, Sato M, Schapira AH, Scharl M, Schatzl HM, Scheper W, Schiaffino S, Schneider C, Schneider ME, Schneider-Stock R, Schoenlein PV, Schorderet DF, Schuller C, Schwartz GK, Scorrano L, Sealy L, Seglen PO, Segura-Aguilar J, Seiliez I, Seleverstov O, Sell C, Seo JB, Separovic D, Setaluri V, Setoguchi T, Settembre C, Shacka JJ, Shanmugam M, Shapiro IM, Shaulian E, Shaw RJ, Shelhamer JH, Shen HM, Shen WC, Sheng ZH, Shi Y, Shibuya K, Shidoji Y, Shieh JJ, Shih CM, Shimada Y, Shimizu S, Shintani T, Shirihai OS, Shore GC, Sibirny AA, Sidhu SB, Sikorska B, Silva-Zacarin EC, Simmons A, Simon AK, Simon HU, Simone C, Simonsen A, Sinclair DA, Singh R, Sinha D, Sinicrope FA, Sirko A, Siu PM, Sivridis E, Skop V, Skulachev VP, Slack RS, Smaili SS, Smith DR, Soengas MS, Soldati T, Song X, Sood AK, Soong TW, Sotgia F, Spector SA, Spies CD, Springer W, Srinivasula SM, Stefanis L, Steffan JS,

Stendel R, Stenmark H, Stephanou A, Stern ST, Sternberg C, Stork B, Stralfors P, Subauste CS, Sui X, Sulzer D, Sun J, Sun SY, Sun ZJ, Sung JJ, Suzuki K, Suzuki T, Swanson MS, Swanton C, Sweeney ST, Sy LK, Szabadkai G, Tabas I, Taegtmeyer H, Tafani M, Takacs-Vellai K, Takano Y, Takegawa K, Takemura G, Takeshita F, Talbot NJ, Tan KS, Tanaka K, Tang D, Tanida I, Tannous BA, Tavernarakis N, Taylor GS, Taylor GA, Taylor JP, Terada LS, Terman A, Tettamanti G, Thevissen K, Thompson CB, Thorburn A, Thumm M, Tian F, Tian Y, Tocchini-Valentini G, Tolkovsky AM, Tomino Y, Tonges L, Tooze SA, Tournier C, Tower J, Towns R, Trajkovic V, Travassos LH, Tsai TF, Tschan MP, Tsubata T, Tsung A, Turk B, Turner LS, Tyagi SC, Uchiyama Y, Ueno T, Umekawa M, Umemiya-Shirafuji R, Unni VK, Vaccaro MI, Valente EM, Van den Berghe G, van der Klei IJ, van Doorn W, van Dyk LF, van Egmond M, van Grunsven LA, Vandenabeele P, Vandenberghe WP, Vanhorebeek I, Vaquero EC, Velasco G, Vellai T, Vicencio JM, Vierstra RD, Vila M, Vindis C, Viola G, Viscomi MT, Voitsekhovskaja OV, von Haefen C, Votruba M, Wada K, Wade-Martins R, Walker CL, Walsh CM, Walter J, Wan XB, Wang A, Wang C, Wang D, Wang F, Wang G, Wang H, Wang HG, Wang HD, Wang J, Wang K, Wang M, Wang RC, Wang X, Wang YJ, Wang Y, Wang Z, Wang ZC, Wansink DG, Ward DM, Watada H, Waters SL, Webster P, Wei L, Weihl CC, Weiss WA, Welford SM, Wen LP, Whitehouse CA, Whitton JL, Whitworth AJ, Wileman T, Wiley JW, Wilkinson S, Willbold D, Williams RL, Williamson PR, Wouters BG, Wu C, Wu DC, Wu WK, Wyttenbach A, Xavier RJ, Xi Z, Xia P, Xiao G, Xie Z, Xu DZ, Xu J, Xu L, Xu X, Yamamoto A, Yamashina S, Yamashita M, Yan X, Yanagida M, Yang DS, Yang E, Yang JM, Yang SY, Yang W, Yang WY, Yang Z, Yao MC,

Yao TP, Yeganeh B, Yen WL, Yin JJ, Yin XM, Yoo OJ, Yoon G, Yoon SY, Yorimitsu T, Yoshikawa Y, Yoshimori T, Yoshimoto K, You HJ, Youle RJ, Younes A, Yu L, Yu SW, Yu WH, Yuan ZM, Yue Z, Yun CH, Yuzaki M, Zabirnyk O, Silva-Zacarin E, Zacks D, Zacksenhaus E, Zaffaroni N, Zakeri Z, Zeh HJ, 3rd, Zeitlin SO, Zhang H, Zhang HL, Zhang J, Zhang JP, Zhang L, Zhang MY, Zhang XD, Zhao M, Zhao YF, Zhao Y, Zhao ZJ, Zheng X, Zhivotovsky B, Zhong Q, Zhou CZ, Zhu C, Zhu WG, Zhu XF, Zhu X, Zhu Y, Zoladek T, Zong WX, Zorzano A, Zschocke J, Zuckerbraun B (2012) Guidelines for the use and interpretation of assays for monitoring autophagy. Autophagy 8:445-544.

Knott AB, Perkins G, Schwarzenbacher R, Bossy-Wetzel E (2008) Mitochondrial fragmentation in neurodegeneration. Nat Rev Neurosci 9:505-518.

Kobayashi S, Xu X, Chen K, Liang Q (2012) Suppression of autophagy is protective in high glucose-induced cardiomyocyte injury. Autophagy 8:577-592.

Kopeikina KJ, Carlson GA, Pitstick R, Ludvigson AE, Peters A, Luebke JI, Koffie RM, Frosch MP, Hyman BT, Spires-Jones TL (2011) Tau accumulation causes mitochondrial distribution deficits in neurons in a mouse model of tauopathy and in human Alzheimer's disease brain. Am J Pathol 179:2071-2082.

Kundu M, Thompson CB (2008) Autophagy: basic principles and relevance to disease. Annu Rev Pathol 3:427-455.

LaFerla FM, Green KN, Oddo S (2007) Intracellular amyloid-beta in Alzheimer's disease. Nat Rev Neurosci 8:499-509.

Lam DW, LeRoith D (2012) The worldwide diabetes epidemic. Curr Opin Endocrinol Diabetes Obes 19:93-96.

Lee J, Kim MS (2007) The role of GSK3 in glucose homeostasis and the development of insulin resistance. Diabetes Res Clin Pract 77 Suppl 1:S49-57.

Leinninger GM, Backus C, Sastry AM, Yi YB, Wang CW, Feldman EL (2006) Mitochondria in DRG neurons undergo hyperglycemic mediated injury through Bim, Bax and the fission protein Drp1. Neurobiol Dis 23:11-22.

Li L, Holscher C (2007) Common pathological processes in Alzheimer disease and type 2 diabetes: a review. Brain Res Rev 56:384-402.

Li ZG, Zhang W, Sima AA (2007) Alzheimer-like changes in rat models of spontaneous diabetes. Diabetes 56:1817-1824.

Liesa M, Shirihai OS (2013) Mitochondrial dynamics in the regulation of nutrient utilization and energy expenditure. Cell Metab 17:491-506.

Liot G, Bossy B, Lubitz S, Kushnareva Y, Sejbuk N, Bossy-Wetzel E (2009) Complex II inhibition by 3-NP causes mitochondrial fragmentation and neuronal cell death via an NMDA- and ROS-dependent pathway. Cell Death Differ 16:899-909.

Liu HY, Yehuda-Shnaidman E, Hong T, Han J, Pi J, Liu Z, Cao W (2009b) Prolonged exposure to insulin suppresses mitochondrial production in primary hepatocytes. J Biol Chem 284:14087-14095.

Liu Y, Liu F, Grundke-Iqbal I, Iqbal K, Gong CX (2009a) Brain glucose transporters, O-GlcNAcylation and phosphorylation of tau in diabetes and Alzheimer's disease. J Neurochem 111:242-249.

Liu Y, Liu F, Iqbal K, Grundke-Iqbal I, Gong CX (2008) Decreased glucose transporters correlate to abnormal hyperphosphorylation of tau in Alzheimer disease. FEBS Lett 582:359-364.

Lizcano JM, Alessi DR (2002) The insulin signalling pathway. Curr Biol 12:R236-238.

Lucas JJ, Hernandez F, Gomez-Ramos P, Moran MA, Hen R, Avila J (2001) Decreased nuclear beta-catenin, tau hyperphosphorylation and neurodegeneration in GSK-3beta conditional transgenic mice. EMBO J 20:27-39.

Ly PT, Wu Y, Zou H, Wang R, Zhou W, Kinoshita A, Zhang M, Yang Y, Cai F, Woodgett J, Song W (2013) Inhibition of GSK3beta-mediated BACE1 expression reduces Alzheimer-associated phenotypes. J Clin Invest 123:224-235.

Maahs DM, West NA, Lawrence JM, Mayer-Davis EJ (2010) Epidemiology of type 1 diabetes. Endocrinol Metab Clin North Am 39:481-497.

Manczak M, Reddy PH (2012) Abnormal interaction between the mitochondrial fission protein Drp1 and hyperphosphorylated tau in Alzheimer's disease neurons: implications for mitochondrial dysfunction and neuronal damage. Hum Mol Genet 21:2538-2547.

Mans AM, DeJoseph MR, Davis DW, Hawkins RA (1988) Brain energy metabolism in streptozotocin-diabetes. Biochem J 249:57-62.

Mastrocola R, Restivo F, Vercellinatto I, Danni O, Brignardello E, Aragno M, Boccuzzi G (2005) Oxidative and nitrosative stress in brain mitochondria of diabetic rats. J Endocrinol 187:37-44.

Mattson MP, Gleichmann M, Cheng A (2008) Mitochondria in neuroplasticity and neurological disorders. Neuron 60:748-766.

Meijer AJ, Codogno P (2006) Signalling and autophagy regulation in health, aging and disease. Mol Aspects Med 27:411-425.

Miki H, Funato Y (2012) Regulation of intracellular signalling through cysteine oxidation by reactive oxygen species. J Biochem 151:255-261.

Mizushima N (2005) The pleiotropic role of autophagy: from protein metabolism to bactericide. Cell Death Differ 12 Suppl 2:1535-1541.

Mizushima N, Levine B, Cuervo AM, Klionsky DJ (2008) Autophagy fights disease through cellular self-digestion. Nature 451:1069-1075.

Moloney AM, Griffin RJ, Timmons S, O'Connor R, Ravid R, O'Neill C (2010) Defects in IGF-1 receptor, insulin receptor and IRS-1/2 in Alzheimer's disease indicate possible resistance to IGF-1 and insulin signalling. Neurobiol Aging 31:224-243.

Moosavi M, Naghdi N, Maghsoudi N, Zahedi Asl S (2006) The effect of intrahippocampal insulin microinjection on spatial learning and memory. Horm Behav 50:748-752.

Moreira PI, Santos MS, Moreno A, Oliveira C (2001) Amyloid beta-peptide promotes permeability transition pore in brain mitochondria. Biosci Rep 21:789-800.

Moreira PI, Santos MS, Moreno AM, Seica R, Oliveira CR (2003) Increased vulnerability of brain mitochondria in diabetic (Goto-Kakizaki) rats with aging and amyloid-beta exposure. Diabetes 52:1449-1456.

Moreira PI, Santos MS, Seica R, Oliveira CR (2007) Brain mitochondrial dysfunction as a link between Alzheimer's disease and diabetes. J Neurol Sci 257:206-214.

Moreira PI, Santos MS, Sena C, Seica R, Oliveira CR (2005) Insulin protects against amyloid beta-peptide toxicity in brain mitochondria of diabetic rats. Neurobiol Dis 18:628-637.

Moreira PI, Santos RX, Zhu X, Lee HG, Smith MA, Casadesus G, Perry G (2010b) Autophagy in Alzheimer's disease. Expert Rev Neurother 10:1209-1218.

Moreira PI, Zhu X, Wang X, Lee HG, Nunomura A, Petersen RB, Perry G, Smith MA (2010a) Mitochondria: a therapeutic target in neurodegeneration. Biochim Biophys Acta 1802:212-220.

Morino K, Neschen S, Bilz S, Sono S, Tsirigotis D, Reznick RM, Moore I, Nagai Y, Samuel V, Sebastian D, White M, Philbrick W, Shulman GI (2008) Muscle-specific IRS-1 Ser->Ala transgenic mice are protected from fat-induced insulin resistance in skeletal muscle. Diabetes 57:2644-2651.

Muratsugu M, Kamo N, Kurihara K, Kobatake Y (1977) Selective electrode for dibenzyl dimethyl ammonium cation as indicator of the membrane potential in biological systems. Biochim Biophys Acta 464:613-619.

Murphy MP (2009) How mitochondria produce reactive oxygen species. Biochem J 417:1-13.

Nakamura N, Kimura Y, Tokuda M, Honda S, Hirose S (2006) MARCH-V is a novel mitofusin 2- and Drp1-binding protein able to change mitochondrial morphology. EMBO Rep 7:1019-1022.

Nelson DL, Cox MM (2004) Lehninger Principles of Biochemistry. W. H. Freeman New York, USA.

Nishida Y, Arakawa S, Fujitani K, Yamaguchi H, Mizuta T, Kanaseki T, Komatsu M, Otsu K, Tsujimoto Y, Shimizu S (2009) Discovery of Atg5/Atg7-independent alternative macroautophagy. Nature 461:654-658.

Noble W, Planel E, Zehr C, Olm V, Meyerson J, Suleman F, Gaynor K, Wang L, LaFrancois J, Feinstein B, Burns M, Krishnamurthy P, Wen Y, Bhat R, Lewis J, Dickson D, Duff K (2005) Inhibition of glycogen synthase kinase-3 by

lithium correlates with reduced tauopathy and degeneration in vivo. Proc Natl Acad Sci U S A 102:6990-6995.

Nordberg J, Arner ES (2001) Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. Free Radic Biol Med 31:1287-1312.

Nunomura A, Perry G, Aliev G, Hirai K, Takeda A, Balraj EK, Jones PK, Ghanbari H, Wataya T, Shimohama S, Chiba S, Atwood CS, Petersen RB, Smith MA (2001) Oxidative damage is the earliest event in Alzheimer disease. J Neuropathol Exp Neurol 60:759-767.

Otera H, Wang C, Cleland MM, Setoguchi K, Yokota S, Youle RJ, Mihara K (2010) Mff is an essential factor for mitochondrial recruitment of Drp1 during mitochondrial fission in mammalian cells. J Cell Biol 191:1141-1158.

Ott A, Stolk RP, van Harskamp F, Pols HA, Hofman A, Breteler MM (1999) Diabetes mellitus and the risk of dementia: The Rotterdam Study. Neurology 53:1937-1942.

Palmeira CM, Rolo AP, Berthiaume J, Bjork JA, Wallace KB (2007) Hyperglycemia decreases mitochondrial function: the regulatory role of mitochondrial biogenesis. Toxicol Appl Pharmacol 225:214-220.

Papon MA, El Khoury NB, Marcouiller F, Julien C, Morin F, Bretteville A, Petry FR, Gaudreau S, Amrani A, Mathews PM, Hebert SS, Planel E (2013) Deregulation of protein phosphatase 2A and hyperphosphorylation of tau protein following onset of diabetes in NOD mice. Diabetes 62:609-617.

Park CR, Seeley RJ, Craft S, Woods SC (2000) Intracerebroventricular insulin enhances memory in a passive-avoidance task. Physiol Behav 68:509-514.

Pasinetti GM (1996) Inflammatory mechanisms in neurodegeneration and Alzheimer's disease: the role of the complement system. Neurobiol Aging 17:707-716.

Pattingre S, Espert L, Biard-Piechaczyk M, Codogno P (2008) Regulation of macroautophagy by mTOR and Beclin 1 complexes. Biochimie 90:313-323.

Paz K, Voliovitch H, Hadari YR, Roberts CT, Jr., LeRoith D, Zick Y (1996) Interaction between the insulin receptor and its downstream effectors. Use of individually expressed receptor domains for structure/function analysis. J Biol Chem 271:6998-7003.

Pei JJ, Sjogren M, Winblad B (2008) Neurofibrillary degeneration in Alzheimer's disease: from molecular mechanisms to identification of drug targets. Curr Opin Psychiatry 21:555-561.

Phiel CJ, Wilson CA, Lee VM, Klein PS (2003) GSK-3alpha regulates production of Alzheimer's disease amyloid-beta peptides. Nature 423:435-439.

Planel E, Tatebayashi Y, Miyasaka T, Liu L, Wang L, Herman M, Yu WH, Luchsinger JA, Wadzinski B, Duff KE, Takashima A (2007) Insulin dysfunction induces in vivo tau hyperphosphorylation through distinct mechanisms. J Neurosci 27:13635-13648.

Pratico D, Uryu K, Leight S, Trojanoswki JQ, Lee VM (2001) Increased lipid peroxidation precedes amyloid plaque formation in an animal model of Alzheimer amyloidosis. J Neurosci 21:4183-4187.

Qing H, He G, Ly PT, Fox CJ, Staufenbiel M, Cai F, Zhang Z, Wei S, Sun X, Chen CH, Zhou W, Wang K, Song W (2008) Valproic acid inhibits Abeta production, neuritic plaque formation, and behavioral deficits in Alzheimer's disease mouse models. J Exp Med 205:2781-2789.

Qiu WQ, Folstein MF (2006) Insulin, insulin-degrading enzyme and amyloid-beta peptide in Alzheimer's disease: review and hypothesis. Neurobiol Aging 27:190-198.

Qu Z, Jiao Z, Sun X, Zhao Y, Ren J, Xu G (2011) Effects of streptozotocin-induced diabetes on tau phosphorylation in the rat brain. Brain Res 1383:300-306.

Querfurth HW, LaFerla FM (2010) Alzheimer's disease. N Engl J Med 362:329-344.

Raina AK, Zhu X, Monteiro M, Takeda A, Smith MA (2000a) Abortive oncogeny and cell cycle-mediated events in Alzheimer disease. Prog Cell Cycle Res 4:235-242.

Raina AK, Zhu X, Rottkamp CA, Monteiro M, Takeda A, Smith MA (2000b) Cyclin' toward dementia: cell cycle abnormalities and abortive oncogenesis in Alzheimer disease. J Neurosci Res 61:128-133.

Reger MA, Watson GS, Frey WH, 2nd, Baker LD, Cholerton B, Keeling ML, Belongia DA, Fishel MA, Plymate SR, Schellenberg GD, Cherrier MM, Craft S (2006) Effects of intranasal insulin on cognition in memory-impaired older adults: modulation by APOE genotype. Neurobiol Aging 27:451-458.

Reger MA, Watson GS, Green PS, Wilkinson CW, Baker LD, Cholerton B, Fishel MA, Plymate SR, Breitner JC, DeGroodt W, Mehta P, Craft S (2008) Intranasal insulin improves cognition and modulates beta-amyloid in early AD. Neurology 70:440-448.

Reggiori F (2006) 1. Membrane origin for autophagy. Curr Top Dev Biol 74:1-30.

Reichard P, Nilsson BY, Rosenqvist U (1993) The effect of long-term intensified insulin treatment on the development of microvascular complications of diabetes mellitus. N Engl J Med 329:304-309.

Reske-Nielsen E, Lundbaek K (1963) Diabetic Encephalopathy. Diffuse and Focal Lesions of the Brain in Long-Term Diabetes. Acta Neurol Scand Suppl 39:SUPPL4:273-290.

Rivera EJ, Goldin A, Fulmer N, Tavares R, Wands JR, de la Monte SM (2005) Insulin and insulin-like growth factor expression and function deteriorate with progression of Alzheimer's disease: link to brain reductions in acetylcholine. J Alzheimers Dis 8:247-268.

Rizzuto R, Bernardi P, Pozzan T (2000) Mitochondria as all-round players of the calcium game. J Physiol 529 Pt 1:37-47.

Rizzuto R, Pinton P, Brini M, Chiesa A, Filippin L, Pozzan T (1999) Mitochondria as biosensors of calcium microdomains. Cell Calcium 26:193-199.

Ryan C, Vega A, Drash A (1985) Cognitive deficits in adolescents who developed diabetes early in life. Pediatrics 75:921-927.

Sabatini BL, Maravall M, Svoboda K (2001) Ca(2+) signaling in dendritic spines. Curr Opin Neurobiol 11:349-356.

Sankar R, Thamotharan S, Shin D, Moley KH, Devaskar SU (2002) Insulin-responsive glucose transporters-GLUT8 and GLUT4 are expressed in the developing mammalian brain. Brain Res Mol Brain Res 107:157-165.

Santel A, Frank S, Gaume B, Herrler M, Youle RJ, Fuller MT (2003) Mitofusin-1 protein is a generally expressed mediator of mitochondrial fusion in mammalian cells. J Cell Sci 116:2763-2774.

Santiago JA, Potashkin JA (2013) Shared dysregulated pathways lead to Parkinson's disease and diabetes. Trends Mol Med 19:176-186.

Santos RX, Cardoso S, Correia S, Carvalho C, Santos MS, Moreira PI (2010b) Targeting autophagy in the brain: a promising approach? Cent Nerv Syst Agents Med Chem 10:158-168.

Santos RX, Correia SC, Cardoso S, Carvalho C, Santos MS, Moreira PI (2011) Effects of rapamycin and TOR on aging and memory: implications for Alzheimer's disease. J Neurochem 117:927-936.

Santos RX, Correia SC, Wang X, Perry G, Smith MA, Moreira PI, Zhu X (2010a) Alzheimer's disease: diverse aspects of mitochondrial malfunctioning. Int J Clin Exp Pathol 3:570-581.

Santos RX, Correia SC, Zhu X, Smith MA, Moreira PI, Castellani RJ, Nunomura A, Perry G (2013) Mitochondrial DNA oxidative damage and repair in aging and Alzheimer's disease. Antioxid Redox Signal 18:2444-2457.

Sato N, Takeda S, Uchio-Yamada K, Ueda H, Fujisawa T, Rakugi H, Morishita R (2011) Role of insulin signaling in the interaction between Alzheimer disease and diabetes mellitus: a missing link to therapeutic potential. Curr Aging Sci 4:118-127.

Scarpulla RC (2006) Nuclear control of respiratory gene expression in mammalian cells. J Cell Biochem 97:673-683.

Scarpulla RC (2008) Transcriptional paradigms in mammalian mitochondrial biogenesis and function. Physiol Rev 88:611-638.

Schechter R, Beju D, Gaffney T, Schaefer F, Whetsell L (1996) Preproinsulin I and II mRNAs and insulin electron microscopic immunoreaction are present within the rat fetal nervous system. Brain Res 736:16-27.

Scheffler IE (2008) Mitochondria. J. Wiley and Sons, Inc., Hoboken, New Jersey, USA.

Schmeichel AM, Schmelzer JD, Low PA (2003) Oxidative injury and apoptosis of dorsal root ganglion neurons in chronic experimental diabetic neuropathy. Diabetes 52:165-171.

Schubert M, Brazil DP, Burks DJ, Kushner JA, Ye J, Flint CL, Farhang-Fallah J, Dikkes P, Warot XM, Rio C, Corfas G, White MF (2003) Insulin receptor substrate-2 deficiency impairs brain growth and promotes tau phosphorylation. J Neurosci 23:7084-7092.

Schubert M, Gautam D, Surjo D, Ueki K, Baudler S, Schubert D, Kondo T, Alber J, Galldiks N, Kustermann E, Arndt S, Jacobs AH, Krone W, Kahn CR, Bruning JC (2004) Role for neuronal insulin resistance in neurodegenerative diseases. Proc Natl Acad Sci U S A 101:3100-3105.

Scorrano L, Ashiya M, Buttle K, Weiler S, Oakes SA, Mannella CA, Korsmeyer SJ (2002) A distinct pathway remodels mitochondrial cristae and mobilizes cytochrome c during apoptosis. Dev Cell 2:55-67.

Scott PH, Brunn GJ, Kohn AD, Roth RA, Lawrence JC, Jr. (1998) Evidence of insulin-stimulated phosphorylation and activation of the mammalian target of rapamycin mediated by a protein kinase B signaling pathway. Proc Natl Acad Sci U S A 95:7772-7777.

Scott RC, Schuldiner O, Neufeld TP (2004) Role and regulation of starvation-induced autophagy in the Drosophila fat body. Dev Cell 7:167-178.

Seibenhener ML, Babu JR, Geetha T, Wong HC, Krishna NR, Wooten MW (2004) Sequestosome 1/p62 is a polyubiquitin chain binding protein involved in ubiquitin proteasome degradation. Mol Cell Biol 24:8055-8068.

Shah K, Desilva S, Abbruscato T (2012) The Role of Glucose Transporters in Brain Disease: Diabetes and Alzheimer’s Disease. Int J Mol Sci 13:12629-12655.

Sharma R, Buras E, Terashima T, Serrano F, Massaad CA, Hu L, Bitner B, Inoue T, Chan L, Pautler RG (2010) Hyperglycemia induces oxidative stress and impairs axonal transport rates in mice. PLoS One 5:e13463.

Shaw JE, Sicree RA, Zimmet PZ (2010) Global estimates of the prevalence of diabetes for 2010 and 2030. Diabetes Res Clin Pract 87:4-14.

Shenouda SM, Widlansky ME, Chen K, Xu G, Holbrook M, Tabit CE, Hamburg NM, Frame AA, Caiano TL, Kluge MA, Duess MA, Levit A, Kim B, Hartman ML, Joseph L, Shirihai OS, Vita JA (2011) Altered mitochondrial dynamics contributes to endothelial dysfunction in diabetes mellitus. Circulation 124:444-453.

Shigenaga MK, Hagen TM, Ames BN (1994) Oxidative damage and mitochondrial decay in aging. Proc Natl Acad Sci U S A 91:10771-10778.

Silva AM, Oliveira PJ (2012) Evaluation of respiration with clark type electrode in isolated mitochondria and permeabilized animal cells. Methods Mol Biol 810:7-24.

Simon D, Garcia-Garcia E, Royo F, Falcon-Perez JM, Avila J (2012) Proteostasis of tau. Tau overexpression results in its secretion via membrane vesicles. FEBS Lett 586:47-54.

Simpson IA, Chundu KR, Davies-Hill T, Honer WG, Davies P (1994) Decreased concentrations of GLUT1 and GLUT3 glucose transporters in the brains of patients with Alzheimer's disease. Ann Neurol 35:546-551.

Sims-Robinson C, Kim B, Rosko A, Feldman EL (2010) How does diabetes accelerate Alzheimer disease pathology? Nat Rev Neurol 6:551-559.

Small GW, Ercoli LM, Silverman DH, Huang SC, Komo S, Bookheimer SY, Lavretsky H, Miller K, Siddarth P, Rasgon NL, Mazziotta JC, Saxena S, Wu HM, Mega MS, Cummings JL, Saunders AM, Pericak-Vance MA, Roses AD, Barrio JR, Phelps ME (2000) Cerebral metabolic and cognitive decline in persons at genetic risk for Alzheimer's disease. Proc Natl Acad Sci U S A 97:6037-6042.

Smirnova E, Griparic L, Shurland DL, van der Bliek AM (2001) Dynamin-related protein Drp1 is required for mitochondrial division in mammalian cells. Mol Biol Cell 12:2245-2256.

Smith MA, Rottkamp CA, Nunomura A, Raina AK, Perry G (2000) Oxidative stress in Alzheimer's disease. Biochim Biophys Acta 1502:139-144.

Soderling TR (2000) CaM-kinases: modulators of synaptic plasticity. Curr Opin Neurobiol 10:375-380.

Song Z, Chen H, Fiket M, Alexander C, Chan DC (2007) OPA1 processing controls mitochondrial fusion and is regulated by mRNA splicing, membrane potential, and Yme1L. J Cell Biol 178:749-755.

Stargardt A, Gillis J, Kamphuis W, Wiemhoefer A, Kooijman L, Raspe M, Benckhuijsen W, Drijfhout JW, Hol EM, Reits E (2013) Reduced amyloid-beta degradation in early Alzheimer's disease but not in the APPswePS1dE9 and 3xTg-AD mouse models. Aging Cell 12:499-507.

Steen E, Terry BM, Rivera EJ, Cannon JL, Neely TR, Tavares R, Xu XJ, Wands JR, de la Monte SM (2005) Impaired insulin and insulin-like growth factor

expression and signaling mechanisms in Alzheimer's disease--is this type 3 diabetes? J Alzheimers Dis 7:63-80.

Stoyanovsky DA, Osipov AN, Quinn PJ, Kagan VE (1995) Ubiquinone-dependent recycling of vitamin E radicals by superoxide. Arch Biochem Biophys 323:343-351.

Strachan MW, Reynolds RM, Marioni RE, Price JF (2011) Cognitive function, dementia and type 2 diabetes mellitus in the elderly. Nat Rev Endocrinol 7:108-114.

Su B, Wang X, Bonda D, Perry G, Smith M, Zhu X (2010a) Abnormal mitochondrial dynamics--a novel therapeutic target for Alzheimer's disease? Mol Neurobiol 41:87-96.

Su B, Wang X, Zheng L, Perry G, Smith MA, Zhu X (2010b) Abnormal mitochondrial dynamics and neurodegenerative diseases. Biochim Biophys Acta 1802:135-142.

Sun XJ, Liu F (2009) Phosphorylation of IRS proteins Yin-Yang regulation of insulin signaling. Vitam Horm 80:351-387.

Swerdlow RH, Khan SM (2004) A "mitochondrial cascade hypothesis" for sporadic Alzheimer's disease. Med Hypotheses 63:8-20.

Swerdlow RH, Khan SM (2009) The Alzheimer's disease mitochondrial cascade hypothesis: an update. Exp Neurol 218:308-315.

Szabadkai G, Simoni AM, Chami M, Wieckowski MR, Youle RJ, Rizzuto R (2004) Drp-1-dependent division of the mitochondrial network blocks intraorganellar Ca2+ waves and protects against Ca2+-mediated apoptosis. Mol Cell 16:59-68.

Taanman JW (1999) The mitochondrial genome: structure, transcription, translation and replication. Biochim Biophys Acta 1410:103-123.

Taguchi N, Ishihara N, Jofuku A, Oka T, Mihara K (2007) Mitotic phosphorylation of dynamin-related GTPase Drp1 participates in mitochondrial fission. J Biol Chem 282:11521-11529.

Takadera T, Sakura N, Mohri T, Hashimoto T (1993) Toxic effect of a beta-amyloid peptide (beta 22-35) on the hippocampal neuron and its prevention. Neurosci Lett 161:41-44.

Takayanagi R, Takeshige K, Minakami S (1980) NADH- and NADPH-dependent lipid peroxidation in bovine heart submitochondrial particles. Dependence on the rate of electron flow in the respiratory chain and an antioxidant role of ubiquinol. Biochem J 192:853-860.

Talbot K, Wang HY, Kazi H, Han LY, Bakshi KP, Stucky A, Fuino RL, Kawaguchi KR, Samoyedny AJ, Wilson RS, Arvanitakis Z, Schneider JA, Wolf BA, Bennett DA, Trojanowski JQ, Arnold SE (2012) Demonstrated brain insulin resistance in Alzheimer's disease patients is associated with IGF-1 resistance, IRS-1 dysregulation, and cognitive decline. J Clin Invest 122:1316-1338.

Taylor RC, Cullen SP, Martin SJ (2008) Apoptosis: controlled demolition at the cellular level. Nat Rev Mol Cell Biol 9:231-241.

Terman A, Kurz T, Navratil M, Arriaga EA, Brunk UT (2010) Mitochondrial turnover and aging of long-lived postmitotic cells: the mitochondrial-lysosomal axis theory of aging. Antioxid Redox Signal 12:503-535.

Thumm M, Egner R, Koch B, Schlumpberger M, Straub M, Veenhuis M, Wolf DH (1994) Isolation of autophagocytosis mutants of Saccharomyces cerevisiae. FEBS Lett 349:275-280.

Todd JA (2010) Etiology of type 1 diabetes. Immunity 32:457-467.

Todde V, Veenhuis M, van der Klei IJ (2009) Autophagy: principles and significance in health and disease. Biochim Biophys Acta 1792:3-13.

Tondera D, Czauderna F, Paulick K, Schwarzer R, Kaufmann J, Santel A (2005) The mitochondrial protein MTP18 contributes to mitochondrial fission in mammalian cells. J Cell Sci 118:3049-3059.

Tsukada M, Ohsumi Y (1993) Isolation and characterization of autophagy-defective mutants of Saccharomyces cerevisiae. FEBS Lett 333:169-174.

Turrens JF (2003) Mitochondrial formation of reactive oxygen species. J Physiol 552:335-344.

Twig G, Hyde B, Shirihai OS (2008) Mitochondrial fusion, fission and autophagy as a quality control axis: the bioenergetic view. Biochim Biophys Acta 1777:1092-1097.

Umegaki H (2012) Neurodegeneration in diabetes mellitus. Adv Exp Med Biol 724:258-265.

Valtorta F, Pennuto M, Bonanomi D, Benfenati F (2004) Synaptophysin: leading actor or walk-on role in synaptic vesicle exocytosis? Bioessays 26:445-453.

van der Heide LP, Ramakers GM, Smidt MP (2006) Insulin signaling in the central nervous system: learning to survive. Prog Neurobiol 79:205-221.

Vekrellis K, Ye Z, Qiu WQ, Walsh D, Hartley D, Chesneau V, Rosner MR, Selkoe DJ (2000) Neurons regulate extracellular levels of amyloid beta-protein via proteolysis by insulin-degrading enzyme. J Neurosci 20:1657-1665.

Verstreken P, Ly CV, Venken KJ, Koh TW, Zhou Y, Bellen HJ (2005) Synaptic mitochondria are critical for mobilization of reserve pool vesicles at Drosophila neuromuscular junctions. Neuron 47:365-378.

Vincent AM, Edwards JL, McLean LL, Hong Y, Cerri F, Lopez I, Quattrini A, Feldman EL (2010) Mitochondrial biogenesis and fission in axons in cell culture and animal models of diabetic neuropathy. Acta Neuropathol 120:477-489.

Virbasius JV, Scarpulla RC (1994) Activation of the human mitochondrial transcription factor A gene by nuclear respiratory factors: a potential regulatory link between nuclear and mitochondrial gene expression in organelle biogenesis. Proc Natl Acad Sci U S A 91:1309-1313.

Wallace DC (2005) A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine. Annu Rev Genet 39:359-407.

Wallace DC, Fan W (2009) The pathophysiology of mitochondrial disease as modeled in the mouse. Genes Dev 23:1714-1736.

Wang X, Su B, Lee HG, Li X, Perry G, Smith MA, Zhu X (2009) Impaired balance of mitochondrial fission and fusion in Alzheimer's disease. J Neurosci 29:9090-9103.

Wang X, Su B, Liu W, He X, Gao Y, Castellani RJ, Perry G, Smith MA, Zhu X (2011) DLP1-dependent mitochondrial fragmentation mediates 1-methyl-4-

phenylpyridinium toxicity in neurons: implications for Parkinson's disease. Aging Cell 10:807-823.

Wang X, Su B, Siedlak SL, Moreira PI, Fujioka H, Wang Y, Casadesus G, Zhu X (2008) Amyloid-beta overproduction causes abnormal mitochondrial dynamics via differential modulation of mitochondrial fission/fusion proteins. Proc Natl Acad Sci U S A 105:19318-19323.

Watson GS, Peskind ER, Asthana S, Purganan K, Wait C, Chapman D, Schwartz MW, Plymate S, Craft S (2003) Insulin increases CSF Abeta42 levels in normal older adults. Neurology 60:1899-1903.

Wei M, Ong L, Smith MT, Ross FB, Schmid K, Hoey AJ, Burstow D, Brown L (2003) The streptozotocin-diabetic rat as a model of the chronic complications of human diabetes. Heart Lung Circ 12:44-50.

Weisiger RA, Fridovich I (1973a) Superoxide dismutase. Organelle specificity. J Biol Chem 248:3582-3592.

Weisiger RA, Fridovich I (1973b) Mitochondrial superoxide simutase. Site of synthesis and intramitochondrial localization. J Biol Chem 248:4793-4796.

Westermann B (2010) Mitochondrial fusion and fission in cell life and death. Nat Rev Mol Cell Biol 11:872-884.

Wild S, Roglic G, Green A, Sicree R, King H (2004) Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes Care 27:1047-1053.

Wojda U, Salinska E, Kuznicki J (2008) Calcium ions in neuronal degeneration. IUBMB Life 60:575-590.

Wu Z, Puigserver P, Andersson U, Zhang C, Adelmant G, Mootha V, Troy A, Cinti S, Lowell B, Scarpulla RC, Spiegelman BM (1999) Mechanisms controlling

mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. Cell 98:115-124.

Xie Z, Klionsky DJ (2007) Autophagosome formation: core machinery and adaptations. Nat Cell Biol 9:1102-1109.

Xu W, Caracciolo B, Wang HX, Winblad B, Backman L, Qiu C, Fratiglioni L (2010) Accelerated progression from mild cognitive impairment to dementia in people with diabetes. Diabetes 59:2928-2935.

Xu X, Kobayashi S, Chen K, Timm D, Volden P, Huang Y, Gulick J, Yue Z, Robbins J, Epstein PN, Liang Q (2013) Diminished autophagy limits cardiac injury in mouse models of type 1 diabetes. J Biol Chem 288:18077-18092.

Yamaguchi R, Perkins G (2009) Dynamics of mitochondrial structure during apoptosis and the enigma of Opa1. Biochim Biophys Acta 1787:963-972.

Yang Y, Ma D, Wang Y, Jiang T, Hu S, Zhang M, Yu X, Gong CX (2013) Intranasal insulin ameliorates tau hyperphosphorylation in a rat model of type 2 diabetes. J Alzheimers Dis 33:329-338.

Yang Y, Zhang J, Ma D, Zhang M, Hu S, Shao S, Gong CX (2013a) Subcutaneous administration of liraglutide ameliorates Alzheimer-associated tau hyperphosphorylation in rats with type 2 diabetes. J Alzheimers Dis 37:637-648.

Yoon Y, Galloway CA, Jhun BS, Yu T (2011) Mitochondrial dynamics in diabetes. Antioxid Redox Signal 14:439-457.

Young AR, Chan EY, Hu XW, Kochl R, Crawshaw SG, High S, Hailey DW, Lippincott-Schwartz J, Tooze SA (2006) Starvation and ULK1-dependent cycling of mammalian Atg9 between the TGN and endosomes. J Cell Sci 119:3888-3900.

Yu T, Robotham JL, Yoon Y (2006) Increased production of reactive oxygen species in hyperglycemic conditions requires dynamic change of mitochondrial morphology. Proc Natl Acad Sci U S A 103:2653-2658.

Yue Z, Friedman L, Komatsu M, Tanaka K (2009) The cellular pathways of neuronal autophagy and their implication in neurodegenerative diseases. Biochim Biophys Acta 1793:1496-1507.

Zhang JY, Peng C, Shi H, Wang S, Wang Q, Wang JZ (2009) Inhibition of autophagy causes tau proteolysis by activating calpain in rat brain. J Alzheimers Dis 16:39-47.

Zhao J, Liu T, Jin S, Wang X, Qu M, Uhlen P, Tomilin N, Shupliakov O, Lendahl U, Nister M (2011) Human MIEF1 recruits Drp1 to mitochondrial outer membranes and promotes mitochondrial fusion rather than fission. EMBO J 30:2762-2778.

Zhao WQ, Chen H, Quon MJ, Alkon DL (2004) Insulin and the insulin receptor in experimental models of learning and memory. Eur J Pharmacol 490:71-81.

Zhu J, Wang KZ, Chu CT (2013) After the banquet: mitochondrial biogenesis, mitophagy, and cell survival. Autophagy 9:1663-1676.

Zimmermann H (1990) Neurotransmitter release. FEBS Lett 268:394-399.

Zuchner S, Mersiyanova IV, Muglia M, Bissar-Tadmouri N, Rochelle J, Dadali EL, Zappia M, Nelis E, Patitucci A, Senderek J, Parman Y, Evgrafov O, Jonghe PD, Takahashi Y, Tsuji S, Pericak-Vance MA, Quattrone A, Battaloglu E, Polyakov AV, Timmerman V, Schroder JM, Vance JM (2004) Mutations in the mitochondrial GTPase mitofusin 2 cause Charcot-Marie-Tooth neuropathy type 2A. Nat Genet 36:449-451.

Zucker RS (1999) Calcium- and activity-dependent synaptic plasticity. Curr Opin Neurobiol 9:305-313.