UNIVERSIDADE D
COIMBRA

Maria Inês Nuno Alves

# EFFECT OF SEX ON BRAIN METABOLISM AND INTRACELLULAR STRESS IN TYPE 2 DIABETES

**Master Dissertation in Cellular and Molecular Biology supervised by Doctor Ana I. Duarte and Professor António Moreno, presented to the Department of Life Sciences, Faculty of Sciences and Technology**

June 2019

Department of Life Sciences

# EFFECT OF SEX ON BRAIN METABOLISM AND INTRACELLULAR STRESS IN TYPE 2 DIABETES

Maria Inês Nuno Alves

Master Dissertation in Cellular and Molecular Biology supervised by Doctor Ana I. Duarte and Professor António Moreno, presented to the Department of Life Sciences, Faculty of Sciences and Technology.

June 2019

FACULDADE DE
CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE D
COIMBRA

This work was performed at the *Metabolism, Mitochondria and Hormones in Brain Disorders Group* from the CNC - Center of Neuroscience and Cell Biology, University of Coimbra, under the scientific guidance of Doctor Ana I. Duarte and Professor António Moreno.

This work was supported by European Regional Development Fund (ERDF), through the Centro 2020 Regional Operational Programme (Projects PTDC/NEU-NMC/0412/2014; PTDC/SAU-TOX/117481/2010), by COMPETE 2020 (Operational Programme for Competitiveness and Internationalization; by Portuguese funds from FCT – Fundação para a Ciência e a Tecnologia (PTDC/NEU-NMC/0412/2014; PTDC/SAU-TOX/117481/2010; UID/NEU/04539/2019), and from Santander-Totta & Faculty of Medicine, University of Coimbra (PEPITA 2018); and by European Social Fund (SFRH/BPD/84473/2012 to Ana I. Duarte).

# Acknowledgements

This Master Dissertation in Cellular and Molecular Biology, from the Department of Life Sciences, Faculty of Sciences and Technology of the University of Coimbra, was only possible to accomplish with the help, support and contribution of several entities/institutions.

As such, I hereby formally thank the CNC - Center for Neuroscience and Cell Biology for providing me the technical facilities to conduct this Dissertation, and to the University of Coimbra for the opportunity to continue my academic growth at the Master in Cellular and Molecular Biology.

To Doctor Ana I. Duarte, my supervisor, I would like to express my sincere gratitude for all the teaching, guidance, patience and trust in the last few years, that allowed me to grow both at the bench and intellectually. I would also like to thank for the constant availability, challenges and scientific discussions that encouraged to defy dogmas and kept the mind sharp.

I would also like to express my gratitude towards Professor António Moreno for accepting to co-supervise this Dissertation, as well as for the help and immense knowledge, especially in the mitochondrial respiratory chain enzymatic techniques.

I would like to express my gratefulness towards Professor Paula Moreira for the opportunity and trust to conduct the work that led to this Dissertation in her group.

I would also like to express my gratitude to Professor Raquel Seiça, Director of the Laboratory of Physiology of the Faculty of Medicine of the University of Coimbra, and her research team for kindly providing us the brain cortical samples and characterization of the Goto-Kakizaki rats.

I would also like to acknowledge Cláudia Almeida and Maria Beatriz Silva for their precious contribution in the determination of some of the parameters measured in this Dissertation.

To Emanuel Candeias, I thank for the previous contribution for this work, availability to help when needed and for the persistent good mood at the workplace.

I would also like to thank all the members of the *Metabolism, Mitochondria and Hormones in Brain Disorders Group* for the support, suggestions and criticism given along the work.

I also acknowledge the technical staff of the CNC for their technical support.

To my “partner in crime”, my lab colleague Débora Mena, I thank for all the precious help, support and good disposition during the making of this Dissertation. I am immensely grateful for all the discussions regarding the work, shared scientific adventures and friendship born at the bench.

In addition to his role in my personal life, I thank Alexandre for the helpful perspective of an outsider, the criticism, support and incentive along this work.

To my close friends, especially Marianas and Alexandra, I am immensely grateful for the care and distraction provided, crucial to face this challenge with a renewed strength.

I leave a special thanks to my parents, for always accompanying me on these battles, believing in me, with love and dedication, and modeling the perseverance needed to keep growing in every aspects of life.

Finally, to anyone I may have forgotten to mention, I thank you in advance for your contribution to this work.

# Resumo

A Diabetes tipo 2 é uma das maiores emergências a nível da saúde do século XXI, maioritariamente devido à sua crescente prevalência e proporções epidémicas. A diabetes tipo 2 pode conduzir a complicações neurocognitivas e neurológicas (tal como a neurodegeneração, declínio cognitivo e demência), sendo considerada um fator de risco para doenças neurodegenerativas (nomeadamente para a doença de Alzheimer). A diabetes tipo 2 e a doença de Alzheimer partilham numerosas características e mecanismos fisiopatológicos, tais como o comprometimento do metabolismo da glucose e mitocondrial, e a ativação de mecanismos de stress intracelulares. O sexo feminino constitui outro fator de risco para doença de Alzheimer, podendo adicionalmente afetar estes mesmos mecanismos (em especial durante a perimenopausa e menopausa).

Como tal, e no seguimento de um estudo recente realizado no nosso laboratório, colocámos a hipótese de que o sexo afeta diferencialmente a função metabólica, a ativação de mecanismos de stress intracelular cerebrais na diabetes tipo 2 e, consequentemente, a suscetibilidade para a doença de Alzheimer durante a meia-idade. Deste modo, no presente estudo pretendemos analisar os mecanismos moleculares envolvidos nas alterações mediadas pelo sexo no metabolismo energético e mecanismos de stress nitrosativo intracelular no córtex cerebral em condições de diabetes tipo 2, durante a meia-idade. Para tal, utilizámos homogeneizados de córtices cerebrais de ratos de meia-idade (8 meses, ex-reprodutores), do sexo masculino e feminino, das estirpes Wistar (controlos) e Goto-Kakizaki (modelo animal de diabetes tipo 2 espontânea e não obesa), com vista à determinação de marcadores-chave do metabolismo cerebral (nomeadamente vias metabólicas relacionadas com a glucose, mitocôndria e corpos cetónicos) e de stress nitrosativo. Estes parâmetros foram medidos através de técnicas de *immunoblotting*, *reverse-phase high-performance liquid chromatography* (HPLC), colorimetria, fluorimetria e ELISA.

Do ponto de vista periférico, os ratos diabéticos tipo 2 GK do sexo feminino de meia-idade parecem encontrar-se numa fase inicial da doença, enquanto os respetivos machos ainda se encontram numa fase pré-diabética. Apesar da maior densidade do transportador de glucose-3 (GLUT3) no córtex cerebral de ambas as fêmeas de ratos controlo e diabéticos tipo 2, a tomada de glucose da periferia para o cérebro poderá estar comprometida e, desta forma, contribuir para a inibição da glicólise e da fase inicial do ciclo de Krebs cerebral nestes animais. Embora tal não tenha sido acompanhado por um desvio da glucose-6-fosfato para a via das pentoses

fosfato, ambos os grupos experimentais de ratos do sexo feminino desenvolveram mecanismos de compensação em diferentes fases do ciclo de Krebs comparativamente aos respetivos machos. Apesar disto, os animais diabéticos tipo 2 do sexo feminino apresentaram uma inibição generalizada da cadeia respiratória mitocondrial sem que tal afetasse os seus níveis cerebrais de ATP, muito provavelmente devido à estimulação do sistema fosfocreatina/creatina cinase. Os perfis cerebrais de corpos cetónicos eram diferentes em ambos os grupos de ratos do sexo feminino, o que sugere um metabolismo divergente destas fontes alternativas de energia. Contudo, estes metabolitos podem também não estar a ser utilizados pelo ciclo de Krebs para produzir energia, uma vez que a fase do ciclo na qual eles poderiam entrar se encontrava atenuada nos cérebros de ambos os grupos de ratos do sexo feminino. Apesar de no nosso estudo anterior os ratos do sexo feminino de meia-idade parecerem mais protegidos contra a acumulação cerebral de marcadores de dano oxidativo e da doença de Alzheimer, no presente estudo os animais diabéticos tipo 2 do sexo feminino poderão estar mais vulneráveis ao stress nitrosativo que os respetivos machos.

Em suma, estes resultados reforçam a hipótese de que a atenuação do metabolismo energético/mitocondrial no cérebro de ratos do sexo feminino, controlos e diabéticos tipo 2, de meia-idade, poderá diminuir a sua suscetibilidade à lesão oxidativa e à doença de Alzheimer. Nesta perspetiva, este estudo enfatiza a necessidade de estabelecer estratégias preventivas e/ou terapêuticas adequadas a cada sexo e fase da vida contra a diabetes tipo 2 e suas complicações crónicas ao nível do sistema nervoso central (nomeadamente o défice cognitivo e a doença de Alzheimer).

**Palavras-chave**: Diabetes tipo 2, disfunção metabólica cerebral, envelhecimento reprodutor, neuroprotecção, sexo, stress intracelular.

# Abstract

Type 2 diabetes constitutes one of the largest health emergencies of the 21st century, mostly due to its epidemic proportions and increasing prevalence. Type 2 diabetes may lead to neurocognitive and neurologic complications (such as neurodegeneration, cognitive impairment and dementia), being considered a risk factor for neurodegenerative disorders (including Alzheimer's disease). Type 2 diabetes and Alzheimer's disease share several pathological features and pathophysiological mechanisms, namely glucose dysmetabolism, mitochondrial dysfunction and activation of intracellular stress mechanisms. Together with aging, female sex is another risk factor for Alzheimer's disease and may itself affect the same mechanisms (especially during perimenopause and menopause).

Following a recent study from our lab, we hypothesized that sex differently affects type 2 diabetic brain metabolic function, intracellular stress mechanisms and ultimately the susceptibility to Alzheimer's disease-like pathophysiology at midlife. Thus, in the present study we aimed to analyze the molecular mechanisms involved in sex-mediated changes in type 2 diabetic brain cortical energy metabolism and intracellular nitrosative stress at midlife. As such, we used brain cortical homogenates from middle-aged (8-month-old, retired breeders) male and female Wistar and non-obese, spontaneously type 2 diabetic Goto-Kakizaki (GK) rats to determine key markers for both brain cortical metabolism (glucose-, mitochondria- and ketone bodies-related) and nitrosative stress, by immunoblotting, reverse-phase high-performance liquid chromatography (HPLC), colorimetric, fluorimetric and ELISA techniques.

Peripheral characterization showed that middle-aged GK female rats were at an early stage of type 2 diabetes, while age-matched GK rat males were still under a pre-diabetic stage. Despite increased brain cortical glucose transporter-3 isoform (GLUT3) density in both control and type 2 diabetic female rats, their glucose uptake towards the brain might be compromised, thereby attenuating their glycolysis and initial stages of Krebs cycle. Though this was not accompanied by a shift of glucose-6-phosphate towards pentose phosphate pathway, both female rat cohorts exhibited compensatory mechanisms at different stages of Krebs cycle compared to the respective male rats. Strikingly, type 2 diabetic female rats showed an overall inhibition of mitochondrial respiratory chain that, nonetheless, did not affect their brain ATP pool – this was most likely due to a stimulation of their phosphocreatine/creatine kinase system. Brain cortical ketone bodies levels profiles were different in both female rat cohorts, suggesting a divergent metabolism of these alternative fuels. However, ketone bodies may not be used by

Krebs cycle to produce energy, since the stage of the cycle whereby they could enter is attenuated in both control and type 2 diabetic female rat brains. Despite the protection against Alzheimer's disease-like and oxidative damage hallmarks described in our previous study, type 2 diabetic female rat brains may be more vulnerable to nitrosative stress than their respective males.

Collectively, these results reinforce the hypothesis that the slowdown of brain energy metabolism in middle-aged control and type 2 diabetic female rats may render them less susceptible to such oxidative damage and Alzheimer's disease-like neuropathology. In this perspective, this study further supports the need to establish sex- and age-specific preventive and/or therapeutic strategies (gender-based Medicine) against chronic type 2 diabetes and its long-term complications affecting the central nervous system (namely cognitive deficits and Alzheimer's disease).

**Keywords**: Brain metabolic dysfunction, intracellular stress, neuroprotection, reproductive aging, sex, type 2 diabetes.

# Table of contents

# List of Tables

# List of Figures

# List of Abreviations

| | |
|---|---|
| 3xTg-AD | Triple-transgenic mouse model of AD |
| α-KGDH | α-ketoglutarate dehydrogenase |
| ΔΨm | Mitochondrial membrane potential |
| Aβ | Amyloid-β |
| AcAc | Acetoacetic acid / acetoacetate |
| Acetil-CoA | Acetil-coenzyme A |
| AD | Alzheimer's Disease |
| ADP | Adenosine diphosphate |
| AGEs | Advanced glycation end products |
| $A_{max}$ | Maximum absorbance |
| AMP | Adenosine monophosphate |
| APOE | Apolipoprotein E |
| APS | Ammonium persulfate |
| ATP | Adenosine triphosphate |
| BBB | Blood-brain barrier |
| BDNF | Brain derived neurotrophic factor |
| BOH | 3-hydroxybutyric acid / 3-β-hydroxybutyrate |
| BSA | Bovine serum albumin |
| $Ca^{2+}$ | Calcium |
| $CaCl_2$ | Calcium chloride |
| CNS | Central nervous system |
| CoA-SH | Coenzyme A |
| CS | Citrate synthase |
| Cytc | Cytochrome c |
| $Cytc_{red}$ | Cytochrome c reduced |
| $Cytc_{ox}$ | Cytochrome c oxidized |
| DCPIP | 2,6-dichlorophenolindophenol |
| DNA | Deoxyribonucleic acid |
| DNTB | 5,5′-dithio-bis-(2-nitrobenzoic acid) |
| Drp1 | Dynamin-related protein-1 |
| DTT | Dithiotreitol |

| | |
|---|---|
| EDTA | Ethylenediamine tetraacetic acid |
| EGTA | Ethylene glycol tetraacetic acid |
| $FADH_2$ | Flavin adenine dinucleotide |
| G6P | Glucose-6-phosphate |
| G6PDH | Glucose 6-phopshate dehydrogenase |
| GK | Goto-Kakizaki |
| GLUT | Glucose transporter |
| GLUT | Glucose transporter isoform-3 |
| GSK3β | Glycogen synthase kinase 3β |
| $H_2O$ | Water |
| $H_2O_2$ | Hydrogen peroxide |
| $HbA_{1c}$ | Glycated hemoglobin / Hemoglobin $A_{1c}$ |
| HBDH | 3-hydroxybutyrate dehydrogenase |
| HCl | Hydrochloric acid |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| HFD | High fat diet-fed |
| KCN | Potassium cyanide |
| $KH_2PO_4$ | Potassium dihydrogen phosphate |
| LDH | Lactate dehydrogenase |
| MCI | Mild cognitive impairment |
| MDH | Malate dehydrogenase |
| $MgCl_2$ | Magnesium chloride |
| $NAD^+$ | Nicotinamide adenine dinucleotide oxidized form |
| NADH | Nicotinamide adenine dinucleotide |
| $NADP^+$ | Nicotinamide adenine dinucleotide phosphate oxidized form |
| NADPH | Nicotinamide adenine dinucleotide phosphate. |
| NaOH | Sodium hydroxide |
| $NO_2^-$ | Nitrite |
| NOS | Nitric oxide synthase |
| $O_2$ | Molecular oxygen |
| OXPHOS | Oxidative phosphorylation system |
| PDH | Pyruvate dehydrogenase |
| PET | Positron-emission tomography |

| | |
|---|---|
| Pi | Inorganic phosphate |
| PMSF | Phenylmethylsulfonyl fluoride |
| ROS | Reactive oxygen species |
| RNS | Reactive nitrogen species |
| SDH | Succinate dehydrogenase |
| SDS | Sodium dodecyl sulpate |
| SOD | Superoxide dismutase |
| STZ | Streptozotocin |
| T1D | Type 1 diabetes |
| T2D | Type 2 diabetes |
| TEA | Triethanolamine |
| TEMED | Tetramethylethylenediamine |
| TNB | 5-thio-2-nitrobenzoic acid |
| TPP | Thiamine pyrophosphate |
| ZDF | Zucker diabetic fatty |

# Introduction

## 1.1. Type 2 diabetes and Alzheimer's disease: linked by metabolism

The increasing life expectancy resulting from the tremendous medical progress over the last decades is one of the biggest triumphs of the modern society, but also one of its main challenges. Considering the demographic shift towards older ages, the raising prevalence of age-related diseases (such as type 2 diabetes and dementia) is not surprising (Lunenfeld and Stratton 2013; World Health Organization 2018).

### *1.1.1 Type 2 diabetes*

*Diabetes mellitus* is a group of chronic, metabolic, ethiologically and clinically heterogeneous disorders, whose hyperglycemia arises from an impaired insulin production and/or action (American Diabetes Association 2014). Its global prevalence has nearly doubled since 1980, with an estimation of 425 million people worldwide living with the disease in 2017 – a number expected to rise to 629 million in 2045 (International Diabetes Federation 2017; World Health Organization 2016). Indeed, only in Portugal, more than 1/4 of the population aged between 60-79 years was diabetic in 2015 (Observatório da diabetes 2016). Estimations point that 90-95% of the cases belong to type 2 diabetes (T2D), rendering it the most common form of the disease (International Diabetes Federation 2017; World Health Organization 2016).

Traditionally, T2D was predominantly a disease of middle-aged and elderly individuals. However, its prevalence is rising among younger adults, in children and adolescents (Alberti *et al.* 2004). This is due to the augmented risk factors associated with *modern* lifestyle (such as sedentarism, unhealthy diet and higher rates of overweight/obesity) that further contribute for the increasing prevalence of T2D worldwide (American Diabetes Association 2019a; Herman and Zimmet 2012; International Diabetes Federation 2017). Hence, T2D has been considered a pandemic by the World Health Organization.

Clinically, T2D is characterized by hyperglycemia due to insulin resistance, that may progress towards a pancreatic β-cell dysfunction and generalized loss of insulin sensitivity and secretion in the later stages (American Diabetes Association 2019a; Sebastião *et al.* 2014). At early stages of T2D, hyperglycemia progresses gradually and, although it often is not accompanied by clinical symptoms, this may be sufficient to cause pathological and functional alterations (American Diabetes Association 2019a). This results in a late T2D diagnosis, often only when patients present chronic complications affecting, *e.g.*, heart, blood vessels, kidney, eyes, nerves, and brain (American Diabetes Association 2019a; Biessels 2014; International

Diabetes Federation 2017; Verdile *et al.* 2015; World Health Organization 2016). Regarding the central nervous system (CNS), neurocognitive and neurologic complications (such as neurodegeneration, cognitive impairment and dementia) are well documented in T2D (Biessels 2014; Moran *et al.* 2013, 2019; Verdile *et al.* 2015).

### *1.1.2 Alzheimer's disease*

Dementia constitutes another large emergency of our century, constituting the 5$^{th}$ global cause of death nowadays. It affects ~50 million people worldwide, a number estimated the reach 152 million in 2050 (World Health Organization 2019).

Alzheimer's disease research has gained an intense focus in the last decades, fueled by its place as the leading cause of dementia (and consequent societal impact). Estimates point that 60-80% of all dementia cases correspond to AD, which affects ~35 million people worldwide (Alzheimer's Association 2019). AD is defined as an age-related, progressive neurodegenerative disease that affects wide areas of cerebral cortex and hippocampus, clinically resulting in progressive memory loss and gradual decline in cognitive function (Harris and Pierpoint 2012; Masters *et al*. 2015). The initial pathological alterations in AD brain may begin >20 years before symptoms appear and may include a subtle cognitive decline that progresses towards profound cognitive deficits, when the brain can no longer compensate for the accumulated changes (Alzheimer's Association 2019). Its neuropathological hallmarks include the deposition of extracellular senile plaques (SPs, mostly composed by amyloid-β (Aβ) peptide) and of intracellular neurofibrillary tangles (NFTs, containing hyperphosphorylated Tau protein), and the downregulation of pre- and postsynaptic proteins, with consequent synaptic loss (Duarte *et al.* 2018). Nevertheless, astrogliosis, nerve cell atrophy and neuronal loss may also occur AD brain (Kametani and Hasegawa 2018; Serrano-Pozo *et al.* 2011).

Sporadic AD is the most common form of the disease, accounting for >95% of all cases and being characterized by a late onset (after 65 years of age) (Masters *et al.* 2015). Like in T2D, this renders aging a risk factor also for sporadic AD, alongside the APOEε4 allele of apolipoprotein E (APOE) gene and the female sex (especially during advanced ages - >60% of AD population are postmenopausal women) (Riedel *et al.* 2016). While this was traditionally attributed to women's greater longevity, recent evidence showed that female sex *per se* is a major player in AD pathogenesis, progression, and clinical manifestations, particularly during

the transition from perimenopause into menopause - a unique feature of female sex (Duarte *et al.* 2018; Mosconi *et al.* 2017; Riedel *et al.* 2016; Zhao *et al.* 2016). Furthermore, the convergence of these three main risk factors (aging, APOEε4 and female sex) creates unique risk profiles for AD for male and female sexes (Riedel *et al.* 2016).

Despite the intense research efforts over the last decades, AD remains incurable and its current pharmacological therapies only attenuate cognitive symptoms, without slowing or halting the neurodegenerative process (Alzheimer's Association 2019). Adding to this, the majority of clinical trials performed to date aimed to address Aβ and/or hyperphosphorylated Tau deposition (*i.e.*, they relied on the amyloid theory) and produced disappointing results (Andrieu *et al.* 2015). Hence, there is an urgent medical and social need to face AD on new perspectives. One of them is to consider the reasons for the constant failure in clinical trials. Regarding sporadic AD, this may rely on the animal models used, since many of the available ones are genetic and mostly based on amyloid pathology (Drummond and Wisniewski 2017; Newman *et al.* 2017). hough these animal models provided crucial insight into some AD-related molecular mechanisms, they do not reflect the initial trigger or the overall biology of its sporadic form (Drummond and Wisniewski 2017). Furthermore, since the disease starts to develop several years prior the clinical manifestations (Alzheimer's Association 2019), research should focus on early interventional or preventive strategies, rather than acting after the onset of symptoms. Finally, sex-related differences across lifespan should be an important element in this equation, since the mechanisms leading to the disease may be different between men and women (Riedel *et al.* 2016).

### *1.1.3 T2D and AD: more than two unrelated diseases*

Epidemiological data revealed a considerable overlap in risk factors, comorbidities and pathophysiological mechanisms between T2D and AD, supporting the hypothesis of a link between both diseases (Moreira 2018). Indeed, T2D is a risk factor for AD, with diabetic patients having nearly twofold increased risk for AD compared with non-diabetic individuals (Ott *et al.* 1999). On the other hand, >80% of AD patients AD also suffer from comorbid T2D and impaired fasting glucose levels (Janson *et al.* 2004). In addition, hyperglycemia, insulin resistance, dyslipidemia and high blood pressure are well-known risk factors for T2D that also increase the risk of progression from mild cognitive impairment (MCI) to AD, particularly in

patients with a higher cognitive and affective decline (Hishikawa *et al.* 2016; de Matos *et al*. 2018; Pal *et al.* 2018; Solfrizzi *et al*. 2011; Viticchi *et al.* 2015).

Pathophysiologically, impaired insulin signaling and glucose metabolism were shown in T2D and AD, and may even trigger, *e.g.*, aging-related injury, mitochondrial dysfunction, intracellular stress mechanisms (such as oxidative/nitrosative stress), pro-inflammatory responses and deposition of amyloidogenic proteins (Candeias *et al.* 2012; Duarte *et al.* 2012). Altogether, these and other evidence support the hypothesis that AD is a metabolic disorder, particularly a "type 3 diabetes" or a "brain insulin resistance state" (Duarte *et al.* 2018).

## 1.2. Mitochondrial (energy) metabolism in T2D brain: possible shortcuts for AD

### *1.2.1 Brain glucose energy dysmetabolism*

Despite accounting for only 2% of total body mass, adult human brain is responsible for ~25% of the total body glucose utilization in the resting awake state, being glucose its main source of energy (Chen and Zhong 2013). As soon as glucose enters the cell, through glucose transporters (GLUTs), it is phosphorylated to glucose-6-phosphate by hexokinase (a regulatory enzyme of glycolysis and the first key point in the regulation of glucose metabolic rate) (Bouché *et al.* 2004). Then, glucose-6-phosphate can follow the remaining glycolytic route or shift towards other metabolic pathways (*e.g.* pentose phosphate or glycogen-forming pathways) (McKenna *et al.* 2012). Pyruvate, the final product of glycolysis, can be further converted into lactate (in a reversible reaction catalyzed by lactate dehydrogenase) or further metabolized by pyruvate dehydrogenase to acetyl-coenzyme A (acetyl-CoA) within the mitochondria (Jha *et al* 2012; Riske *et al.* 2017). This may in turn enter the Krebs cycle to generate reducing equivalents for the mitochondrial respiratory chain (Osellame *et al.* 2012).

Although ATP can be generated from glycolysis, under physiological conditions the high energy demanding neuronal cells produce it mostly from the oxidative metabolism (through Krebs cycle and mitochondrial oxidative phosphorylation) (Bélanger *et al.* 2011; Falkowska *et al.* 2015). From the total energy produced, 3/4 are used for signaling functions (including to control membrane potential) and the remaining are used for basic cellular activities (such as the turnover of proteins, nucleotides and phospholipids, and for axoplasmic transport) (McKenna *et al.* 2012). Thus, disruption of systemic glucose metabolism and the deficient supply of glucose to the brain may contribute to the impaired cerebral glucose metabolism that

characterizes T2D (including glucose uptake, glycolysis and Krebs cycle), ultimately affecting brain and cognitive function. Indeed, numerous studies showed that T2D patients exhibit brain glucose hypometabolism (especially in AD brain signature regions) that may result, at least in part, from peripheral and/or brain insulin resistance (Baker *et al.* 2011; García-Casares *et al.* 2014; Roberts *et al.* 2014; Willette *et al.* 2015a; Willette *et al.* 2015b). In addition, Baker *et al.* (2011) correlated the reduced cerebral glucose metabolic rate with insulin resistance in adults with prediabetes or T2D, whereas a more recent study showed that such hypometabolism was already present in diabetic individuals even before the onset of cognitive deficits (Roberts *et al.* 2014). Thus, brain hypometabolism may partially underlie the cognitive decline arising from T2D – a hypothesis further supported by its correlation with a worse cognitive performance (including in diabetic MCI patients), independently from amyloid deposition (García-Casares *et al.* 2014; Roberts *et al.* 2014).

It is increasingly accepted that T2D disrupts cerebral glucose metabolism, but the precise nature and mechanisms affected remain poorly understood. Studies in T2D rodent models suggest that these may involve disturbances in both glycolysis and oxidative metabolism (Andersen *et al.* 2017a; Carvalho *et al.* 2013; Girault *et al.* 2017; Leffa *et al.* 2017; Mechlovich *et al.* 2014; Shi *et al.* 2011; Sickmann *et al.* 2010; Zheng *et al.* 2017). Accordingly, Andersen *et al.* (2017)a observed a downregulation of glycolysis in cortical and hippocampal slices from *db/db* mice. Mechlovich *et al.* (2014) reported a slight decrement in mRNA levels of the glycolytic enzymes enolase-1 and aldolase in frontal cortex of *ob/ob* mice and in hippocampus of high fat-fed (HFD) mice. These data suggest an attenuation of brain glycolysis in T2D.

Results on the impact of T2D on brain Krebs cycle are contradictory. Nevertheless, a major portion of data point towards an impairment/inhibition of this metabolic pathway. In line with this, tow recent studies demonstrated a higher neuronal rate and lower astrocytic rate of Krebs cycle in *db/db* mice, while an inverse profile was observed in Goto-Kakizaki (GK) rats (Girault *et al.* 2017; Zheng *et al.* 2017). The later was in agreement with an overall impairment in brain glucose metabolism from Zucker diabetic fatty (ZDF) rats, being their Krebs cycle more downregulated than glycolysis, including in the cerebral cortex (Sickmann *et al.* 2010). Similarly, enzymes from Krebs cycle (*e.g.,* citrate synthase, aconitase) were inhibited in mice fed with an insulin resistance-inducing diet or with a 20% glucose solution (Carvalho *et al.* 2013; Leffa *et al.* 2017). Furthermore, hippocampal pyruvate dehydrogenase subunit PDHE1α (critical for the control of this enzyme activity) was downregulated in a T2D rat model resulting from the combination of a high-fat diet with streptozotocin (STZ) injection, further supporting

the inhibition of brain Krebs cycle in T2D (Shi *et al.* 2011). From the above, it is plausible that the apparently contradictory data on this issue may arise from the different T2D models and/or from the methodologies used.

Strikingly, brain glucose dysmetabolism is also an early pathophysiological feature in AD brain that may exacerbate its progression, most likely due to a deficient mitochondrial energy production (Blonz 2017; Chen and Zhong 2013; Duarte *et al.* 2018; Mamelak 2012). As detailed for T2D, a characteristic hypometabolic state was already present in memory-related brain regions from MCI patients (Chen and Zhong 2013) and may, therefore, constitute an effective biomarker for the cognitive deficits linked to progressive neurodegeneration, and to distinguish AD from other forms of dementia (Chen and Zhong 2013; Mosconi 2005; Silverman *et al.* 2001; Valotassiou *et al.* 2018). Also similar to T2D, AD-related hypometabolism may arise from a reduced glucose uptake by the brain (due to decreased GLUTs expression (Harr *et al.* 1995; Liu *et al.* 2008; Mooradian *et al.* 1997; Simpson *et al* 1994)), and/or from impaired downstream metabolic pathways. In line with this, both glycolysis and Krebs cycle were compromised in AD brain, as given by the downregulation of hippocampal genes and the altered activities of numerous brain enzymes from both pathways in AD (Brooks *et al.* 2007; Bubber *et al.* 2005; Iwangoff *et al.* 1980; Mastrogiacomo *et al.* 1996; Mastrogiacomo *et al.* 1993; Rex Sheu *et al.* 1985; Sorbi *et al.* 1983; Spillane *et al.* 1979).

### *1.2.2 Mitochondrial energy dysmetabolism*

Mitochondria are central organelles in many biological processes. They are involved in energy and reactive oxygen species (ROS) production, biosynthesis of macromolecules (*e.g.* amino acids, nucleotides, cholesterol, nitric oxide), calcium ($Ca^{2+}$) handling, antioxidant activity, apoptosis and other signaling processes (Chandel 2015; Herst *et al.* 2017). Most of the energy is generated in this organelle as adenosine triphosphate (ATP), via the mitochondrial oxidative phosphorylation (OXPHOS) (Herst *et al.* 2017; Wallace *et al.* 2010). In this process, electrons from the nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) (produced by Krebs cycle, fatty acids β-oxidation or from the conversion of ketone bodies to acetyl-CoA) are transferred to mitochondrial respiratory chain NADH-ubiquinone oxidoreductase (complex I) or to succinate: ubiquinone oxidoreductase (complex II), respectively, being then transferred to cytochrome c reductase (complexes III) and cytochrome c oxidase (complex IV), to be finally accepted by oxygen. The energy released as the protons

flow through the complexes is stored in the form of a proton gradient that generates an electric potential across the inner mitochondrial membrane. When the protons flow back into mitochondria through ATP synthase (complex V), their energy is used for ATP generation (Herst *et al.* 2017; Wallace *et al.* 2010). Neurons are highly differentiated cells with a high energy-demand, particularly for signaling functions (*e.g.*, maintenance of membrane ion gradients and neurotransmission) (McKenna *et al.* 2012; Moreira 2018). Thus, disruption in mitochondrial function may severely compromise neuronal activity, as described in T2D and AD brains (Moreira *et al.* 2010; Moreira 2018). Indeed, several studies showed a reduction in mitochondrial respiratory chain complexes expression and activities in HFD and *db/db* mice, and ZDF rats across distinct brain regions (Huang *et al.* 2015; Petrov *et al.* 2015; Raza *et al.* 2015). In addition, a wide range of T2D-induced brain mitochondrial defects (including energy impairment) have been considered as potential triggers and/or accelerators of AD in these patients (Moreira 2018). These include, *e.g.*, impaired brain mitochondrial respiratory chain (namely decreased respiratory control rate, complexes I–III and V activities), and decreased mitochondrial membrane potential ($\Delta\Psi_m$), ADP-induced depolarization and ATP/ADP in synaptosomes and brain vessels from mature sucrose-fed T2D male mice, which paralleled those suffered by the 3xTg-AD mouse model for AD (Carvalho *et al.* 2012, 2013). However, others found little or no signs of hippocampal and brain cortical mitochondrial decline in young *db/db* mice and GK rats, respectively (Huang *et al.* 2015; Santos *et al.* 2014). This suggested that the defects may arise upon aging and/or T2D progression, an hypothesis further supported by evidence that aging impairs mitochondrial respiratory chain and uncouples OXPHOS in GK rats' brain (Moreira *et al.* 2003). Strikingly, 4-month-old *db/db* mice brains appeared to have developed compensatory mechanisms to counteract their glucose hypometabolism and fulfill their energetic needs, through the maintenance of glutamate and glutamine oxidative metabolism (including an increased mitochondrial ADP-stimulated respiration upon administration of pyruvate and malate) (Andersen *et al.* 2017a, b).

Although the precise mechanisms underlying brain mitochondrial dysfunction in T2D remain poorly understood, Huang *et al.* (2015) suggested that it could result, at least in part, from an imbalance between mitochondrial fusion and fission, most likely mediated by glycogen synthase kinase 3β (GSK3β)/dynamin-related protein-1 (Drp1). In line with this, Drp1 was shown to be affected by hyperglycemia and insulin resistance (Akhtar *et al.* 2016; Sims-Robinson *et al.* 2010). Nevertheless, since the first defects in mitochondrial function occurred prior to the depression of AKT signaling in primary cortical rat neurons, it seems reasonable to

hypothesize that hyperglycemia may be the initial trigger of brain mitochondrial deficits (Peng *et al.* 2016). Conversely, mitochondrial dysfunction itself may contribute to insulin resistance (Gonzalez-Franquesa and Patti 2017; Kim *et al.* 2008) (namely through a higher production of ROS) and the subsequent creation of a vicious cycle of brain injury.

Mitochondrial energy defects are also an early event in sporadic AD pathology. At its initial stages, the inhibition of mitochondrial respiratory complexes I, II and V were detected in AD patients' entorhinal cortex, that were accompanied by a downregulation in the expression of several subunits from complexes I, II, IV and V in the later stages (Armand-Ugon *et al.* 2017). Additionally, Birnbaum *et al.* (2018) showed recently abnormalities in the composition OXPHOS complexes from induced pluripotent stem cells (iPSC)-derived neuronal cells from late-onset AD (a cellular model that mimics the early stage of AD), even before the detection of mitochondrial dynamics deficits and independent from Aβ or Tau protein levels. Hence, abnormalities in mitochondrial energetics may precede those in dynamics, and may even promote the later (Morán *et al.* 2012). Accordingly, numerous studies reported alterations in the expression/activities of the mitochondrial respiratory complexes in AD patients' brains (Carvalho *et al.* 2013; Chai *et al.* 2017; Pedrós *et al.* 2014), affecting especially complex IV (Parker Jr. *et al.* 1994; Salminen *et al.* 2015), with their regional pattern of downregulation mirroring that of reduced cerebral glucose metabolism (Liang *et al.* 2008). Thus, the AD characteristic brain glucose hypometabolism (discussed in *Section 1.2.1.*) may either arise from mitochondrial energy-related abnormalities and/or contribute to them through a negative feedback mechanism. Nevertheless, MCI patients showed an increased hippocampal expression of the same genes that suggested that compensatory mechanisms may occur in early stages of the disease (Mastroeni *et al.* 2017).

### *1.2.3. Brain pentose phosphate dysmetabolism*

Alternatively to glycolysis, glucose-6-phosphate may be metabolized by the pentose phosphate pathway that occurs in the cytosol (Baquer *et al.* 1988). This pathway can be subdivided in two distinct reactions (Baquer *et al.* 1988; Stincone *et al.* 2015): the **oxidative branch**, whose rate-limiting enzyme is glucose-6-phosphate dehydrogenase and generates nicotinamide adenine dinucleotide phosphate (NADPH) and ribose 5-phospate (a pentose required for the biosynthesis of nucleic acids) (Baquer *et al.* 1988; Stincone *et al.* 2015), and the **non-oxidative branch**, whose rate-limiting enzyme is transketolase and is critical for the

interchange of metabolites between glycolysis and the pentose phosphate pathway (Baquer *et al.* 1988).

Although pentose phosphate pathway contributes to only 2-5% of the cerebral metabolic glucose consumption (Ulusu *et al.* 2003), it is extremely important in physiological conditions, by providing genetic substrates and the reducing equivalent NADPH for antioxidant defenses (Bolaños and Almeida 2010; Chen and Zhong 2013; Stincone *et al.* 2015). Indeed, NADPH grants the reducing power for most antioxidant and redox regulatory enzymes (such as glutathione/glutaredoxin, thioredoxin, heme oxygenase-1 and aldo-ketoreductases - the main systems for cellular redox homeostasis) in adult brain (Bolaños and Almeida 2010; Fernandez-Marcos and Nóbrega-Pereira 2016; Mamelak 2012; Stanton 2012; Stincone *et al.* 2015), participating also in hydrogen peroxide ($H_2O_2$) detoxification (Baquer *et al.* 1988). Hence, it is not surprising that, upon $H_2O_2$-induced oxidative stress, glucose metabolism was shifted towards the pentose phosphate pathway to reinsure the replenishment of antioxidant defenses at the expense of ATP (Godon *et al.* 1998; Ralser *et al.* 2007). Since hyperglycemia and insulin resistance are well-known promoters of oxidative stress, the pentose phosphate pathway may therefore be stimulated in diabetic brain. However, increased (Gok *et al.* 2016; Ulusu *et al.* 2003) and decreased (Nakhaee *et al.* 2010) glucose-6-phosphate dehydrogenase activity were observed in the brains of the type 1 diabetes (T1D) STZ rat model, while in alloxan-induced diabetic rats the enzyme was stimulated (Abdel-Rahim *et al.* 1992; Lakhman *et al.* 1994). These apparently contradictory data may be due to the stage of the disease, experimental design and sex of the animals used (Martins 1986b). Regarding the T2D, despite no available data on the pentose phosphate pathway in brains from human patients, an inhibition in glucose-6-phosphate dehydrogenase was detected in serum. Additionally, evidences exist that reduced enzyme activity levels and the genetic disorder glucose-6-phosphate dehydrogenase deficiency may constitute risk factors for T2D development (Aouacheri *et al.* 2015; Lai *et al.* 2017; Wan *et al.* 2002), thus supporting a connection between pentose phosphate pathway and T2D pathophysiology. However, this enzyme may be affected differently from tissue to tissue, as reported in T1D animal models (Gok *et al.* 2016; Ulusu *et al.* 2003). Indeed, Shi *et al.* (2011), reported an inhibition of hippocampal glucose-6-phosphate dehydrogenase of T2D rats (by mass spectrometry), that was mirrored by a slight decrease in its mRNA and protein levels (Shi *et al.* 2011). Conversely, astrocytic and neuronal pentose phosphate pathway was stimulated after acute or chronic hyperglycemia (although at different extents) that, nonetheless, only

rescued the elevation of ROS in astrocytes and in mixed cultures, possibly by counteracting neuronal oxidative stress through the astrocytic antioxidant defenses (Takahashi *et al.* 2012).

Pentose phosphate pathway is also altered in AD, as given by the increased glucose-6-phosphate dehydrogenase levels and activity in different brain regions (including frontal and inferior temporal cortices) from MCI and AD patients (Balazs and Leon 1994; Iwangoff *et al.* 1980; Martins *et al.* 1986a; Palmer 1999; Russell *et al.* 1999; Sultana *et al.* 2008), in line with the increased oxidative stress detected early in the disease (Butterfield *et al.* 2006; Keller *et al.* 2005; Wang *et al.* 2006). In addition, 6-phosphogluconate dehydrogenase (the enzyme responsible for the second NADPH producing-reaction of the pathway) was stimulated in frontal and inferior temporal cortex from AD patients (Martins *et al.* 1986a; Palmer 1999). Strikingly, studies in AD rodent models provided variable results, ranging from a stimulation of glucose-6-phosphate dehydrogenase (Chakrabarty *et al.* 2012; van Gijsel-Bonnello *et al.* 2017; Huang *et al.* 2010; Soucek *et al.* 2003) towards a reduced pentose phosphate pathway flux in hippocampal slices and whole brain tissue from young and aged APP/PS1 mice, respectively (Cisternas *et al.* 2018; Tiwari and Patel 2014). Besides the deficits in the oxidative branch of pentose phosphate pathway upon AD, Chen and Zhong (2013) an inhibition of both peripheral and cerebral transketolase in AD patients that, together with dysfunctional pyruvate dehydrogenase and α-ketoglutarate dehydrogenase, may hamper the exchange of intermediates between glycolysis and pentose phosphate pathway.

### *1.2.4. Ketone bodies: an alternative brain fuel*

Ketone bodies are mainly produced in the liver from the oxidation of fatty acids and ketogenic amino acids (*i.e.*, leucine, lysine, phenylalanine, isoleucine, tryptophan, tyrosine, threonine) (Camandola and Mattson 2017; Puchalska and Crawford 2017). Once in circulation, ketone bodies can cross the brain-blood barrier (BBB) through monocarboxylic acid transporters (Morris 2005; Newman and Verdin 2014), thus rendering liver their principal supplier for the brain (Camandola and Mattson 2017; Puchalska and Crawford 2017). Nevertheless, astrocytes are also ketogenic cells that can generate small quantities of ketone bodies from leucine and fatty acids (Camandola and Mattson 2017). Once in the CNS, ketone bodies constitute its main alternative metabolic fuel (most likely via the conversion into acetyl-CoA to enter Krebs cycle for energy synthesis) (Morris 2005; Newman and Verdin 2014) thus reinforcing their crucial role during development and disease, or even when glucose availability

is limited (*i.e.*, after prolonged fasting, prolonged hypoglycemia, or due to T2D-associated insulin resistance) (Morris 2005). The most metabolically significant ketone bodies are acetoacetate and 3-β-hydroxybutyrate, that can be interconverted by 3-β-hydroxybutyrate dehydrogenase to provide up to 60% of brain energy and/or to be used in the biosynthesis of, *e.g.*, sterols (such as cholesterol in myelin), fatty acids and amino acids (Camandola and Mattson 2017; Morris 2005).

Ketone bodies appear also to exert neuroprotective effects, and may be therapeutically promising against neurodegenerative diseases (namely in AD) (Lange *et al.* 2017; Włodarek 2019). More specifically, administration of ketone bodies increased cell surviving and neurite number/outgrowth in an AD cellular model (Kashiwaya *et al.* 2000). Accordingly, ketogenic/ketosis-inducer diet mitigated amyloid pathology and phosphorylated tau protein levels, recovered the levels glycolytic and Krebs cycle intermediates and mitochondrial function in the brains from AD rodent models, ultimately restoring their cognition (Kashiwaya *et al.* 2013; Studzinski *et al.* 2008; Yao *et al.* 2011; Yin *et al.* 2016). Moreover, Lange *et al.* (2017) described that ketogenic diets ameliorated cognitive performance in AD patients, particularly in non-ApoE4 carriers. In 2004, Guzmán and Blázquez (2004) also reported that starvation (and its consequent increment in plasma ketone bodies) and 3-β-hydroxybutyrate protected the brain against hypoxia/ischemia-associated damage in animal models. This could be of the outmost relevance, since *1)* ischemic brain infarction is a chronic vascular complication of T2D, a disorder where *2)* glucose metabolism is compromised and *3)* that constitutes a major risk factor for AD, thus rendering ketone bodies a pivotal brain energy source in elderly T2D patients (Nakayama *et al*. 2015). Indeed, despite their glucose hypometabolism, hippocampal slices from T2D *db/db* mice had an increased acetoacetate and β-hydroxybutyrate metabolism that may in turn improve their mitochondrial function (Andersen *et al.* 2017a). Although the precise mechanisms underlying the neuroprotection by ketone bodies remain elusive, it has been hypothesized that their function as signaling metabolites may alter the expression of genes related with oxidative stress, energy metabolism, memory and learning (Mamelak 2017; Newman and Verdin 2014).

One may emphasize that, in spite of the putative benefits of ketone bodies' to the CNS in the short-term, if the pool of peripherally-produced ketone bodies brain becomes chronically limited, the brain may start to metabolize its own fatty acids and other ketone bodies sources (such as axonal myelin), ultimately contributing to neurodegeneration and to the exacerbation of AD susceptibility (Brinton *et al.* 2015; Duarte *et al.* 2018).

### 1.3. Intracellular stress in T2D brain: a potential trigger for AD

ROS and reactive nitrogen species (RNS) are inevitable byproducts of cellular metabolism, with ~1–2% of the $O_2$ consumed by mitochondria being used for ROS formation, mainly through the mitochondrial respiratory complexes I and III (Chen *et al.* 2018; Dalle-donne *et al.* 2005). RNS derive from the nitric oxide produced from *L*-arginine, in a reaction catalyzed by the nitric oxide synthase (Dalle-donne *et al.* 2005). ROS and RNS play crucial biological roles as signaling molecules in numerous processes, but they can be equally destructive (Dalle-donne *et al.* 2005; Ray *et al.* 2012). Under physiological conditions, the detrimental effects of ROS and RNS are counteracted by the antioxidant systems. However, when the redox balance is lost (due to an overproduction of ROS/RNS and/or impaired antioxidant defenses), oxidative/nitrosative stress and its related damage and dysfunction of macromolecules occur (*e.g.*, proteins, lipids, DNA). This oxidative/nitrosative stress has been widely linked to the pathophysiology of several age-related diseases, including T2D and AD (de Matos *et al.* 2018; Moreira 2012).

Chronic hyperglycemia promotes oxidative/nitrosative stress, namely by facilitating the formation of ROS and RNS via *1)* an excessive flux of mitochondrial respiratory chain and its consequent dysfunction; *2)* the increased formation of advanced glycation end products (AGEs); *3)* the activation of protein kinase C (PKC) pathway; *4)* the stimulation of hexosamine pathway flux (Kandimalla *et al.* 2017; Panigrahy *et al*. 2017). These may in turn lead to insulin resistance (including in the brain), further exacerbating ROS and RNS generation and creating a vicious cycle of oxidative/nitrosative stress-related injury (Kandimalla *et al.* 2017; de la Monte 2017). Indeed, increased ROS and RNS levels together with defective antioxidant defenses were associated with T2D progression and related complications (namely the increased risk for AD) (Butterfield *et al.* 2014; Hamed 2017; de Matos *et al.* 2017). This was supported by the higher ROS and RNS levels, increased accumulation of markers for oxidative damage (*e.g.*, lipid peroxidation, protein carbonylation and DNA damage) in the brains from T2D animal models (Carvalho *et al.* 2014; Pu *et al.* 2018; Raza *et al.* 2015; Santos *et al.* 2014; Zhang *et al.* 2019). Moreover, the increase in the inducible nitric oxide synthase isoform in T2D ZDF rats suggests that their higher RNS levels may arise from an overproduction due to mitochondrial OXPHOS dysfunction, rather than from an impaired antioxidant system (Raza *et al.* 2015). This renders mitochondrial impairment a pivotal contributor and a target of ROS and RNS (Islam 2017; Moreira 2012; Singh *et al.* 2019).

Notably, data on the role of T2D on antioxidant defenses remain debatable. Previous studies from our laboratory showed that the antioxidant system could counteract T2D-related oxidative/nitrosative stress until a certain extent, as evidenced by increments in the non-enzymatic antioxidant vitamin E and in enzymes from the glutathione redox cycle in the brain synaptosomes and homogenates of T2D GK rats, respectively *(Carvalho et al. 2014; Correia et al.* 2008; Duarte *et al.* 2000; Santos *et al.* 2000).Nevertheless, other studies reported a decrement in glutathione redox cycle, total and manganese superoxide dismutase and in catalase activities, as well as in the non-enzymatic antioxidant coenzyme Q content in T2D rat and mouse brain (Carvalho *et al.* 2014; Correia *et al.* 2008; Raza *et al.* 2015; Santos *et al.* 2001; Zhang *et al.* 2019). The later observations were mirrored by reduced nuclear factor erythroid-2-related factor 2 (Nrf2) levels (Carvalho *et al*. 2015; Pu *et al.* 2018), a redox-sensitive master regulator of antioxidant genes (namely those encoding superoxide dismutase, catalase ore even glucose-6-phosphate dehydrogenase) (Thimmulappa *et al.* 2002; Zhu *et al.* 2005). As referred above, T2D-induced oxidative stress may also alter glucose metabolism, possibly via the oxidative modification and consequent blunting of metabolic enzymes, as described also for AD (Butterfield and Halliwell 2019). Indeed, several authors demonstrated that 4-hydroxy-2-nonenal (a product of lipid peroxidation) inhibits α-ketoglutarate dehydrogenase and pyruvate dehydrogenase (Gibson *et al.* 2008; Tretter and Adam-Vizi 2005).

Oxidative/nitrosative stress is also an early event in AD, likely constituting one of the motors of its onset and progression, since the accumulation of markers for their damage occurs already in MCI and early stage AD individuals (Butterfield *et al.* 2006; Keller *et al.* 2005; Tönnies and Trushina 2017; Wang *et al.* 2006) and preceded Aβ pathology (Nunomura *et al.* 2001). These markers included, *e.g.*, increased levels of oxidized proteins, glycosylated products, lipid peroxidation, free carbonyls, and oxidative modifications in RNA, and nuclear and mitochondrial DNA (Tönnies and Trushina 2017). Furthermore, antioxidant defenses were impaired in AD patients (Tönnies and Trushina 2017; Wojsiat *et al.* 2018). Thus, the early impairment of glucose metabolism and mitochondrial function observed in the disease may promote these intracellular stress mechanisms and the subsequent neuronal damage, even before the onset of Aβ or Tau pathology (Moreira 2012; Tönnies and Trushina 2017) (further detailed in *Sections 1.2.2, 1.2.3*).

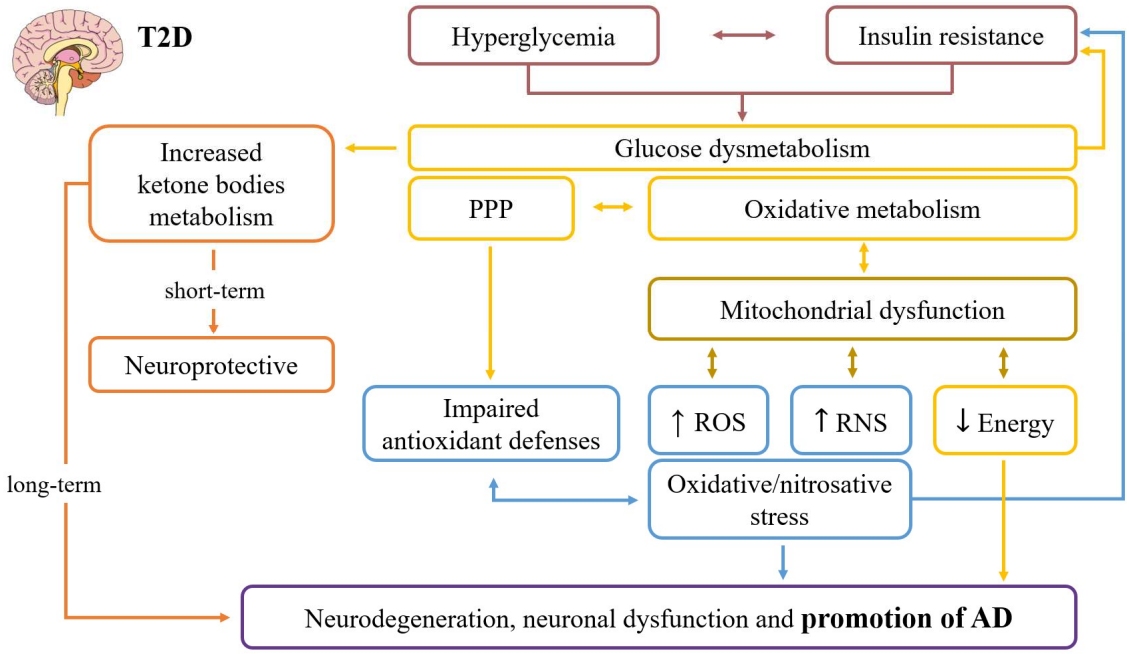



**Figure 1.1: Hyperglycemia and insulin resistance, the two main hallmarks of T2D, promote a vicious cycle of brain damage, culminating in AD.** Hyperglycemia and insulin resistance impact brain glucose metabolism in different ways (*e.g.* glucose uptake, glycolysis, Krebs cycle). Altered glucose mitochondrial metabolism and increased reactive oxygen and nitrogen species (ROS and RNS, respectively) that arise from hyperglycemia aggravate mitochondrial dysfunction, that in turn accentuates glucose dysmetabolism and further increases the production of ROS and RNS. These may in turn exacerbate insulin resistance. Under these conditions, the uptake of glucose by the brain decreases, leading to an hypometabolic state, in which the decrement in brain glucose, in the activity of enzymes from metabolic pathways (due, *e.g.*, to oxidative stress), together with mitochondrial dysfunction may blunt energy production. All these mechanisms, either by themselves and/or through their crosslinks, may promote neurodegeneration, neuronal dysfunction and, ultimately, increase the susceptibility to AD. Since glucose metabolism is compromised in T2D and AD, the brain may utilize ketone bodies as an alternative fuel. Although these may exert a neuroprotective effect, in the long run they may also contribute to neurodegeneration. Abbreviations: PPP, pentose phosphate pathway; ROS, reactive oxygen species; RNS, reactive nitrogen species.

## 1.4. Female sex and reproductive aging: effects on the brain

As recently referred by Nebel *et al.* (2018), medically the term *sex* refers to the biological differences (such as chromosomal or hormonal factors), whereas *gender* refers to differences in the impact of psychosocial, cultural and environmental influences on biological factors between men and women. Importantly, both can impact brain function, but through different means – this reinforces the need to distinguish and evaluate their effects in separate. Indeed, female and male brains age differently, with female brains undergoing age-related changes considerably earlier than males (including the entrance in a hypometabolic state that increases their susceptibility to AD) (Zhao *et al.* 2016). For instance, female sex affects brain energy metabolism and intracellular stress mechanisms (Duarte *et al.* 2018).

As mentioned in *Section 1.1.3*, female sex is itself a risk factor for AD and, albeit the mechanisms underlying such an increased risk in women remain unclear, the few studies available point towards a regulatory effect of estrogen (Rettberg *et al.* 2014). Estrogen is a master regulator that controls several metabolic systems: it may stimulate brain glucose uptake and metabolism, promoting glucose transport and upregulating neuronal aerobic glycolysis (by upregulating the glycolytic enzymes hexokinase and posphofructokinase 1), Krebs cycle and mitochondrial OXPHOS towards ATP synthesis, while it may inhibit brain fatty acid and ketone bodies metabolism (Rettberg *et al.* 2014). Furthermore, estrogen increased the maximal mitochondrial respiratory rate in neurons and glia (Rettberg *et al.* 2014), protected against DNA oxidative damage and increased the expression of antioxidant enzymes (such as manganese superoxide dismutase) (Rettberg *et al.* 2014). This and other neuroprotective actions from estrogen are most likely mediated by its receptors network.

Classical estrogen receptors (ERα and ERβ), and G protein-couple estrogen receptors are ubiquitously distributed across brain regions (including those involved in cognition) and interact with different neuroprotective intracellular signaling cascades (like the insulin-like growth factor-1 (IGF-1) receptor, Wnt and BDNF signaling pathways) (Arevalo *et al.* 2015; Brinton *et al.* 2015). Thus, estrogen binding to its receptors initiates a highly coordinated network of signaling and transcriptional pathways towards the upregulation of anti-apoptotic and downregulation of pro-apoptotic and pro-inflammatory genes and proteins, to promote spinogenesis, synaptogenesis and neuronal survival (Arevalo *et al.* 2015; Hara *et al.* 2015).

During several decades, women benefit from estrogen protection. However, at a certain point the estrogen/estrogen receptors become dysfunctional and menopause arises (Brinton *et al.* 2015). Strikingly, individuals of the female sex also undergo a unique phase of reproductive transition during midlife termed perimenopause that, nonetheless, ultimately culminates in reproductive aging/senescence (Brinton *et al.* 2015). From an endocrine perspective, perimenopause is characterized by irregular menstrual cycles and variable levels of circulating hormones. The secretion of the ovarian hormones estrogen and progesterone stabilize at low levels in the final stage of perimenopause - the menopause -, which is completed after 1 year of amenorrhea (Brinton *et al.* 2015). During this period, typical symptoms may arise, many of them of neurological nature (namely cognitive decline) that somehow indicate a disruption in multiple estrogen-related systems, and render perimenopause a neuroendocrine transition state (Brinton *et al.* 2015).

Upon perimenopause, the network of estrogen receptors becomes uncoupled from the bioenergetic system, thereby promoting a hypometabolic state, neurological dysfunction and increased risk for neurodegenerative disorders (Brinton *et al.* 2015). Indeed, perimenopause may downregulate the energy mechanisms that were previously operational: the decline in neuronal GLUTs coincides with the entrance of brain into a hypometabolic state that leads to a glucose dysmetabolism and deficits in mitochondrial energetics, increased fatty acid catabolism, Aβ accumulation and synaptic dysfunction (Ding *et al.* 2013a; Duarte *et al.* 2018; Lejri *et al.* 2018; Mosconi *et al.* 2017; Yin *et al.* 2015). As a consequence, the perimenopausal brain may shift to a compensatory ketone-based metabolism (as shown by an increment in ketones levels, transporters and enzymes) to provide the Krebs cycle with the acetyl-CoA required to synthesize ATP via mitochondrial OXPHOS (Ding *et al.* 2013a; Yao *et al.* 2010). Furthermore (and potentially arising from the loss of mitochondrial function and antioxidant defenses), the perimenopausal brain also undergoes oxidative stress (Lejri *et al.* 2018). Hence, it is not surprising that perimenopausal women become more susceptible to neurodegenerative processes and AD.

Although alterations in genes involved in energy and amyloidogenic metabolism started earlier in female mice hippocampi than in males, only their ERβ was altered, suggesting a role in the premature aging suffered by females (Zhao *et al.* 2016). This was accompanied by a downregulation of insulin signaling-related genes, in line with evidence for insulin resistance under such conditions (Duarte *et al.* 2018). Interestingly, CNS insulin signaling may have different primordial roles according to sex: while men were more sensitive to insulin-related acute anorexigenic effect, its beneficial effects on hippocampal-dependent memory functions were more marked in women (Benedict *et al.* 2008). Moreover, peripheral metabolic control in women during and after this period may also influence their cognitive performance, since postmenopausal women with a poor control had a worse cognitive decline, thus emphasizing the importance of energy metabolism on cognitive function (Rettberg *et al.* 2016).

Regarding the interactions between sex and T2D, in postmenopausal women T2D was associated with a higher cognitive decline (Espeland *et al.* 2011). However, some controversies exist regarding the effects of hormone replacement therapy (estrogen alone or conjugated with other hormones) on T2D incidence, with studies reporting lower T2D incidence in treated women (Bonds *et al.* 2006; Margolis *et al.* 2004; Rettberg *et al*. 2014), whereas others reported that higher endogenous estrogen levels after menopause increased the risk for insulin resistance and T2D (Ding *et al.* 2007; Espeland *et al.* 2015; Kalish *et al.* 2003). Interestingly, young-adult

and mature female Wistar (control) and T2D GK rat brains showed a rise in phosphorylated Tau and a reduction of synaptic proteins upon aging, while increased Aβ levels was detected only in aged GK rats (Pintana *et al.* 2017). This pointed towards a role for aging on female rat susceptibility to AD that was exacerbated by T2D. However, the authors did not provide results for male controls, thus failing to demonstrate a sex-specific effect. Hence, recent findings from our group showed that T2D may anticipate the peripheral impairment in gonadal estrogen production and, thus, the menopause (Candeias *et al.* 2017). This was accompanied by altered peripheral and brain cholesterol levels in middle-aged female rats, with the subsequent compromise of estrogen uptake from the periphery into the CNS and/or its local brain steroidogenic metabolism (Candeias *et al.* 2017). Nevertheless, these animals developed adaptive mechanisms to delay AD-like neuropathology, thus reinforcing the hypothesis that different steroid hormone profiles in middle-aged male and female rats differently impact their brain across T2D progression (Candeias *et al.* 2017). However, further studies are needed to evaluate the molecular mechanisms involved in sex-mediated changes in brain mitochondrial (energy) metabolism and intracellular stress pathways upon T2D progression and reproductive aging.

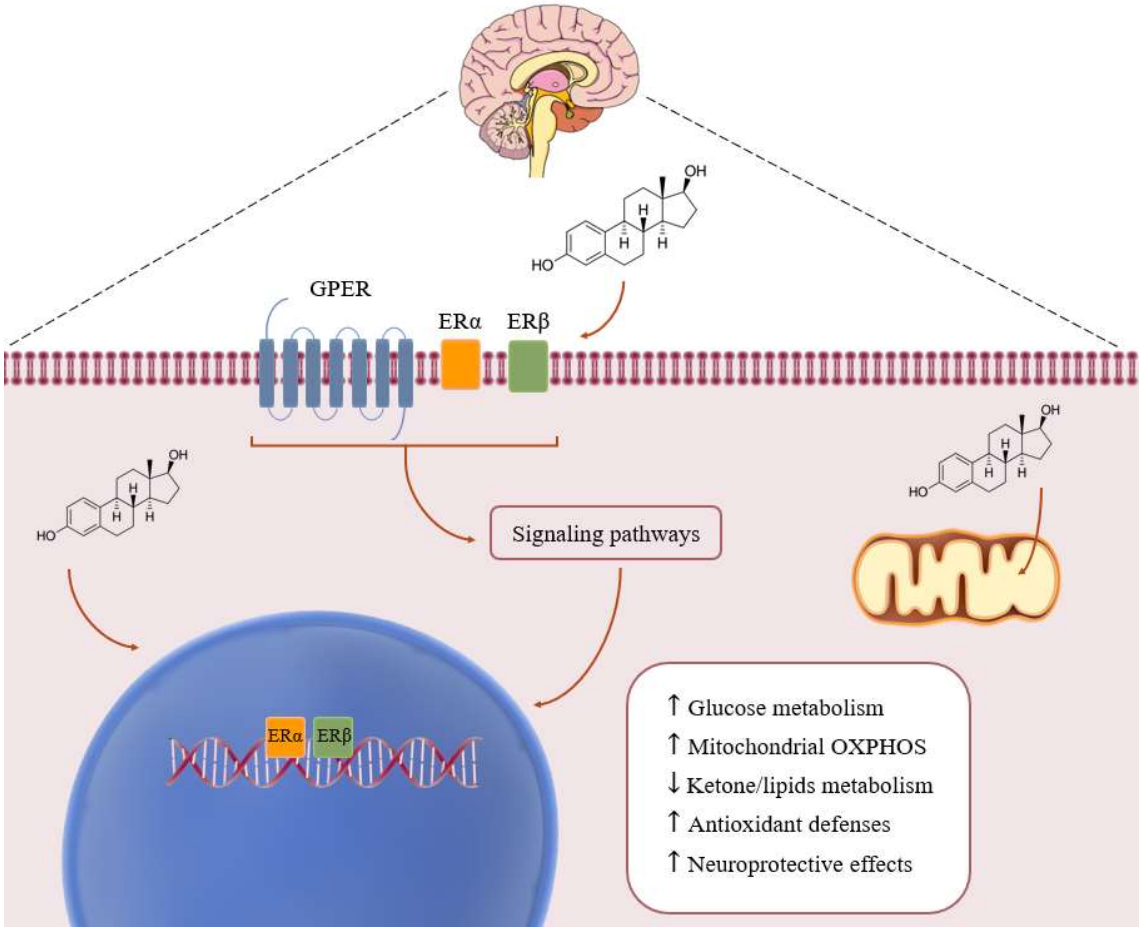



**Figure 1.2: Estrogen/Estrogen receptor network and beneficial effects in CNS.** Estrogen binds to its receptors located at the plasma membrane, nucleus and mitochondria, activating downstream signaling pathways that promote changes at transcriptional level. Transcriptional pathways may be also activated by a direct action of estrogen in the nucleus. Estrogen may also modulate mitochondrial genes. These interactions may stimulate glucose metabolism and mitochondrial function (at the expense of lipid and ketone metabolism), antioxidant systems and other neuroprotective effects (*e.g.*, synaptogenesis, cell survival). Upon perimenopause these responses are inhibited, possibly as a result from the uncoupling between estrogen/estrogen receptor network and the bioenergetic system. Abbreviations: Erα, estrogen receptor α; Erβ, estrogen receptor β; GPER, G protein-couple estrogen receptor.

## 1.5. GK rat: a valuable model to study T2D-related CNS changes

Goto-Kakizaki (GK) rats are considered one of the best polygenic animal model for spontaneous T2D (Akash *et al.* 2013). They were first generated by Goto and collaborators, upon the repetitive, selective breeding of Wistar rats with slightly increased blood glucose levels after an oral glucose tolerance test (Goto *et al.* 1975).

GK rats are a non-obese model of T2D, allowing the study of the pathophysiological mechanisms underlying T2D *per se.* They are also a non-ketotic rodent model, characterized by mild to moderate (but stable) hyperglycemia, hyperinsulinemia and hyperleptinemia at

younger ages, that progress towards a deficient insulin secretion and peripheral insulin/leptin resistance at later ages (Akash *et al.* 2013; Candeias *et al.* 2017; Portha *et al.* 2012). Their hyperglycemia appears after a prediabetic period (often during the first 3 weeks of age, mostly after weaning) (Movassat *et al.* 2008). Portha (2005) demonstrated that the T2D state of GK rats was stable over 72 weeks, with no apparent differences between males and females. However, females were also described to present slightly lower postprandial plasma glucose levels than their male cohorts (Portha 2005). Of note, postprandial plasma glucose levels in GK males often range between 10-14 mM, while in age-matched Wistar rats range from 6-8 mM (Portha 2005). Importantly, the decreased pancreatic β-cell mass and function in GK rats may arise from the interaction between their genetic profiles associated with defective insulin secretion, their gestational metabolic impairment that may decrease β-cell neogenesis, and their secondary loss due to the chronic exposure to hyperglycemia/glucotoxicity (Portha 2005).

GK rats often present several characteristics found in human T2D patients and develop human-like T2D complications, thus rendering them a widely used model for the study of T2D-related chronic complications and pathophysiological mechanisms, including those affecting the brain (Candeias *et al.* 2017; Wang *et al.* 2013). Among such brain changes, we emphasize the perturbed expression of genes related to neurotransmission, lipid metabolism, oxidative stress, DNA damage signaling, cell cycle regulation, neuronal development, insulin secretion and feeding behavior in hippocampus and prefrontal cortex (Abdul-Rahman et al. 2012). Though as far as we know there is no direct proof of AD-like pathology in GK rats, some components of AD-susceptible phenotypes were reported previously, including cortical neuronal loss and increased microglia activation in 13-month-old, and alterations in brain energy metabolism in middle-aged (6-7 month-old) GK rat males (Girault et al. 2017; Hussain et al. 2014). Furthermore, an age-dependent redox imbalance occurred in brain synaptosomes and vessels of male GK rats that may render their brains more vulnerable to degeneration (Carvalho et al. 2014). These rats also had less exploratory activity, most likely due to learning deficits (Moreira *et al.* 2007). On the other hand, sex also influenced the alterations suffered by GK rat brains over disease progression (Candeias *et al.* 2017).

From all the above, GK rats constitute a valuable model for the study of T2D-related CNS alterations, especially to evaluate the impact of sex herein.

# Hypothesis and Aim

From the above, and taking into account that:

*1)* the precise pathophysiological mechanisms underlying chronic T2D-related brain and cognitive dysfunction and AD-like neuropathology remain poorly understood (but may involve, *e.g.*, mitochondrial dysfunction (Moreira 2018), oxidative stress (Ahmad *et al.* 2017; Moreira 2012), neurodegeneration and death, mainly in brain cortex and hippocampus (Duarte *et al.* 2013));

*2)* T2D and AD share similar characteristics (namely the compromise in peripheral and brain glucose metabolism (Chen and Zhong 2013) and cognitive decline (Verdile *et al.* 2015));

*3)* we recently demonstrated that, although changes in sex steroid hormones may start in the brains from middle-aged rat females and then spread towards the ovaries (being accelerated by T2D), both control and T2D female rats were less vulnerable to the accumulation of oxidative stress and AD-like neuropathological features (Candeias *et al.* 2017);

*4)* alterations in brain and peripheral metabolism may underlie such sexual dimorphism along T2D progression,

in the present study we hypothesized that sex differently affects brain cortical (energy) metabolism and intracellular stress pathways in T2D at midlife.

Thus, we aimed to study the role of metabolic function and intracellular stress on the differential effects of sex in middle-aged (8-month-old, retired breeders) T2D GK rat brains. More specifically, we aimed to evaluate the impact of sex on middle-aged Wistar (control) and T2D GK male and female rat brain cortical:

*1)* glucose (energy) metabolism, especially on glycolysis, pentose phosphate pathway, TCA cycle, mitochondrial respiratory chain, ketone bodies and phosphocreatine/creatine system;

*2)* intracellular nitrosative stress markers, with a special emphasis on nitrites, a reactive nitrogen species.

With this study, we expect to open new avenues on the differential role of sex on T2D brain cortical glucose (energy) metabolism and oxidative/nitrosative stress vulnerability during perimenopause. Moreover, this study may contribute to the adoption of sex-specific preventive/therapeutic strategies (the so-called personalized or gender-based Medicine) against chronic T2D and its long-term complications affecting the CNS (namely cognitive deficits and AD), especially among in the high-risk elderly women.

# Materials and Methods

### 3.1. Materials

All materials and reagents were of the highest purity commercially available. Rabbit polyclonal GLUT3 antibody was obtained from Abcam (Cambridge, UK). Anti-rabbit IgG secondary antibody was obtained from GE Healthcare (Chicago, IL, USA). Rabbit anti-α-tubulin antibody (1:1000, Cell Signaling Technology, Leiden, The Netherlands). QuantiChrom™ Glucose Assay kit (DIGL-100) and EnzyChrom™ Ketone Body assay kit were purchased to BioAssay Systems (Hayward, CA, USA). PicoProbe™ Fructose-6-Phosphate Fluorimetric Assay kit, Pyruvate Colorimetric/Fluorometric Assay kit, Lactate Colorimetric/Fluorometric Assay kit, $NAD^+$/NADH Quantification Colorimetric kit were purchased to BioVision (Milpitas, CA, USA).

### 3.2. Animals

Following EU and Portuguese legislation (Directive 2010/63/EU; DL113/2013, August 7$^{th}$), 8 month-old (middle-aged, retired breeders) male and female Wistar control and T2D GK rats (a non-obese, spontaneously T2D since early age that result from the selective breeding of Wistar rats with high glucose levels) (Akash *et al.* 2013; Goto *et al.* 1975) (Table 3.1) were used upon ethical approval by the Animal Welfare Committee of the Center for Neuroscience and Cell Biology and Faculty of Medicine, University of Coimbra. Following "3Rs" Reduction principle established by FELASA, we used the brain cortical levels of estrogen E obtained in our previous study (Candeias *et al.* 2017) to estimate the number of animals required for the present one. Briefly, by using the *F* test, ANOVA 1-way on the G-Power software (Faul *et al.* 2007), with the above-mentioned means, α error of 0.05, power of 80%, and equal sample size, we estimated that a total of 12 rats should be used for the overall study (Candeias *et al.* 2017). In line with this and in order to increase the power of our hypothesis, in the current study we used at least n = 4 rats/group. Animals were kindly provided by Professor Raquel Seiça (Institute of Physiology, Faculty of Medicine, University of Coimbra), and housed in our local animal facilities (conventional animal facilities of Faculty of Medicine, University of Coimbra). Rats were kept in pairs from the same sex in a static microisolator cage with a filter top, and bedding and nesting materials, under controlled light (12h day/night cycle) and humidity (45-65%), *ad libitum* standard hard pellets chow, and sterilized and acidified water (pH 2.5-3). Signs of distress were carefully monitored and glucose tolerance tests were used as

selection index. Although not expected, a rapid decrease of body weight >15-20% was considered as a humane endpoint for the study.

**Table 3.1: Experimental groups of animals.**

| **Age** | Middle-aged (8-month-old) | | | |
|---|---|---|---|---|
| **Strain** | Wistar | | Goto-Kakizaki | |
| **Sex** | Male | Female | Male | Female |

### 3.3. Peripheral blood collection and routine biochemical analyses

After an overnight fasting, blood samples were collected by terminal cardiac puncture from anesthesized [ketamine chloride (75 mg/kg, i.p.) and chlorpromazine chloride (2.65 mg/kg, i.m., Lab. Vitória, Portugal)] rats, according to a previously described method (Matafome *et al.* 2011).

Blood glucose levels were measured by the glucose oxidase reaction, using a glucometer (*Glucometer Elite*, Bayer SA, Portugal) and compatible reactive stripes. Results were expressed as mg glucose/dL blood.

Blood glycated hemoglobin ($HbA_{1c}$) was determined with the Multi-Test $HbA_{1c}$ (A1C Now$^{+}$, Bayer SA, Portugal) and results expressed as %.

### 3.4. Isolation and preparation of cerebral cortex homogenates

Animals were weighed, anesthesized, and euthanized by decapitation. Brains were immediately removed and transferred onto 0.01 M phosphate buffer (composed by: 80 mM $Na_2HPO_4$, 20 mM $NaH_2PO_4$, 150 mM NaCl, pH 7.5), at 4ºC, to remove the meninges. Then, both hemispheres were separated by a longitudinal cut the inter-hemispheric sulcus, the cortices were dissected and snap-frozen at -80ºC for further studies.

Immediately before the experiments, brain cortices were homogenized at 0-4°C in 500 µL of lysis buffer containing (in mM): 25 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2 $MgCl_2$, 1 ethylenediamine tetraacetic acid (EDTA), 1 ethylene glycol tetraacetic acid (EGTA) (pH 7.4), supplemented with 2 mM dithiotreitol (DTT), 0.1 mM phenylmethanesulfonyl fluoride (PMSF) and cocktails of protease and phosphatase inhibitors (SigmaFast Protease Inhibitor Tablets, Sigma-Aldrich; and PhosSTOP – Phosphatase Inhibitor Cocktail Tablets, Roche), using a Teflon-glass homogenizer (~10 strokes) of Potter-Elvejhem

type, according to our previously described method (Candeias *et al.* 2017). The homogenates were then centrifuged at 17,968 ×*g* for 10 min, at 4 °C, in a refrigerated Sigma 2-16K centrifuge (SciQuip Ltd, Newton, UK), to remove the nuclei. The resulting supernatant ($S_1$) was collected and the pellet ($P_1$) resuspended in 500 µL of supplemented lysis buffer, and centrifuged again under the same conditions. Then, the supernatant $S_2$ was added to the previously obtained one ($S_1$) and protein levels were measured as described below.

### 3.5. Protein quantification by the Sedmak method

Protein levels from cerebral cortex homogenates were measured by the Sedmak colorimetric assay (Sedmak and Grossberg 1977), adapted for 96-well microplate. This method is based on the conversion, in acidic conditions, of Coomassie brilliant blue G250 from a brownish-orange color to an intense blue when coupled to protein (Fig. 3.1.). The absorbance of this protein-dye complex peaks at 620 nm (Sedmak and Grossberg 1977).

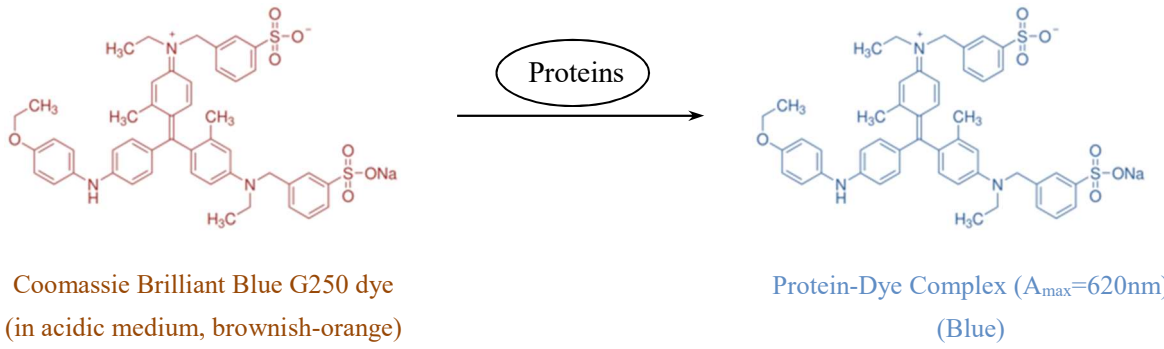



**Figure 3.1: Principle of the Sedmak colorimetric assay for protein quantification.** Free coomassie brilliant blue G250 possess a brownish-orage color that turns to blue upon coupling with protein basic/aromatic side chains. The protein-dye complex is detected at 620nm. Abbreviation: $A_{max}$, maximum absorbance.

Briefly, 2 µL of each brain cortical homogenate were added, in a 96-well plate, to 78 µL of type 1 water. A standard curve was prepared using known concentrations of bovine serum albumin (BSA) 0.1% (Sigma-Aldrich, St. Louis, MO, USA): 0, 10, 20, 40, 80, 95 µg/µL, together with the supplemented lysis buffer used to prepare the samples (Table 3.2). Concentrated Sedmak reagent (0.06% Coomassie Blue G250 prepared in 3% (w/v) perchloric acid) was diluted in a proportion of 30 mL of concentrated Sedmak reagent to 70 mL of 3% perchloric acid, and then 200 µL were added to each well. The plate was incubated at room temperature for 15 min, protected from light, and then read in a SpectraMax Plus 384

spectrophotometer (Molecular Devices, San Jose, CA, USA), at 620 nm. Protein concentration was calculated by extrapolation from the standard curve and expressed as µg protein/µL.

**Table 3.2: Protocol for the determination of protein levels by the Sedmak colorimetric assay.**

| | [BSA] (µg/µL) | BSA 0.1% (µL) | Sample (µL) | $H_2O$ (µL) | Supplemented Lysis Buffer (µL) | Sedmak Reagent (diluted) (µL) |
|---|---|---|---|---|---|---|
| $P_0$ | 0 | 0 | - | 98 | 2 | 200 |
| $P_{10}$ | 10 | 10 | - | 88 | 2 | 200 |
| $P_{20}$ | 20 | 20 | - | 78 | 2 | 200 |
| $P_{40}$ | 40 | 40 | - | 58 | 2 | 200 |
| $P_{80}$ | 80 | 80 | - | 18 | 2 | 200 |
| $P_{95}$ | 95 | 95 | - | 3 | 2 | 200 |
| Sample | ? | - | 2 | 98 | - | 200 |

Volumes of the reagents pippeted into 96-well microplates for subsequent measurement of absorbance at 620nm and protein levels analysis.

## 3.6. Determination of brain cortical glucose transporter 3 (GLUT3) isoform protein levels

GLUT3 is a high-affinity glucose transporter isoform, mainly expressed on neuronal membranes (although also found in astroglial and endothelial cells), that allows the transport of glucose into the neuron (Rettberg 2014).

Brain cortical GLUT3 protein levels were determined by Western blotting analysis, as described by (Reagan *et al.* 2000), modified by (Duarte *et al.* 2008). Briefly, samples containing 50 µg of brain cortical lysates each were denatured with sodium dodecylsulfate (SDS) sample buffer, containing: 0.5 M Tris–HCl, 0.4% SDS (pH 6.8), supplemented with 30% glycerol, 10% SDS, 0.6 M DTT and 0.012% bromophenol blue, at 100ºC, for 5 min. Then, samples containing denatured proteins were loaded onto a 4% polyacrylamide stacking gel on top of a 10% polyacrylamide resolving gel (see Table 3.3) and electrophoretically separated by SDS/PAGE according to their molecular weight, using a running buffer composed by 25 mM Tris, 192 mM bicine, 0.1% SDS (pH 8.3).

**Table 3.3: Composition of SDS-PAGE gels for Western Blot (2 gels).**

| Components | Resolving Gel 10% | Stacking Gel 4% |
|---|---|---|
| **0.5 mM Tris-HCl, pH 6.8** | - | 2.5 mL |
| **1.5 mM Tris-HCl, pH 8.8** | 5 mL | - |
| **$H_2O$** | 8.2 mL | 6.1 mL |
| **Acrilamide** | 6.6 mL | 1.3 mL |
| **SDS 10%** | 200 µL | 100 µL |
| **APS 20%** | 100 µL | 50 µL |
| **TEMED** | 10 µL | 10 µL |

The initial electrophoretic separation phase was performed at 80 V and once the proteins reached the resolving gel, the voltage was increased to 140 V until the system was stopped. Afterwards, the stacking gel was removed and proteins were electrotransferred onto polyvinyl difluoride (PVDF) membranes at 1A for 90 min in a CAPS solution (10 mM CAPS (pH 11), 10% methanol). Then, membranes were blocked for 1h at room temperature in Tris-buffered saline (TBS) (20 mM Tris, 137 mM NaCl, pH 7.4) plus 1% BSA and 0.1% Tween 20 (TBS-T). Membranes were then incubated overnight at 4ºC, with rabbit polyclonal anti-GLUT3 antibody (1:2000, Abcam, Cambridge, UK), diluted in 1% BSA prepared in TBS-T. Subsequently, the membranes were washed 3 times with TBS-T and incubated with anti-rabbit IgG secondary antibody (1:10 000, GE Healthcare, Chicago, IL, USA), for 2h, at room temperature. After that, the membranes were washed again with TBS-T and the proteins detected using an alkaline phosphatase substrate for enhanced chemifluorescence (ECF, GE Healthcare). Immunoreactive bands were visualized by the VersaDoc Imaging System (Bio-Rad, Hercules, CA, USA). Fluorescence signal was analyzed using the QuantityOne software (BioRad) and the results given as INT/mm$^2$.

Of note, membranes were then reprobed with rabbit anti-α-tubulin (1:1000) antibody to normalize the protein in the samples. They were washed 3 times with TBS-T for 5 min to remove the previous primary and secondary antibodies from the membrane. Then, the membranes were incubated overnight, at 4ºC, with rabbit anti-α-tubulin antibody (1:1000, Cell Signaling Technology, Leiden, The Netherlands) antibody and the remaining procedure was repeated as described above. Results were presented as GLUT3 protein expression (corresponding to the ratio of GLUT3 *vs.* α-tubulin).

## 3.7. Determination of brain cortical glucose levels

Brain cortical glucose levels were colorimetrically determined by QuantiChrom™ Glucose Assay kit (DIGL-100) (BioAssay System; Hayward, CA, USA), according to manufacturer's instructions with slight modifications. This assay relies on the formation of a blue-green complex between *D*-glucose and *o*-toluidine (Eq. 3.1). More specifically, the carbonyl group of the open form of *D*-glucose condenses with the amino group of o-toluidine, generating a stoichiometric colored imino adduct.

$$\text{Glucose} + \text{o-toluidine} \longrightarrow \text{Imine} \quad (3.1)$$

Briefly, 1.25 µL of each brain cortical homogenate were incubated in 250 µL reaction buffer and then heated to 100ºC in a boiling water bath, for 8 min. Then, the mixes were immediately cooled down in ice for 4 min. Absorbance was read at 630 nm, in a SpectraMax Plus 384 microplate reader. Brain cortical glucose levels were expressed as mg/mg protein.

## 3.8. Determination of markers for the glycolytic metabolism

Glycolysis was given by the activity of hexokinase (the rate-limiting step of the glycolytic pathway), the rate of formation of glucose-6-phosphate, the levels of fructose-6-phosphate, pyruvate, lactate and NADH, and by the activity of lactate dehydrogenase in rat brain cortical lysates.

### *3.8.1. Determination of hexokinase activity*

Hexokinase activity was determined by a colorimetric method previously described (Crabtree *et al.* 1979). This method is based on the reduction of $NADP^+$ to NADPH, as result of the following reactions (Eq. 3.2; 3.3):

$$\text{Glucose} + \text{ATP} \xrightarrow{\textbf{Hexokinase}} \text{Glucose-6-phosphate} + \text{ADP} \quad (3.2)$$

$$\text{Glucose-6-phosphate} + NADP^+ \xrightarrow{\textbf{G6PDH}} \text{6-phosphogluconolactone} + \text{NADPH} + H^+ \quad (3.3)$$

Briefly, 5 µL of each brain cortical homogenate were added to 90.7 µL reaction buffer (composed by 50 mM Tris-HCl (pH 8.0), supplemented with 10 mM $MgCl_2$, 1.1 mM ATP-

$Mg^{2+}$, 1.2 mM $NADP^+$ and 2 U/mL glucose-6-phosphate dehydrogenase) in an UV microplate. Absorbance was continuously read at 340 nm, 37ºC, for 2 min with 30 s intervals, in a SpectraMax Plus 384 microplate reader. Then, the reaction was initiated by the addition of 216 mM *D*-glucose and absorbance was read again for 5 min, with intervals of 30 s. Hexokinase activity was calculated through the slopes obtained during the linear phase. Hexokinase specific activity was determined as the difference between basal activity in the absence and presence of *D*-glucose. An $\varepsilon_{340nm}$ = 6220 $M^{-1}$ $cm^{-1}$ and normalization to protein amount were applied. Hexokinase activity was expressed as pM/min/mg protein.

### *3.8.2. Determination of the rate of glucose 6-phosphate formation*

The rate of glucose-6-phosphate production was determined colorimetrically, according to a previously described method (Lamprecht *et al.* 1974), with some modifications. This method is based on the reaction represented by Eq. 3.3, whereas glucose-6-phosphate is converted to 6-phosphogluconolactone, by glucose-6-phosphate dehydrogenase, using $NADP^+$ as coenzyme. Absorbance is proportional to glucose consumption to generate glucose-6-phosphate.

Briefly, 5 µL of each brain cortical homogenate were incubated in 72.45 µL triethanolamine (TEA) buffer, containing (in mM): 50 triethanolamine-hydrochloride and 22 NaOH (pH 7.5), supplemented with 0.2 mM β-$NADP^+$ sodium salt and 8.35 mM $MgCl_2$, in a 96-well UV plate. Absorbance was continuously read at 339 nm, 37ºC, for 2 min, with 30 s intervals, in a colorimetric plate reader SpectraMax Plus 384. Then, the reaction was initiated by the addition of 743.75 U/L glucose-6-phosphate dehydrogenase from baker's yeast (*S. cerevisæ*), type VII, ammonium sulphate suspension, and the absorbance continuously read at 339 nm, 37°C, for 3 min, with 30 s intervals. The rate of glucose-6-phosphate formation was calculated using an $\varepsilon_{339nm}$=1 $mol^{-1}mm^{-1}$, from the extrapolation of absorbance, according the formula $\Delta A = A_2 - A_1$, where $A_2$ corresponds to the absorbance measured after the addition of glucose-6-phosphate dehydrogenase and $A_1$ corresponds to the basal reading. Results were normalized to the amount of protein and the rate of glucose-6-phosphate formation expressed as µmol/min/mg protein.

### *3.8.3. Determination of fructose 6-phosphate levels*

Fructose-6-phosphate levels were determined by the PicoProbe™ Fructose-6-Phosphate Fluorimetric Assay Kit (BioVision; Milpitas, CA, USA) according to manufacturers' instructions with slight modifications. The assay is based on the formation of a fluorescent product, resulting from the conversion of fructose-6-phosphate into glucose-6-phosphate that is subsequently oxidized to a fluorescent product, whose fluorescence intensity can be determined with an excitation and emission wavelengths of 535 nm and 587 nm, respectively.

Briefly, 5 µL of each brain cortical homogenate were incubated in 45 µL of fructose-6-phosphate assay buffer. Then, the reaction was initiated by the addiction of 50 µL of reaction mix, followed by an incubation of 5 min, at 37ºC, protected from light. Fluorescence intensity was measured using excitation and emission wavelengths of 535 nm and 587 nm, respectively, in a SpectraMax Gemini EM multiplate fluorescence reader (Molecular Devices, San Jose, CA, USA). Fructose 6-phosphate levels were expressed as nmol/mg protein.

### *3.8.4. Determination of pyruvate levels*

Pyruvate levels were accessed colorimetrically by the Pyruvate Colorimetric/Fluorometric Assay Kit (BioVision; Milpitas, CA, USA), according to manufacturer's instructions. This colorimetric assay is based on the oxidation of pyruvate by pyruvate oxidase to generate a colored product that can be detected at 570 nm.

Briefly, 5 µL of each brain cortical homogenate were incubated in 45 µL assay buffer and 50 µL reaction mix, for 30 min, at room temperature, protected from light. Absorbance was read at 570 nm, in a SpectraMax Plus 384 microplate reader. Pyruvate levels were expressed as nmol/mg protein.

### *3.8.5. Determination of lactate dehydrogenase activity*

Lactate dehydrogenase (LDH) activity was colorimetrically determined at 340 nm, by following the oxidation of NADH after the addition of monosodic pyruvate (Eq. 3.4), according to a previously described method (Bergmeyer and Bernt 1974).

$$\text{Pyruvate} + \text{NADPH} + \text{H}^+ \xleftrightarrow{\textbf{LDH}} \text{Lactate} + \text{NAD}^+ \qquad (3.4)$$

Briefly, 5 µL of each brain cortical homogenate were incubated in 50 µL Tris-NaCl buffer (composed by 81.3 mM Tris and 203.3 mM NaCl, pH 7.2), supplemented with 1.5 mM monosodic pyruvate, in an UV microplate. Absorbance was continuously read at 340 nm, 37ºC, for 3 min, with 30 s intervals, in a SpectraMax Plus 384 microplate reader. Then, the reaction was initiated by the addition of 1.2 mM NADH, and the absorbance continuously read at 340 nm, for 5 min, with 30 s intervals. Lactate dehydrogenase activity was calculated through the slopes obtained during the linear phase. Lactate dehydrogenase specific activity was determined as the difference between basal activity in the absence and presence of NADH. An $\varepsilon_{340nm}$= 0.63 $mmol^{-1}$ $mm^{-1}$ and normalization to the amount of protein were applied. Lactate dehydrogenase activity was expressed as µM/min/mg protein.

### *3.8.6. Determination of lactate levels*

Lactate levels were determined colorimetrically by the Lactate Colorimetric/Fluorometric Assay Kit (BioVision; Milpitas, CA, USA), according to manufacturer's instructions. In this colorimetric assay, lactate specifically reacts with an enzyme mix to generate a product that further interacts with lactate probe to produce color, detected at 570 nm.

Briefly, 5 µL of each brain cortical homogenate were incubated in 45 µL assay buffer (working dilution 1:10). Absorbance was read at 570 nm, in a SpectraMax Plus 384 microplate reader. Lactate levels were expressed as nmol/mg protein.

### *3.8.7. Determination of NADH levels*

NADH levels were determined colorimetrically by $NAD^+$/NADH Quantification Colorimetric kit (BioVision; Milpitas, CA, USA), according to manufacturer's instructions with slight modifications. This colorimetric assay relies on the capacity of the NAD Cycling Enzyme Mix (component of the kit) to specifically recognize NADH/NAD in an enzyme cycling reaction that can be measured at 450 nm.

Briefly, 10 µL of each brain cortical homogenate were first incubated in 50 µL extraction buffer and decomposed by heating to 100ºC in a water bath. This allows the specific detection of NADH in the homogenates. Then, 50 µL of each previous mixture were incubated in 50 µL cycling buffer for 5 min, at room temperature. Then, 10 µL of NADH developer were added and the ideal time of reaction was accessed by intercalating an incubation at room temperature

with periodic absorbance measurements at 450 nm, until reaching an absorbance of 0.3. The ideal reaction time was accorded to be 3 h, thus, at the end of that period the final absorbance was read at 450 nm, in a SpectraMax Plus 384 microplate reader. NADH levels were expressed as pmol/mg protein.

### 3.9. Determination of glucose-6-phosphate dehydrogenase activity

Glucose-6-phospate dehydrogenase activity, the rate limiting enzyme of the oxidative branch of pentose phosphate pathway, was colorimetrically measured according to the procedure described by (García-Nogales *et al.* 1999), with slight modifications, to yield the function of this metabolic pathway. This method relies on the measurement, at 340 nm, of the reduction of $NADP^+$ to NADPH that occurs as glucose-6-phosphate is converted to 6-phosphogluconolactone (Eq. 3.3).

Briefly, 5-10 µL of the previously-referred brain cortical homogenates were added to the reaction buffer, composed by 50 mM Tris-HCl (pH 7.5), supplemented with 5 mM $MgCl_2$ and 0.3 mM $NADP^+$, in a 96-well UV microplate, to a volume of 95 µL/well. Absorbance was continuously read at 340 nm, 37ºC, for 2 min, with 30 s intervals, in a SpectraMax Plus 384 spectrophotometer. The reaction was initiated by the addition of 5 µL of 10 mM glucose-6-phosphate, to perfume a final volume of 100 µL/well, and the absorbance continuously read for 5 min, with 30 s intervals. Glucose-6-phosphate dehydrogenase activity was calculated from the slopes obtained during the linear phase, using an $\varepsilon_{340nm}$ = 6220 $M^{-1}cm^{-1}$. Glucose-6-phosphate dehydrogenase activity was expressed as pmol glucose-6-phosphate/min/mg protein.

### 3.10. Determination of activities from Krebs cycle

Brain cortical Krebs cycle function was given by the activity of the enzymes pyruvate dehydrogenase, citrate synthase, α-ketoglutarate dehydrogenase, succinate dehydrogenase and malate dehydrogenase.

### *3.10.1. Determination of pyruvate dehydrogenase activity*

Pyruvate dehydrogenase activity was monitored at 340 nm, by following the reduction of $NAD^+$ upon the conversion of pyruvate to acetyl coenzyme A (acetyl-CoA) (Eq.3.5), as previously described (Humphries and Szweda 1998).

$$\text{Pyruvate} + \text{CoA} + NAD^+ \xrightarrow{\textbf{PDH}} \text{Acetyl-CoA} + CO_2 + \text{NADPH} + H^+ \quad (3.5)$$

Briefly, 5 - 12.5 µL of each brain cortical homogenate were added to the reaction medium containing (in mM): 35 $KH_2PO_4$, 5 $MgCl_2$, 2 KCN, 0.5 EDTA, 0.25% Triton X-100 (pH=7.25), supplemented with 2.5 µM rotenone, 0.5 mM $NAD^+$, 0.2 mM thiamine pyrophosphate (TPP), 0.13 mM coenzyme A (CoA) and 1 mM cysteine, in a 96-well UV microplate, to a volume of 95 µL/well. Of note, rotenone is a well-known inhibitor of mitochondrial respiratory chain complex I and was used to prevent the immediate consumption of the NADH formed. This allowed the calculation of the specific activity of pyruvate dehydrogenase. Absorbances were continuously read at 340 nm, at 37ºC, for 2 min, with 30 s intervals, in a SpectraMax Plus 384 spectrophotometer. Then, the reaction was initiated by the addition 5 µL pyruvate 80 mM, and the absorbances read again for 5 min, with 30 s intervals. Pyruvate dehydrogenase activity was calculated trough the slopes obtained during the linear phase. Pyruvate dehydrogenase activity was calculated using an $\varepsilon_{340nm}$=6220 $M^{-1}$ $cm^{-1}$ and expressed as pM/min/mg protein.

### *3.10.2. Determination of citrate synthase activity*

Citrate synthase (CS) activity was determined according to a previously described method (Coore *et al*. 1971), with some modifications. This method relies on the colorimetric measurement of the mercaptide ion 5-thio-2-nitrobenzoic acid (TBN) resulting from the reaction between reaction of coenzyme A (CoA-SH) and the Ellman's reagent - 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) (Eq. 3.6). This allows the detection of the rate of formation CoASH, produced in the presence of oxaloacetate and acetyl-CoA in a reaction catalyzed by citrate synthase (Eq. 3.7) at 412 nm.

$$\text{DTNB} + \text{CoA-SH} \longrightarrow \text{CoA-S-S-TNB} + \text{TNB} \quad (3.6)$$

$$\text{Oxaloacetate} + \text{Acetyl-CoA} + \text{H2O} \xrightarrow{\textbf{CS}} \text{Citrate} + \text{CoA-SH} \quad (3.7)$$

Briefly, 12.5 µg of each brain cortical homogenate were added to buffer A (composed by 200 mM Tris (pH=8.0), 10 mM acetyl-CoA, 10 mM DTNB) to a volume of 96 µL/well, in a 96-well microplate. Basal absorbance was continuously read at 412 nm, 37ºC, for 3 min with 30 s intervals, in a SpectraMax Plus 384 spectrophotometer. Then, the reaction was initiated by the addition of 2 µL oxaloacetate 10 mM, and the absorbance was read again for 6 min, with 30 s intervals. Finally, a negative control was performed with 1% Triton X-100 (a nonionic detergent that solubilizes mitochondria, inhibiting citrate synthase) (Gurtubay *et al*. 1980) and the absorbance read again for 6 min, with 30 s intervals. Citrate synthase activity was calculated trough the slopes obtained during the linear phase. Citrate synthase specific activity was determined as the difference between basal activity in the absence and presence of Triton X-100. Citrate synthase activity was calculated using an $\varepsilon_{412nm} = 13.6\ mM^{-1}\ cm^{-1}$ and expressed as nmol/min/mg protein.

### *3.10.3. Determination of α-ketoglutarate dehydrogenase activity*

α-Ketoglutarate dehydrogenase (α-KGDH) activity was measured as described by Starkov *et al.* (2004), with some modifications. This method is based on the colorimetric assay, at 340 nm, of the reduction of $NAD^+$ resulting from the conversion of α-ketoglutarate to succinyl-CoA, catalyzed by α-ketoglutarate dehydrogenase (Eq. 3.8).

$$\text{Oxaloacetate} + \text{Acetyl-CoA} + H2O \xrightarrow{\textbf{α-KGDH}} \text{Citrate} + \text{CoA-SH} \qquad (3.8)$$

Briefly, 5-12.5 µL of each brain cortical homogenate were added to reaction medium containing 25 mM $KH_2PO_4$, 5 mM $MgCl_2$, 2 mM KCN, 0.5 mM EDTA, 0.25% Triton X-100 (pH=7.25), supplemented with 2.5 µM rotenone, 0.2 mM $NAD^+$, 0.3 mM TPP, 0.13 mM CoA and 1 mM cysteine, in a 96-well UV microplate. Basal absorbance was continuously read at 340 nm, 37ºC, for 2 min, with 30 s intervals, in a SpectraMax Plus 384 microplate reader. Then, the reaction was initiated by the addition of 5 mM α-ketoglutarate, and the absorbance read again for 5 min, with 30 s intervals. α-Ketoglutarate dehydrogenase activity was calculated through the slopes obtained during the linear phase. α-Ketoglutarate dehydrogenase specific activity was determined as the difference between basal activity in the absence and presence of α-ketoglutarate. An $\varepsilon_{340nm} = 6220\ M^{-1}\ cm^{-1}$ and normalization to protein amount were applied. α-Ketoglutarate dehydrogenase activity was expressed as pM/min/mg protein.

### *3.10.4. Determination of succinate dehydrogenase activity*

Succinate dehydrogenase (SDH) activity was determined colorimetrically at 550 nm, as previously described by (Munujos *et al.* 1993) and Niatsetskaya *et al.* (2010), with some modifications. This assay is based on the formation of formazan due to the reduction of a tetrazolium salt reduction (in this case, the nitro blue tetrazolium, NBT), catalyzed by succinate dehydrogenase, in the presence of a high concentration of succinate (Eq. 3.9).

$$\text{NBT} \xrightarrow[\text{electron donor (}e.g.\text{ NADH/FADH}_2\text{)}]{\textbf{SDH}} \text{Formazan} \qquad (3.9)$$

Briefly, 5-10 µL of each brain cortical homogenate were added to the reaction medium (composed by 100 mM Tris (pH=7.5), supplemented with 0.5 mM EDTA, 2 µM antimycin A, 2 µM rotenone, 10 mM sodium azide and 2 mM NBT), in a 96-well microplate. Absorbance was read after the addition of 20 mM succinate and a 60 min incubation, at 550 nm, 37ºC, in a SpectraMax Plus 384 spectrophotometer. Then, the reaction was stopped by the addition of 2% sodium dodecyl sulfate (SDS), to perform a final volume of 100 µL/well, and the absorbance read again. Succinate dehydrogenase activity was calculated from the slope obtained during the linear phase. Succinate dehydrogenase specific activity was determined as the difference between basal activity in the absence and presence of SDS. An $\varepsilon_{550nm}$ = 21 000 $M^{-1}$ $cm^{-1}$ and normalization for protein amount were used. Succinate dehydrogenase activity was expressed as pM/min/mg protein.

### *3.10.5. Determination of malate dehydrogenase activity*

Malate dehydrogenase (MDH) activity was determined by following the reduction of $NAD^+$, at 340 nm, that results from the conversion of malate to oxaloacetate (Eq. 3.10), according to a previously described method (Nulton-Persson and Szweda 2001), with some modifications.

$$\text{Malate} + \text{NAD}^+ \xrightarrow{\textbf{MDH}} \text{Oxaloacetate} + \text{NADH} + \text{H}^+ \qquad (3.10)$$

Briefly, 5-10 µL of each brain cortical homogenate were added to lysis buffer (containing: 25 mM $KH_2PO_4$ (pH=7.25), 0.5 mM EDTA, 0.01% Triton X-100), supplemented with 9.7 µL

of reaction buffer (containing: 10 mM rotenone, 5 mM $MgCl_2$, 1 U/mL citrate synthase, 0.3 mM acetyl-CoA, 10 mM $NAD^+$), in a 96-well UV microplate, to perform a volume of 95 µL/well. Basal absorbance was continuously read at 340 nm, 37ºC, for 2 min with 30 s intervals, in a SpectraMax Plus 384 microplate reader. Then, the reaction was initiated by the addition of 5 µL malate 25 mM, and the absorbances read again for 20 min, with 1 min intervals. Malate dehydrogenase activity was calculated through the slopes obtained during the linear phase. Malate dehydrogenase specific activity was determined as the difference between basal activity in the absence and presence of malate (substrate). Malate dehydrogenase activity was calculated using an $\varepsilon_{340nm} = 6220\ M^{-1}\ cm^{-1}$ and normalization to protein amount was applied. Malate dehydrogenase activity was expressed as pM/min/mg protein.

## 3.11. Determination of mitochondrial respiratory chain complexes I-IV activities

### *3.11.1. Determination of complex I activity*

Mitochondrial complex I (NADH-ubiquinone oxidoreductase) activity was measured by a previously described method (Long *et al.* 2009), with some modifications. This method measures the reduction of 2,6-dichlorophenolindophenol (DCPIP) (instead of exogenous ubiquinone) upon electron transfer from NADH mediated by complex I (Janssen *et al.* 2007; Long *et al.* 2009). This approach has the advantage of preventing the accumulation of ubiquinol and the subsequent inhibition of complex I (Bénit *et al.* 2008), together with the mitigation of the signal obtained from other unspecific dehydrogenases, since the absorbance is read at 600 nm (from DCPIP, instead of 340 nm, as the experimental procedures used to measure complex I activity in isolated mitochondria based on NADH oxidation) (Long *et al.* 2009).

Briefly, 5 µL of each brain cortical homogenate were diluted in 91.83 µL reaction buffer containing (in mM): 25 $KH_2PO_4$ (pH 7.5), 5 $MgCl_2$, 0.3 KCN, 0.004 antimycin A, supplemented with 3 mg/mL BSA fatty acid-free, 60 µM coenzyme $Q_1$ and 160 µM DCPIP. Complex I activity was continuously measured at 600 nm, for 6 min with 30s intervals, in a SpectraMax Plus 384 spectrophometer, by following the decrease in absorbance of DCPIP at 37ºC, upon addition of freshly-prepared 100 µM NADH. Mitochondrial complex I activity was calculated through the slopes obtained during the linear phase. Mitochondrial complex I specific activity was determined as the difference between the activities in the absence and presence of 10 µM rotenone (specific inhibitor of complex I). Complex I activity was further

calculated using an $\varepsilon_{600nm}$ = 19.1 $mM^{-1}$ $cm^{-1}$. Results were normalized to the amount of protein and expressed as nmol $_{DCPIP}$/min/mg protein.

### *3.11.2. Determination of complexes II/III activities*

Mitochondrial complexes II/III (succinate-cytochrome c reductase) activities were determined by a modification of a method previously described (Tisdale 1967). This assay relies on the reduction of cytochrome c by electrons initially derived from the conversion of succinate to fumarate by complex II (Tisdale 1967).

Briefly, 5 µL of each brain cortical lysate were preincubated at 37 °C, for 5 min, in 60 µL of phosphate buffer (composed by 166 mM $KH_2PO_4$, pH 7.4), supplemented with 1 mM KCN and 33.2 mM sodium succinate. The reaction was initiated by the addition of 10 µL phosphate buffer supplemented with 0.1 mM oxidized cytochrome c (substrate of complex III) plus 0.3 mM EDTA-$2K^+$ (a calcium chelator that regulates its levels for a proper oxidative phosphorylation) (Glancy *et al.* 2013). Mitochondrial complex II/III activities were measured by following the reduction of cytochrome c, at 550 nm, for 5 min with 30s intervals, using a SpectraMax Plus 384 microplate reader. Activities were calculated through the slopes obtained during the linear phase. Mitochondrial complex II/III specific activities were determined as the difference between basal activity in the absence and presence of 36.5 µM antimycin A (specific inhibitor of complex III). An $\varepsilon_{550nm}$ = 19.1 $mM^{-1}$ $cm^{-1}$ and normalization to protein amount were applied. Complex II/III activities were expressed as nmol $cytc_{ox}$/min/mg protein.

### *3.11.3. Determination of complex III activity*

Mitochondrial complex III (cytochrome c reductase) activity was assayed by a previously described method (Luo *et al.* 2008), with some modifications. This method evaluates the reduction of cytochrome c, using decylubiquinol as the reducing agent (Luo *et al.* 2008).

Briefly, 5 µL of each brain cortical homogenate were incubated in 87.62 µL reaction buffer containing: 50 mM $KH_2PO_4$ (pH 7.5), 3.75 µM rotenone, 0.05% Tween-20, 0.2 µM freshly-prepared decylubiquinol, at 37 °C, and basal absorbance was read at 550 nm, for 3 min with 30 s intervals, using a SpectraMax Plus 384 microplate reader. Then, complex III activity was determined by following the reduction of cytochrome c after the addition of freshly prepared 75 µM oxidized cytochrome c, for 5 min with 30s intervals. Mitochondrial complex III activity

was calculated through the slopes obtained during the linear phase. Mitochondrial complex III specific activity was determined as the difference between basal activity in the absence and presence of 2.5 µM antimycin A (specific inhibitor of complex III). An $\varepsilon_{550nm}$ = 19.1 $mM^{-1}$ $cm^{-1}$ and normalization to protein amount were applied. Mitochondrial complex III activity was expressed as nmol $cytc_{ox}$/min/mg protein.

### *3.11.4. Determination of complex IV activity*

Mitochondrial complex IV (cytochrome c oxidase) activity was measured by a previously described method (Brautigan *et al.* 1978), with some modifications. This method is based on the quantification of the oxidation of cytochrome c by complex IV, in the presence of an excess of reduced cytochrome c (Brautigan *et al.* 1978).

Briefly, 5 µL of each brain cortical homogenate were incubated at 37 °C, in 71.55 µL reaction buffer (composed by 75 mM $KH_2PO_4$ (pH 7.0), 3.65 µM antimycin A and 0.05% n-dodecyl-β-*D*-maltoside). Basal absorbance was read at 550 nm, for 3 min with 30 s intervals, in a SpectraMax Plus 384 spectrophotometer. Then, we measured the enzymatic activity given by the oxidation of cytochrome c upon addition of 7.2 µM of freshly-prepared reduced cytochrome c, for 6 min with 30 s intervals. Mitochondrial complex IV activity was calculated through the slopes obtained during the linear phase. Mitochondrial complex IV specific activity was determined as the difference between basal activity in the absence and presence of 10 mM KCN (specific inhibitor of complex IV). An $\varepsilon_{550nm}$ = 19.1 $mM^{-1}$ $cm^{-1}$ plus normalization to protein amount were applied. Mitochondrial complex IV activity was expressed as nmol $cytc_{red}$/min/mg protein.

## 3.12. Determination of adenine nucleotides levels and phosphocreatine/creatine kinase system

### *3.12.1. Determination of adenine nucleotides levels*

Brain cortical levels of adenine nucleotides (ATP, ADP and AMP) were determined in 5 µL of each brain cortical homogenate, diluted into 25 µL supplemented lysis buffer, by separation in a reverse-phase HPLC, as previously described (Stocchi *et al.* 1985). The HPLC apparatus

was a Beckman-System Gold, consisting of a 126 Binary Pump Model and 166 Variable UV detector controlled by a computer. The detection wavelength was 254 nm, and the column was a Lichrospher 100 RP-18 (5 µm) from Merck (Darmstadt, Germany). An isocratic elution with 100 mM phosphate buffer ($KH_2PO_4$, pH 6.5) and 1.0% methanol was performed with a flow rate of 1 mL/min. Each analysis had the duration of 6 min. ATP, ADP and AMP were identified by their chromatographic behavior (retention time, absorption spectra, and correlation with standards). Results were expressed as nmol or pmol/mg protein.

### *3.12.2. Determination of phosphocreatine/creatine kinase system*

The rate of phosphocreatine formation was determined similarly to the colorimetric protocol used to determine glucose-6-phosphate formation (Lamprecht *et al.* 1974), and mostly based on the following reaction (Eq. 3.11):

$$\text{Creatine phosphate} + \text{ADP} \xleftrightarrow{\textbf{CK}} \text{Creatine} + \text{ATP} \qquad (3.11)$$

Briefly, 5 µL of each brain homogenate were incubated in a 96-well UV plate with 72.45 µL of reaction medium containing (in mM): 50 triethanolamine-hydrochloride and 22 NaOH (pH 7.5), supplemented with 0.2 mM β-$NADP^+$ sodium salt, 8.35 mM $MgCl_2$, 41.75 mM *D*-glucose, 0.18 mM ADP disodium salt, 743.75 U/L glucose-6-phosphate dehydrogenase from baker's yeast (*S. cerevisæ*), type VII, ammonium sulphate suspension, and 595 U/L hexokinase from baker's yeast (*S. cerevisæ*), type F-300, sulfate-free. Absorbance was continuously read at 339 nm, 37ºC, for 2 min with 30 s intervals, in a SpectraMax Plus 384 microplate reader. Then, the reaction was started with the addition of 31.35 kU/L creatine kinase, from rabbit muscle, and the absorbance read again for 3 min, with 30 s intervals. The rate of phosphocreatine formation was calculated by using an $\varepsilon_{339nm} = 1\ mol^{-1}\ mm^{-1}$, from the extrapolation of absorbance, according to the formula: $\Delta A = A_2 - A_1$, where $A_2$ was the reading after the addition of creatine kinase and $A_1$ was the basal reading. The rate of phosphocreatine formation was expressed as nmol/min/mg protein.

### 3.13. Measurement of ketone bodies levels

Acetoacetic acid (AcAc) and 3-hydroxybutyric acid (BOH) concentrations were determined by the EnzyChrom™ Ketone Body assay kit (BioAssay Systems; Hayward, CA, USA), according to manufacturer's instructions. This kit allows the measurement of AcAc and BOH levels in separate, based on the reactions catalyzed by 3-hydroxybutyate dehydrogenase (HBDH), in which the change in NADH absorbance is directly related with the ketone bodies concentrations (Eq. 3.12).

$$AcAc + NADPH + H^{+} \underset{\text{pH=9.5}}{\overset{\text{pH=7}}{\rightleftharpoons}} \textbf{HBDH} \rightleftharpoons BOH + NAD^{+} \quad (3.12)$$

For both AcAc and BOH assays, the protocol only differed in the components used and incubation time (5 min for AcAc assay and 15 min for BOH assay). Briefly, 5 µL of each cortical homogenate were incubated with 95 µL of working reagent, consisting of 91.03 µL AcAc or BOH buffer, 3.73 µL AcAc or BOH reagent and 0.23 µL HBDH enzyme. After incubation at room temperature, the absorbance was read in a UV 96-well plate, at 340 nm, in a SpectraMax Plus 384 multiplate reader. Results were expressed as mM/mg protein for both acetoacetic acid and 3-hydroxybutyric acid.

### 3.14. Determination of nitrites levels

Nitrite ($NO_2^-$) is a product from the oxidative cleavage of nitric oxide and its levels provide an indirect measure of nitric oxide formation in biological systems (Kevil and Lefer 2010). Nitrites levels were measured according to the method described by Green *et al.* (1981). This relies on the colorimetric measurement of the chromophoric azo product from the reaction, under acidic conditions, between nitrites and the Griess reagent. This azo product strongly absorbs at 550 nm.

Briefly, 5 µL of each brain cortical homogenate were incubated in 45 µL 1x phosphate-buffered saline (PBS) (composed by 8 g/L NaCl, 1.15 g/L $Na_2HPO_4$, 0.2 g/L $KH_2PO_4$ (anhydrous), 0.2 g/L KCl] (pH 7.3)), plus 50 µL Griess reagent (composed by 1:1 of 1% sulfanilamide in 5% $H_3PO_4$ and 0.1% N-(1-naphthyl) ethylenediamine), for 30 min, protected from light. Absorbance was read at 530 nm in a spectrophotometer SpectraMax Plus 384. Nitrites levels were expressed as µmol/mg protein.

### 3.15. Statistical analysis

Results were presented as scatter-dot plot with bar (mean ± S.E.M.) of the indicated number of rats/group. Statistical analysis and graphic artwork were obtained using the *GraphPad Prism 6.0* software. After the identification of outliers with the ROUT test, and the Gaussian distribution analysis with the Kolmogorov-Smirnov normality test, statistical significance was determined using the two-way ANOVA with Fisher LSD post-test, the one-way ANOVA test with Fisher LSD post-test for multiple comparisons (for a Gaussian distribution) or the Kruskal-Wallis test, with Dunn's post-test (for a non-Gaussian distribution). Differences with a *P*-value $<0.05$ were considered statistically significant.

# Results

## 4.1. Effect of sex on peripheral T2D features in middle-aged Wistar and GK rats

GK rats are a polygenic, non-obese, spontaneously T2D animal model, characterized by mild to moderate (but stable) hyperglycemia (Akash *et al.* 2013; Candeias *et al.* 2017).

In the present study, we observed that sex affects body weight in middle-aged rats ($P<0.01$), with both female cohorts weighing (at least tendentiously) less than their male counterparts (by 22% and 27%, $P<0.05$, in Wistar and GK rat females, respectively) (Table 4.1). This was accompanied by an effect of sex and T2D on fasting blood glucose levels ($P<0.0001$), most likely due to a marked hyperglycemia in GK rat females (by 125% and 136%, $P<0.0001$) compared with the respective male and non-diabetic female cohorts, respectively (Table 4.1). Surprisingly, fasting blood glucose levels were not significantly different between male Wistar and GK rats (Table 4.1). However, sex and T2D significantly affected blood $HbA_{1c}$ levels, as given by the 48% and 53% ($P<0.05$ and $P=0.0529$) higher $HbA_{1c}$ content in male and female GK rats than in their respective non-T2D cohorts (Table 4.1).

These results suggest that middle-aged male GK rats may undergo a pre-diabetes state, whereas their female counterpart may have recently reached an early T2D stage.

**Table 4.1: Peripheral characterization of middle-aged male and female Wistar and GK rats**

| | Wistar rats | | GK rats | |
|---|---|---|---|---|
| | **Male** | **Female** | **Male** | **Female** |
| **Body weight** (g) **(Sex$^{**}$)** | 429.4 ± 35.91 (n=5) | 334.7 ± 19.06 (P=0.0994) (n=3) | 390.2 ± 15.59 (n=10) | 283.0 ± 4.93 $^{\$}$ (n=3) |
| **Blood glucose levels** (mg/dL blood) **(Sex$^{****}$ x Diabetes$^{****}$)** | 89.10 ± 7.30 (n=10) | 86.67 ± 5.05 (n=9) | 91.10 ± 5.59 (n=10) | 204.7 ± 22.18 $^{\$\$\$\$}$ ££££ (n=10) |
| **$HbA_{1c}$** (%) **(Sex$^{**}$ x Diabetes$^{***}$)** | 4.15 ± 0.28 (n=4) | 4.00 ± 0.20 (n=5) | 6.37 ± 0.43 $^{£}$ (n=3) | 4.30 ± 0.13 (*P*=0.0529) (n=5) |

**Diabetes diagnosis criteria: fasting plasma glucose test ≥126 mg/dL or $HbA_{1c}$ ≥ 6.5%** (American Diabetes Association 2019a). Data are mean ± SEM of the indicated number of animals. Statistical significance: $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$ by two-way ANOVA for the interaction between the independent variables T2D and sex; $^{\$}P<0.05$, $^{\$\$\$\$}P<0.0001$ *vs.* male GK rats; $^{£}P<0.05$, $^{££££}P<0.0001$ *vs.* respective Wistar rat cohort, by one-way ANOVA for multiple comparisons, with Fisher's LSD post-test (blood glucose levels) or by Krustal-Wallis with Dunn's post-test (body weight, $HbA_{1c}$).

## 4.2. Despite the slightly increased brain cortical GLUT3 density in middle-aged female rats, brain glucose levels dropped in Wistar female rats

Glucose transport into the central nervous system is critical for its subsequent intracellular metabolism within the brain (Kumagai 1999; Mergenthaler *et al.* 2013; Shah *et al.* 2012). Both T2D and estrogen affect brain glucose uptake and glucose transporters (GLUTs) density (Rettberg *et al*. 2014; Shah *et al.* 2012). Since we previously found that brain estrogen levels were altered in middle-aged GK female rats (Candeias *et al.* 2017), in the present study we aimed to evaluate the effect of sex on brain cortical expression of GLUT3 (the main neuronal GLUT isoform within the brain, although it can be found also in astroglial and endothelial cells) (Szablewski 2017) and glucose levels in middle-aged T2D rats.

We observed that sex slightly affected brain cortical GLUT3 levels, most likely due to a 94% and 22% increased expression in Wistar and GK female rats when compared to the respective males (although this was only statistically significant in Wistar females; $P<0.05$) (Fig. 4.1.A). Nevertheless, brain glucose levels were massively decreased (by 87%, $P<0.05$; and 97%, $P<0.0001$) in Wistar female and GK male rats in comparison with the age-matched Wistar male rats, thereby accounting for the significant effect of sex ($P<0.05$) and T2D ($P<0.01$) observed (Fig. 4.1.B). Surprisingly, despite the markedly higher glycemia in T2D female rats described above (Table 4.1), their brain glucose levels were only tendentiously increased (by 115%) or even slightly decreased (~54%) compared with control female and T2D male rats, respectively (Fig. 4.1.B).

These results suggest that, despite a slight increment in brain cortical GLUT3 protein expression in both female rat cohorts, the Wistar ones may have either an impairment in peripheral glucose uptake and/or an increase in its downstream metabolism.

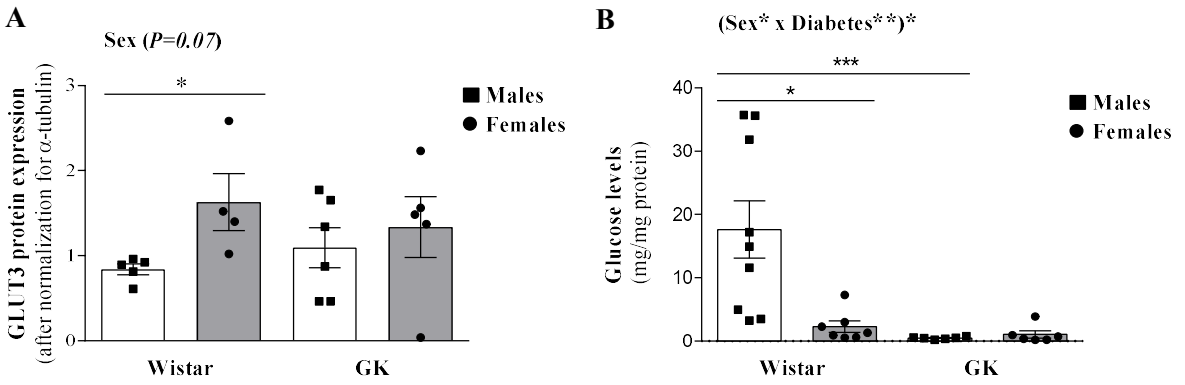



**Figure 4.1: Effect of sex on brain cortical GLUT3 density and glucose levels in middle-aged T2D rats.** Brain cortical GLUT3 protein expression (A) and glucose levels (B) were given by immunoblotting and colorimetric assay, performed in brain cortical lysates isolated from middle-aged male and female Wistar (control) and T2D GK rats. Data are the mean ± S.E.M. of the indicated number of animals/group. Statistical significance: **P*<0.05, ***P*<0.01, by two-way ANOVA for the interaction between the independent variables T2D and sex; **P*<0.05, ****P*<0.001, by the Kruskal-Wallis test, with Dunn's post-test.

## 4.3. Female sex attenuates glycolysis in middle-aged control and T2D rat brain cortices

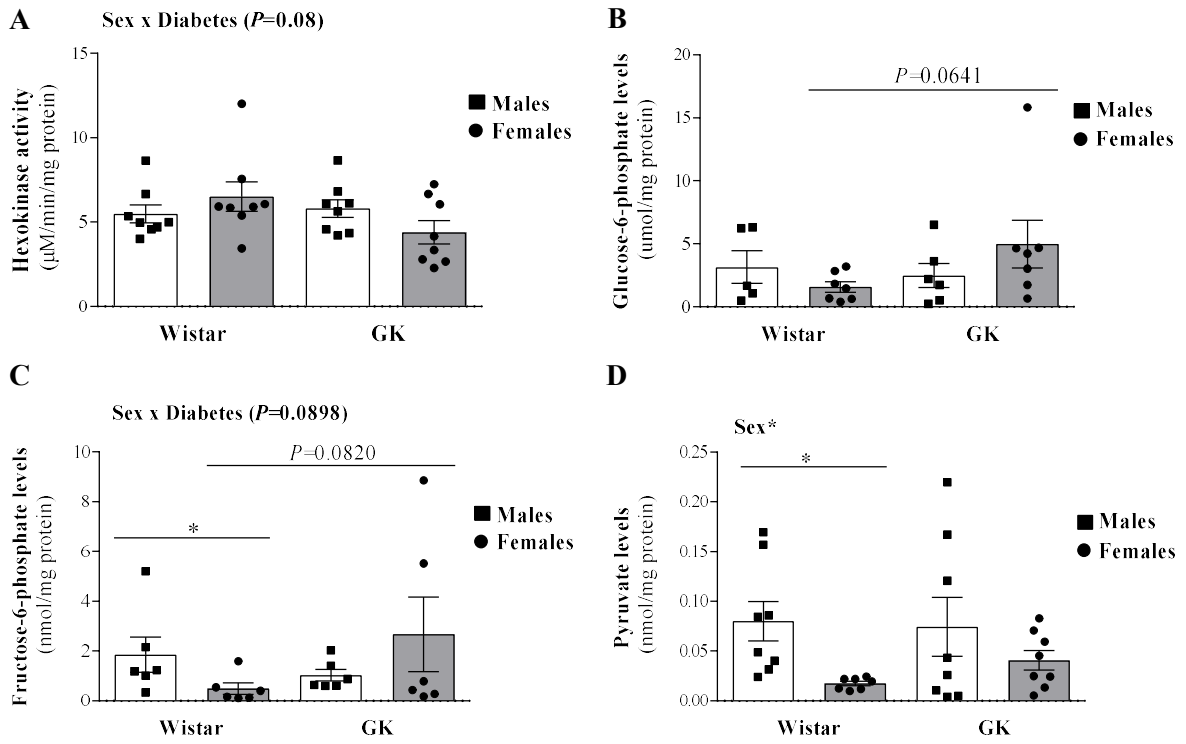
Since the above alterations in brain cortical glucose levels in both female rat cohorts can result from changes in its transport and/or an immediate metabolism, as previously observed for both sex/estrogen and T2D (Andersen *et al.* 2017a; Brinton *et al.* 2015; Rettberg *et al.* 2014; Sickmann *et al.* 2010), we aimed to evaluate the role of sex on markers for glycolysis in middle-aged T2D rat brain cortices.

Immediately after entering the cells, glucose is metabolized by hexokinase, the first and one of the rate-limiting enzymes of glycolysis (Bouché *et al.* 2004). We observed that sex slightly interacts with T2D (*P*=0.08) to affect hexokinase activity, mostly due to a tendentious inhibition of hexokinase in female GK rat brains (by 24% and 33%) compared with their respective males and non-diabetic female rats (Fig. 4.2.A). Nevertheless, T2D female rats had tendentiously increased brain cortical glucose-6-phosphate (by 101%; Fig. 4.2.B) and fructose-6-phosphate levels (by 160%; Fig. 4.2.C) compared with age-matched T2D male rats. Conversely, both glucose-6-phosphate (Fig. 4.2.B) and fructose-6-phosphate levels (Fig. 4.2.C) were at least slightly lower (by 50%; and 73%, *P*=0.0641) in female Wistar rat brains than in their respective male cohort. This was accompanied by a significant effect of sex on brain cortical levels of pyruvate (the *ultima* product of glycolysis) (*P*<0.05), as given by the lower pyruvate content in both female rat groups than the respective male cohorts (Fig. 4.2.D). However, it only attained statistical significance for female Wistar rats (*P*<0.05). This was

mirrored by an effect of sex on brain cortical activity of lactate dehydrogenase (which catalyzes the reversible conversion of pyruvate in lactate), mostly due to a lactate dehydrogenase inhibition (by 65% and 83%, $P<0.05$) in both Wistar and GK rat female cohorts compared to respective males (Fig. 4.2.E). Sex and T2D also significantly affected ($P<0.01$) brain cortical lactate levels, most likely due to a reduction in lactate content in GK female rats (by 25% and 34%, $P<0.01$) compared with their respective males and with non-diabetic female rats (Fig. 4.2.F). Interestingly, Wistar female rats had significantly increased brain lactate levels (by 23%, $P<0.05$) compared with their age-matched control males (Fig. 4.2.F).

In agreement with the activity of lactate dehydrogenase, brain cortical NADH levels (a cofactor used by this and other enzymes) were tendentiously decreased in both Wistar and GK female rats (by 23% and 44%, respectively) relatively to their respective male groups (Fig. 4.2.G). Interestingly, the significantly lower NADH content in both T2D male and female rats *versus* the respective control cohorts may account for the significant effect of T2D observed ($P<0.001$) (Fig. 4.2.G).

These results point towards an attenuation of brain cortical glycolytic metabolism in both Wistar and GK female rats at midlife.



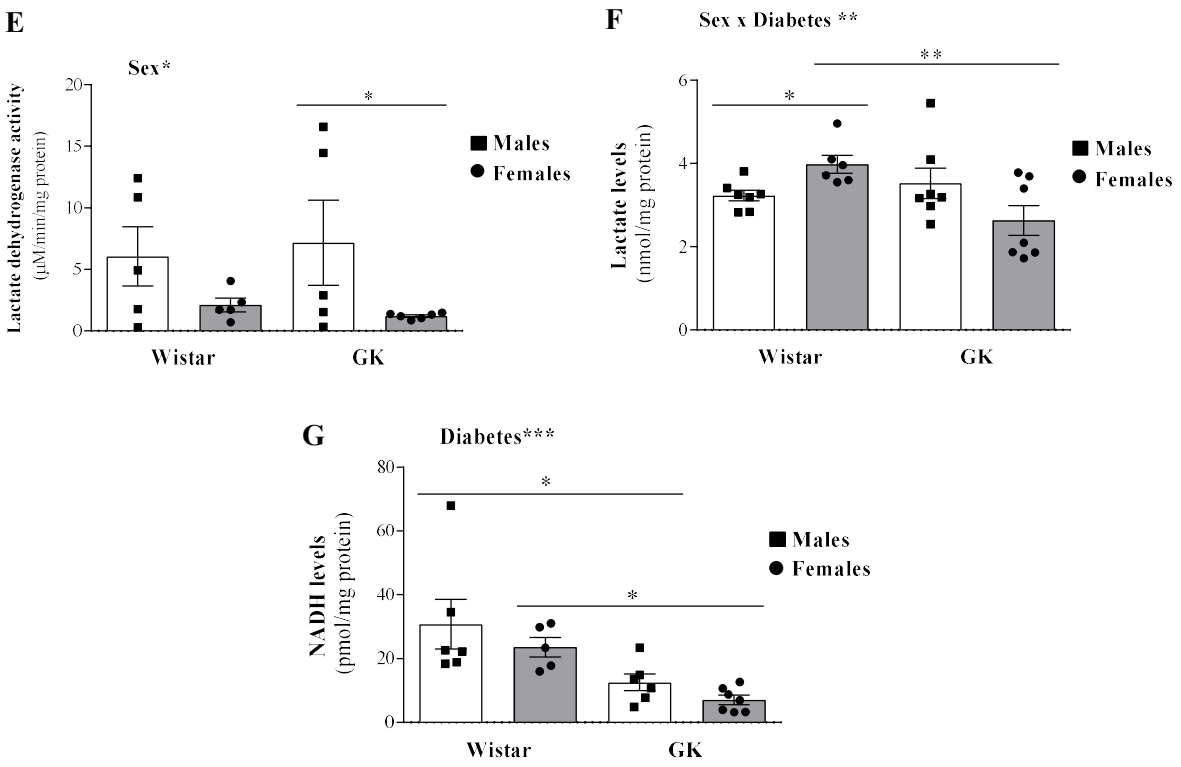



**Figure 4.2: Effect of sex on brain cortical markers for glycolysis in middle-aged T2D rats.** Brain cortical hexokinase activity (A), glucose 6-phosphate levels (B), fructose 6-phosphate levels (C), pyruvate levels (D), lactate dehydrogenase activity (E), lactate levels (F) and NADH levels (G) were given by colorimetric assays, in brain cortical homogenates from middle-aged male and female Wistar (control) and T2D GK rats. Data are the mean ± S.E.M. of the indicated number of animals/group. Statistical significance: **P*<0.05, ***P*<0.01, ****P*<0.001, by two-way ANOVA for the interaction between the independent variables T2D and sex; **P*<0.05, ***P*<0.01, by one-way ANOVA for multiple comparisons, with Fisher LSD post-test (D, E), or by Kruskal-Wallis test, with Dunn's post-test (A-C, F, G).

## 4.4. Female sex attenuates brain cortical pentose phosphate pathway in both middle-aged Wistar and GK rats

Alternatively to glycolysis, glucose can be also metabolized through the pentose phosphate pathway, that starts from glucose-6-phosfate. This pathway is also pivotal in the maintenance of cellular antioxidant defenses (Baquer *et al.* 1988; Mamelak 2012; Shi *et al.* 2011). Previous studies reported that the pentose phosphate pathway is altered in diabetic brain (Gok e*t al.* 2016; Shi *et al.* 2011; Ulusu *et al.* 2003). Hence, we aimed to evaluate the role of sex on pentose phosphate pathway function in middle-aged T2D rat brain cortices, as given by the activity of glucose-6-phosphate dehydrogenase (the rate limiting enzyme of its oxidative branch) (Baquer *et al.* 1988).

We observed that sex affects significantly ($P$<0.05) the activity of brain cortical glucose-6-phosphate dehydrogenase, most likely due to the 68% ($P$<0.05) and 46% decrease in its activity in both Wistar and GK female rats, respectively, when compared to the respective males (Fig. 4.3.).

These results suggest an attenuation of brain metabolism through (at least) the oxidative branch of the pentose phosphate pathway in both middle-aged female rat cohorts compared to their age-matched male rats. Therefore, their lower glycolytic profile may not involve the shift of glucose-6-phosphate towards this oxidative branch of the pentose phosphate pathway. Even though, since glucose-6-phosphate dehydrogenase was slightly stimulated (by 69%) in the brains from T2D female rats compared to their non-diabetic cohort (Fig. 4.3.), they may have a higher metabolic flux via the pentose phosphate pathway than Wistar rat females.

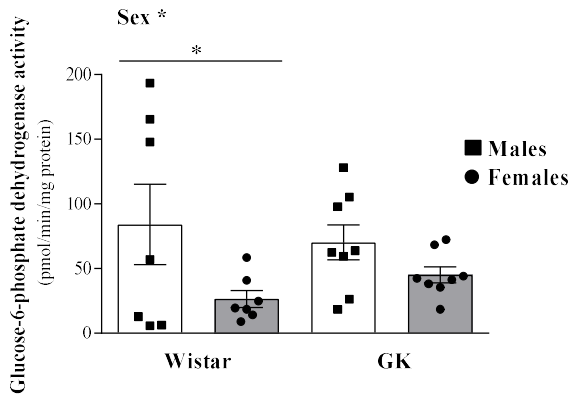



**Figure 4.3: Effect of sex on brain cortical glucose-6-phosphate dehydrogenase activity in middle-aged T2D rats.** Brain cortical glucose-6-phosphate dehydrogenase activity (the rate-limiting enzyme of the oxidative branch of the pentose phosphate pathway) was colorimetrically determined in brain cortical homogenates from middle-aged male and female Wistar (control) and T2D GK rats Data are the mean ± S.E.M. of the indicated number of animals/group. Statistical significance: *$P$<0.05, by two-way ANOVA for multiple comparisons, with Fisher LSD post-test.

## 4.5. T2D differently affects the activities of enzymes from brain cortical Krebs cycle in middle-aged female rats

Since pyruvate can be transported into the mitochondria to be further metabolized via the Krebs cycle to provide substrates for the mitochondrial respiratory chain function, and given the aforementioned inhibition of the glycolytic metabolism in both Wistar and GK rat females' brains, we aimed to evaluate the effect of sex on markers for the Krebs cycle in middle-aged

T2D rat brain cortices. These included the activity of pyruvate dehydrogenase, citrate synthase, α-ketoglutarate dehydrogenase, succinate dehydrogenase and malate dehydrogenase (Fig. 4.4.).

Although non-statistically significant, brain cortical pyruvate dehydrogenase activity was 69% higher in Wistar female rats than in the respective male rats (Fig. 4.4.A). This was accompanied by a significant effect of sex and T2D in brain citrate synthase activity, most likely due to its lower activity (by 62%, $P<0.01$; and 47%) in both Wistar and GK female rats than in the corresponding male cohorts (Fig. 4.4.B). In addition, citrate synthase activity was 67% lower ($P<0.05$) in brain cortices from male GK rats compared with male Wistar rats (Fig. 4.4.B).

Although non-statistically significant, brain cortical α-ketoglutarate dehydrogenase activity was 110% and 88% higher in Wistar and GK female rats, respectively, in comparison with the respective males, thereby accounting for a slight effect of sex herein (Fig. 4.4.C). Conversely, this was followed by a 45% higher (though non-significant) succinate dehydrogenase activity in middle-aged Wistar female rat brains compared to their respective males (Fig. 4.4.D). In addition, brain malate dehydrogenase activity was 72% and 61% lower in Wistar female and GK male rats compared to Wistar male animals, being 23% higher (though non-statistically significant) in GK females than in the respective males (Fig. 4.4.F).

These results suggest that female sex exerts differential effects on Krebs cycle in middle-aged rat brain. Despite the (tendentious) inhibition of citrate synthase in both control and T2D female rats, they may have developed some compensatory pathways towards the maintenance (or even the slight stimulation) of the activity of subsequent enzymes (except for the last enzyme, malate dehydrogenase, whose activity was massively decreased in control female rat brains).

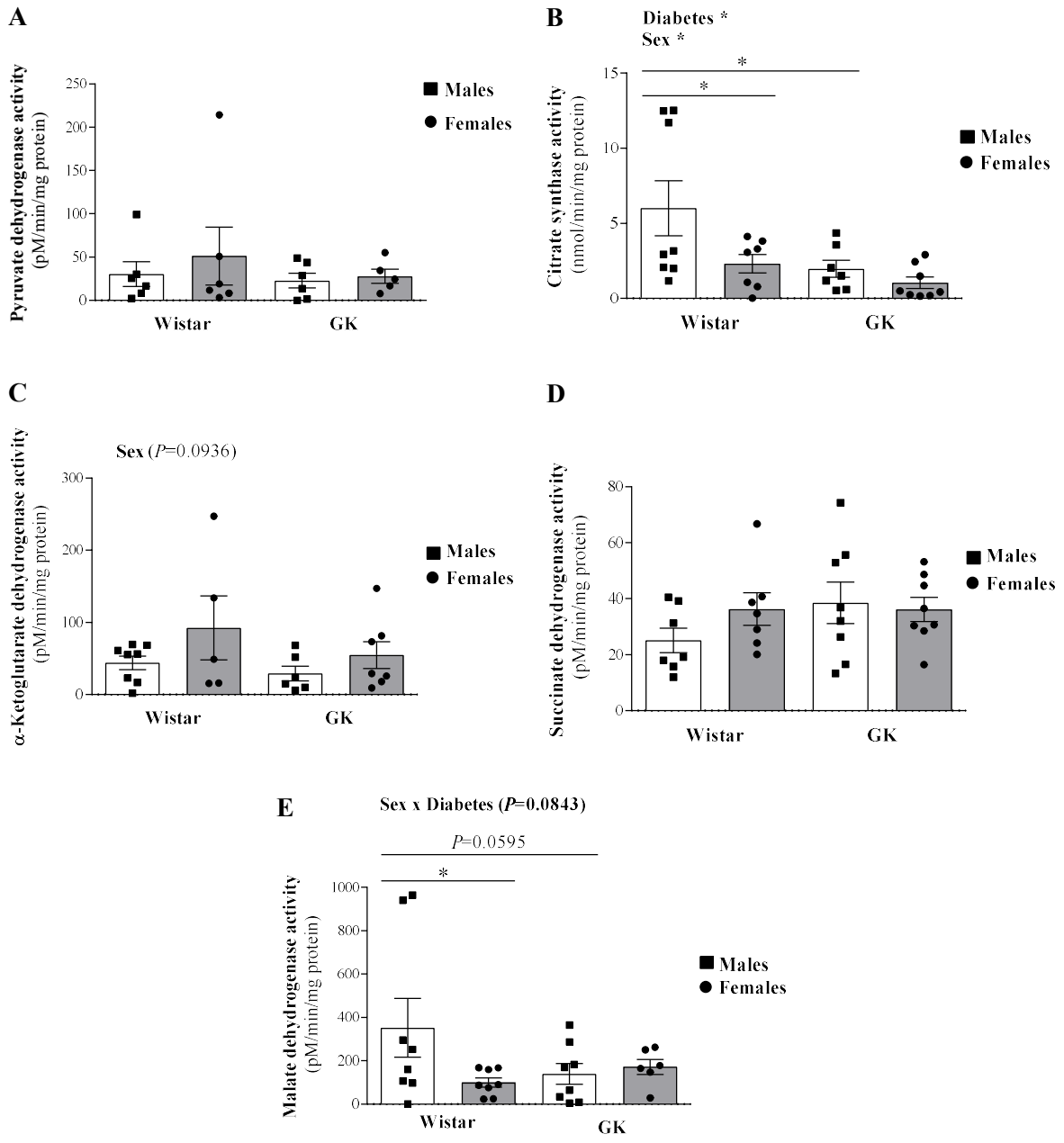



**Figure 4.4: Effect of sex on the activities of brain cortical enzymes from the Krebs cycle in middle-aged T2D rats.** Brain cortical pyruvate dehydrogenase (A), citrate synthase (B), α-ketoglutarate dehydrogenase (C), succinate dehydrogenase (D) and malate dehydrogenase activities (E) were given by colorimetric assays, in brain cortical homogenates from middle-aged male and female Wistar (control) and T2D GK rats. Data are the mean ± S.E.M. of the indicated number of animals/group. Statistical significance: *$P$<0.05, by two-way ANOVA for the interaction between the independent variables T2D and sex; *$P$<0.05 **$P$<0.01, ***$P$<0.001, by one-way ANOVA for multiple comparisons, with Fisher LSD post-test (D), by two-way ANOVA with Fisher LSD post-test (B), or by Kruskal-Wallis test, with Dunn's post-test (A, C, E).

### 4.6. Brain cortical mitochondrial respiratory chain function is attenuated in middle-aged T2D female rats

Oxidative phosphorylation at mitochondrial respiratory chain is one of the major sources of the energy used for brain function. In addition, mitochondrial energy metabolism was shown to be altered by T2D (Moreira 2018) and female sex (Brinton *et al.* 2015; Duarte *et al.* 2018; Rettberg *et al.* 2014). This, together with our previous results, prompted us to evaluate the effect of sex on mitochondrial respiratory chain function (more specifically, on complexes I-IV activities) in middle-aged T2D rat brain cortices.

We found an overall attenuation of brain cortical mitochondrial respiratory chain function in GK female rats, as given by the (at least tendentious) inhibition in mitochondrial respiratory complexes I-IV activities (by 59%; 61%; 75%, $P<0.01$; and 49%) compared to their respective male cohorts (Fig. 4.5.A-D). Interestingly, only brain mitochondrial respiratory complexes I and IV activities were slightly decreased (by 47% and 28%) in Wistar female rats *versus* their respective males, whereas their complexes II/III and III activities were at least tendentiously augmented (by 195%, $P<0.05$; and 152%, respectively) (Fig. 4.5.A, D). This, together with the significant reduction in complex II/III activity (by 79%, $P<0.01$) exhibited by T2D female rat brains compared to Wistar female rats contributed for the interaction between sex and T2D herein (Fig. 4.5.B).

We also found an interaction of sex and T2D in mitochondrial respiratory complex III activity, resulting from its slight stimulation (by 152%) in Wistar female rat brains compared to their respective males, while in T2D female rats there was a massive inhibition (by 75%, $P<0.01$) compared to GK rats (Fig. 4.5.C). In addition, brain mitochondrial respiratory complex III activity was markedly raised (by 1196%, $P<0.001$) in T2D male rats *versus* non-diabetic controls (Fig. 4.5.C). Finally, we observed a tendentious effect of sex in mitochondrial respiratory complex IV activity, mostly due to its slight reduction (by 28% and 49%) in brain cortices from both female rat cohorts (control and GK, respectively) (Fig. 4.5.D).

These results suggest that mitochondrial respiratory chain may be downregulated in T2D female rats, whereas control ones may try to compensate at the level of mitochondrial respiratory complexes II/III and III.

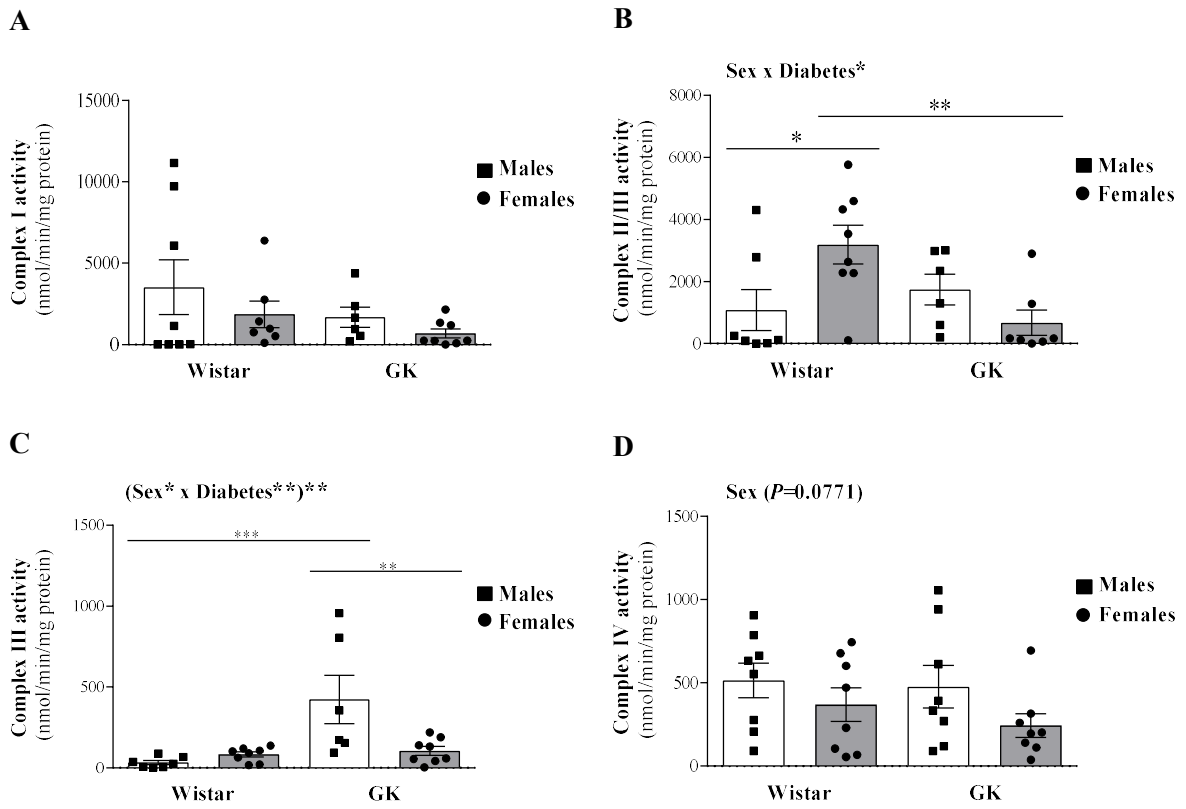



**Figure 4.5: Effect of sex on middle-aged T2D rat brain cortical mitochondrial respiratory complexes I-IV activities.** Brain mitochondrial respiratory chain function was given by the colorimetric measurement of the activities of complex I (A), II/III (B), III (C) and IV (D) in brain cortical homogenates from middle-aged male and female Wistar (control) and T2D GK rats. Data are the mean ± S.E.M. of the indicated number of animals/group. Statistical significance: **P*<0.05, ***P*<0.01, ****P*<0.001, by two-way ANOVA for multiple comparisons, with Fisher LSD post.

## 4.7. T2D differently affects brain cortical adenine nucleotides and phosphocreatine levels in middle-aged female rats

Since the overall glucose energy metabolism appears to be compromised in the brain cortices from both control and T2D female rats, we aimed to evaluate the role of sex on adenine nucleotides levels (ATP, ADP and AMP) in middle-aged T2D rat brain cortices.

We observed a significant interaction between sex and T2D ($P < 0.05$) in brain cortical ATP levels, mostly attributed to its massive reduction in both control female and T2D male rats compared to Wistar male rats (Fig. 4.6.A). Nevertheless, brain ATP levels were tendentiously increased (by 53% and 121%) in T2D female rats compared to GK male and non-diabetic female rats, respectively (Fig. 4.6.A). On the other hand, these GK female rats showed a drastic reduction (by 85%, $P<0.05$) in brain ADP levels compared to their respective males (Fig. 4.6.B). This could result from its dephosphorylation into AMP, as given by their higher brain cortical AMP levels (by 108%; and 554%, $P<0.05$) in comparison with their respective male

rat cohort, as well as with age-matched control female rats (Fig. 4.6.C). Thus, these differences may account for the significant interaction between sex and T2D ($P$<0.05) on brain cortical AMP levels. Strikingly, Wistar female rats showed tendentiously lower (by ~71%) brain cortical AMP levels than age-matched male rats (Fig. 4.6.C).

These results suggest that brain cortices from middle-aged control female rats may have either a downregulation in the synthesis of adenine nucleotides (ATP, ADP and AMP) and/or an increase in their downstream metabolism towards the formation of purines (hypoxanthine, inosine and adenosine) in comparison with age-matched control rats. Conversely, the slightly increased brain cortical ATP and ADP levels in GK rat females compared to T2D male rats suggest the development of compensatory mechanisms to provide energy for their brain function.

To further obtain a more detailed perspective herein, we analyzed the phosphocreatine/creatine kinase system, another cellular ATP-generating system (besides mitochondrial oxidative phosphorylation, the major source of ATP under physiological conditions) (Andres *et al.* 2008) (Fig. 4.6.D). Despite the above-mentioned inhibition of brain cortical mitochondrial respiratory chain in T2D female rats (Fig. 4.5), their phosphocreatine levels were maintained compared to their age-matched GK male rats (Fig. 4.6.D). This suggests that phosphocreatine/creatine kinase system may account for the slightly higher levels of ATP in T2D female rat brains (Fig. 4.6.A). Despite the 184% increase (though non-significant) in brain phosphocreatine levels in Wistar female rats compared to control male rats (Fig. 4.6.D) and their partial compensatory mechanisms involving complexes II/III and III (Fig. 4.5.), these were not sufficient to restore their brain ATP content (Fig. 4.6.A).

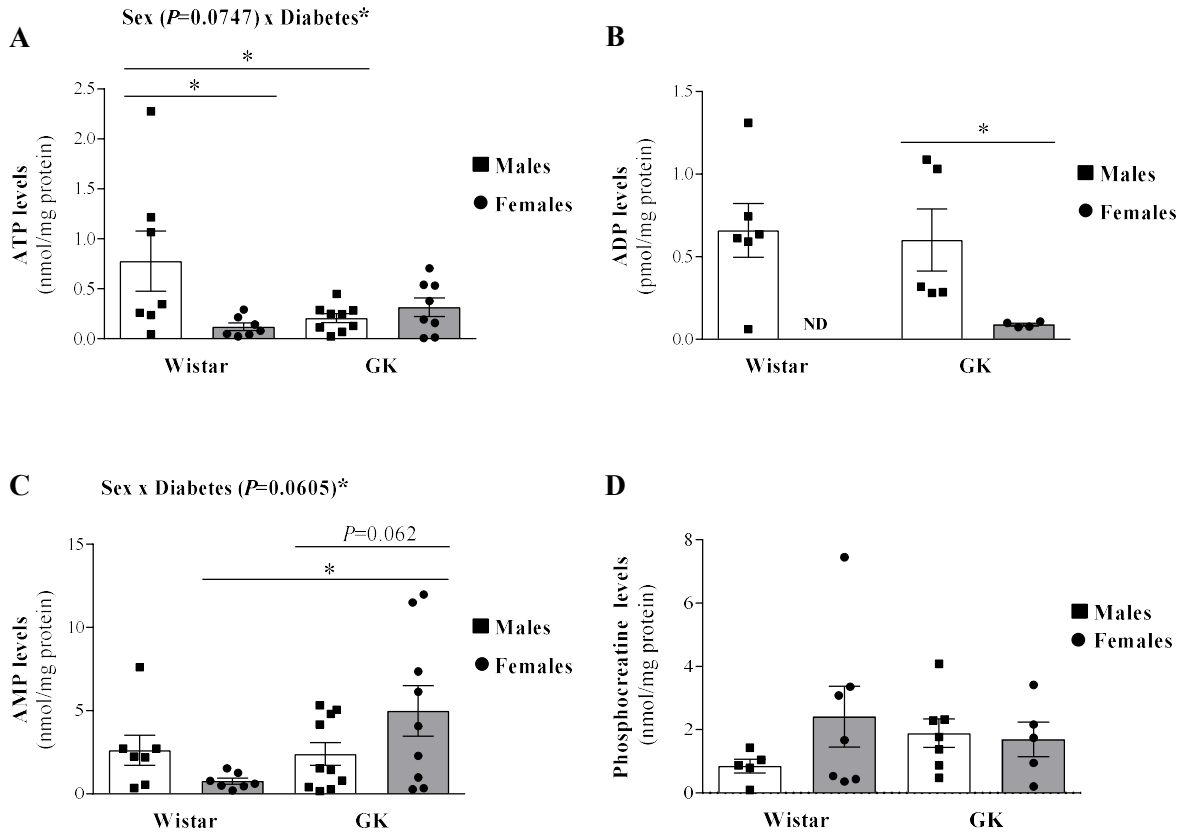



**Figure 4.6: Effect of sex on middle-aged T2D rat brain cortical levels of adenine nucleotides and phosphocreatine.** Brain cortical ATP (A), ADP (B), AMP levels (C) were assessed by reverse-phase HPLC, while phosphocreatine levels (D) were obtained by colorimetry, in brain cortical homogenates from middle-aged male and female Wistar (control) and T2D GK rats. Data are the mean ± S.E.M. of the indicated number of animals/group. Statistical significance: **P*<0.05, by two-way ANOVA for the interaction between the independent variables T2D and sex; **P*<0.05 by one-way ANOVA for multiple comparisons, with Fisher LSD post-test (A, D), or by Kruskal-Wallis test, with Dunn's post-test (B, C).

## 4.8. T2D differently affects brain cortical ketone bodies levels in middle-aged female rats

Ketone bodies are an alternative fuel for the brain, especially when glucose metabolism is compromised (Morris 2005; Newman and Verdin 2014). Previous studies showed that ketone bodies are altered in T2D (Andersen *et al.* 2017a) and perimenopause (Brinton *et al.* 2015). Therefore, we aimed to determine the role of sex on ketone bodies levels in middle-aged T2D rat brain cortices, by measuring the levels of 3-hydroxybutyric acid and acetoacetic acid.

We observed a slight decrement (by 26%) in brain cortical content of 3-hydroxybutyric acid in Wistar female rats (Fig. 4.7.A), whereas their brain acetoacetic acid levels were slightly increased (by 25%) in comparison with their respective males (Fig. 4.7.B). Despite no significant alterations in brain 3-hydroxybutyric acid levels in GK female rats (Fig. 4.7.A),

their acetoacetic acid levels were tendentiously lower (by 27%) than in the respective male cohort (Fig. 4.7.B). Furthermore, GK female rats showed a decrement (by 41%, $P<0.01$) in brain cortical acetoacetic acid content compared to non-diabetic female rats (Fig. 4.7.B). Importantly, these differences may be responsible for the observed effect of sex and/or diabetes ($P<0.05$) on brain cortical acetoacetic acid levels.

These results suggest that acetoacetic acid formation may be attenuated (or its consumption increased) in the brain cortices from middle-age T2D female rats. Moreover, the slightly lower slightly 3-hydroxybutiric levels in control females may result from a higher conversion in acetoacetic acid, which would explain the increasing levels of the later.

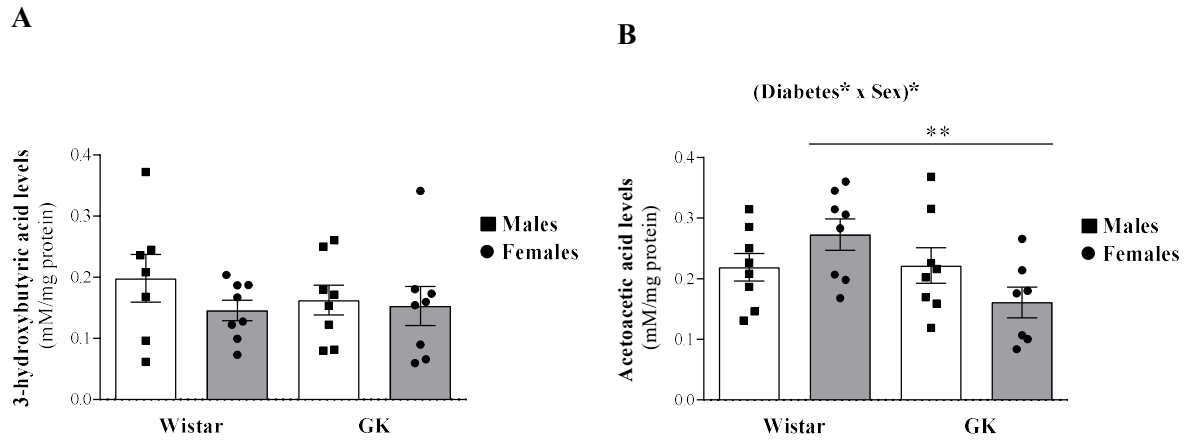



**Figure 4.7: Effect of sex on brain cortical ketone bodies in middle-aged T2D rats.** Brain cortical 3-hydroxybutyric acid (A) and acetoacetic acid (B) levels were given by colorimetric kits, in brain cortical homogenates from middle-aged male and female Wistar (control) and T2D GK rats. Data are the mean ± S.E.M. of the indicated number of animals/group. Statistical significance: *$P<0.05$, *$P<0.01$ by two-way ANOVA for multiple comparisons, with Fisher LSD post-test.

## 4.9. T2D differently affects brain cortical nitrites levels in middle-aged female rats

T2D-associated hyperglycemia promotes oxidative and nitrosative stress in the periphery and brain (Kandimalla *et al.* 2017; Panigrahy*et al.* 2017; Pu *et al.* 2018; Zhang *et al.* 2019), which were also affected by estrogen levels and/or female sex (Duarte *et al.* 2018; Rettberg *et al.* 2014). Additionally, we previously showed that middle-aged female T2D and control rat brains were more protected against DNA and lipid oxidative damage (Candeias *et al.* 2017). Hence, we aimed to complement our previous data by determining the role of sex on nitrosative stress in middle-aged T2D rat brain cortices, by measuring their levels of nitrites.

We observed a tendency for the interaction between sex and T2D ($P=0.065$) in brain cortical levels of nitrites, possibly due to their (at least tendentious) increase (by 49%; and 96%,

*P*<0.05) in GK female rats compared with their respective male rats and the non-diabetic female cohort (Fig. 4.8). Conversely, Wistar female rats had slightly lower (by 37%) brain nitrite levels than their respective male rat cohort (Fig. 4.8.).

Our results suggest that, despite the previously described lower accumulation of markers for lipid and DNA oxidative in both T2D and control female rat brains at midlife (Candeias *et al.* 2017), the later are still somewhat protected against nitrosative stress, while the T2D ones may have started to loose such protection.

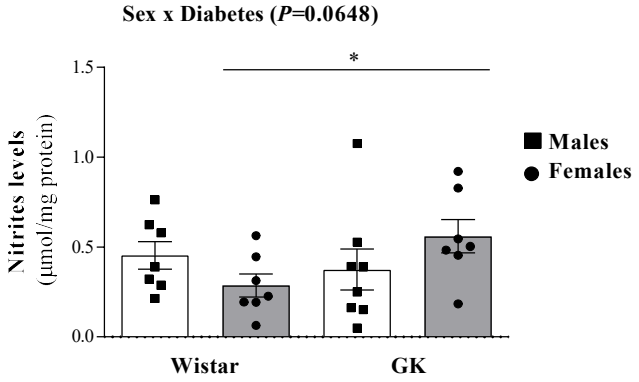



**Figure 4.8: Effect of sex on brain cortical nitrite levels in middle-aged T2D rats.** Brain cortical nitrites levels were determined colorimetrically in brain cortical homogenates from middle-aged male and female Wistar (control) and T2D GK rats. Data are the mean ± S.E.M. of the indicated number of animals/group. Statistical significance: **P*<0.05, by two-way ANOVA for multiple comparisons, with Fisher LSD post-test.

In sum, our results suggest that, despite the slightly increased brain GLUT3 density in middle-aged control female rats, their glucose uptake from periphery must be already impaired, contributing for the slowdown in brain cortical glycolysis that, nonetheless, may not involve a shift of glucose-6-phosphate towards the oxidative branch of the pentose phosphate pathway. Alternatively, the slight increment in brain acetoacetic acid levels in Wistar female rats could provide extra-substrates for Krebs cycle. However, this may not to be the case, since this metabolic pathway was attenuated in these female rats. Furthermore, their tendentiously higher mitochondrial respiratory complexes II/III and III activities, and phosphocreatine/creative kinase system were not sufficient to replenish the intracellular pools of adenine nucleotides. Importantly, this overall slowdown in brain cortical glucose (energy) metabolism may ultimately render middle-aged Wistar female rats less susceptible against nitrosative and oxidative stresses, and AD-like neuropathological hallmarks (Candeias *et al.* 2017).

Conversely, middle-aged T2D female rats may be undergoing an early T2D stage, while their male counterparts were still under a pre-diabetes stage. Despite this and the slightly increased brain GLUT3 density in T2D female rats, their brain glucose content was only slightly higher than in T2D male rats. This was accompanied by a slowdown in brain cortical glycolysis in these female rats that may not involve the shift of glucose-6-phosphate towards the oxidative branch of the pentose phosphate pathway. Although Krebs cycle function was maintained in T2D female rat brains, this may not rely on their acetoacetic acid levels, as these were decreased under such conditions. Even if the lower acetoacetic acid pool resulted from higher conversion into acetyl-CoA, this would not contribute to the cycle since the stage at which the metabolite would enter is attenuated. Moreover, increased phosphocreatine/creatine kinase system may restore T2D female rat brain ATP levels, despite the overall inhibition of their mitochondrial respiratory chain function. In contrast to their apparent protection against oxidative damage (Candeias *et al.* 2017), these females may be more vulnerable to brain nitrosative stress. However, the later may still not be sufficient to alter the lower susceptibility of middle-aged T2D female rat brains to the accumulation of makers for oxidative injury to lipids and DNA nor AD-like neuropathological features (Candeias *et al.* 2017).

Chapter 5

# Discussion

In this study, we observed that: *1)* despite the increased GLUT3 density in brain cortices from both control and T2D female rats at midlife, their glucose uptake may be compromised, thereby *2)* attenuating their glycolysis and initial stages of Krebs cycle that, nonetheless, was not accompanied by a shift of glucose-6-phosphate towards pentose phosphate pathway. However, *3)* both female rat cohorts exhibited compensatory mechanisms at different stages of Krebs cycle compared to the respective males. Despite this, *4)* T2D female rats showed an overall attenuation of mitochondrial respiratory chain, but no alterations occurred in their ATP levels. *5)* The different ketone bodies levels profiles in both female rat groups suggests a divergent metabolism of this fuel. *6)* While the pool of ketone bodies could be used to produce energy via the Krebs cycle, this may not be the case in none of the female rat groups, since the stage of the cycle whereby they could enter is attenuated. Finally, *6)* despite the protection against AD-like and oxidative damage hallmarks, T2D female rats may be more vulnerable to nitrosative stress than their respective males. Collectively, these results reinforce the hypothesis that the slowdown of brain energy metabolism in both control and T2D middle-aged female rats may contribute for their reduced susceptibility to oxidative damage and AD-like neuropathology.

In the last decade, and similarly to T2D, AD consistently became considered a metabolic disorder that affects both brain and peripheral energy metabolism (Kandimalla *et al.* 2017; Neth and Craft 2017). Furthermore, perimenopause is a transition neurological state (whose main symptoms include, *e.g.*, cognitive deficits) that further increases women's risk for AD later on (Brinton *et al.* 2015). Interestingly, changes in brain metabolism appear to be an early feature in both perimenopause and AD ethiogenesis (Brinton *et al.* 2015; Ding *et al.* 2013a; Yao *et al.* 2010, 2009;-Yin *et al.* 2015). Thus, targeting energy metabolism may constitute a promising therapeutic or preventive strategy to slowdown the AD onset and progression, especially among women (Cholerton *et al.* 2013; Craft 2012; Duarte *et al.* 2018; Zilberter and Zilberter 2017). Accordingly, several studies demonstrated that recovery of peripheral energy metabolism attenuated both central and peripheral pathology in AD animal models (Batista *et al.* 2018; Bomfim *et al.* 2012; Pawlosky *et al.* 2017; Yin *et al.* 2016). In addition, the interaction between T2D and female sex (particularly in pre- and perimenopausal phases) may modulate brain metabolic function and, ultimately, their susceptibility to T2D-associated neurodegeneration and AD. Hence, the success of (sex-specific) preventive/therapeutic approaches against T2D-associated AD risk relies on the establishment of the precise molecular mechanisms underlying sex-associated changes in brain energy metabolism under such

conditions, using appropriate models for each phase of life. Although surgical models of menopause allowed some insights on its metabolic effects, they present several limitations, namely they: *1)* abolish all ovary-derived hormone, instead of promoting a selective loss of, *e.g.*, estrogen; *2)* lack an early, progressive perimenopause; and *3)* do not consider a role for brain-derived sexual hormones during perimenopause.

To the best of our knowledge, this is the first study analyzing the molecular mechanisms underlying sex-mediated changes in brain cortical energy metabolism and intracellular stress (more precisely, nitrosative stress) in middle-aged (8-month-old) T2D GK rats. According to the literature, at this age female rats often undergo a pre-perimenopause/early perimenopause (Maffucci and Gore 2006; Yin *et al.* 2015), with metabolic changes already reported (Ding *et al.* 2013a). Accordingly, we described recently that circulating estrogen levels were still high in middle-aged control female rats, whereas in their brain cortical it remained similar to their male counterparts (Candeias *et al.* 2017). This suggests that these females were still under their fertile phase. Conversely, such hormonal impairment appeared to be accelerated in T2D female rats, that already showed decreased brain estrogen levels, while at periphery its levels remained similar to the respective males, pointing towards a perimenopause-like condition (Candeias *et al.* 2017). These observations further suggest that, in female rats, sex steroid hormones dysmetabolism may start in their brain and then affect the ovaries, being accelerated by T2D (Candeias *et al.* 2017).

Although previous studies showed no differences in blood glucose levels between middle-aged GK male and female rats (Candeias *et al.* 2017; Díaz *et al.* 2019), here we observed that GK female rats were markedly hyperglycemic compared to their respective male cohorts. Despite this apparent discrepancy, according to the $HbA_{1c}$ levels, our male GK rats fitted in a pre-diabetic diagnosis ($HbA_{1c}$ between 5.7-6.4%) (American Diabetes Association 2019a). Surprisingly, the hyperglycemia observed in GK female rats was probably recent and, therefore, not prolonged enough to yield $HbA_{1c}$ levels typical of T2D ($HbA_{1c}$ >6.5%), since this parameter usually reflects the blood glucose levels from the last 3 months (American Diabetes Association 2019b).

Given these differences in peripheral glucose homeostasis and previous evidence that sexual dimorphism in brain glucose uptake occurs already in young adult humans, with the effects varying among different parts of brain cortex (Hu *et al.* 2013), we next evaluated if this also occurred in middle-aged control and T2D rats. Indeed, brain cortical GLUT3 expression was upregulated in both Wistar and GK female rats, suggesting that they could be

attempting to increase glucose uptake towards brain cells (particularly neurons). This is supported by studies demonstrating that GLUT3 was modulated by estrogen, namely by a decrease in this transporter's mRNA and protein levels in the brains from ovariectomized Wistar female rats, *Rhesus* monkeys and in the 3xTgAD mouse model for AD - an effect attenuated upon estrogen treatment (Cheng *et al.* 2001; Ding *et al*. 2013b; López-Grueso *et al.* 2014). Since the Wistar female rats used herein were still under their fertile phase (Candeias *et al.* 2017), the available estrogen may still upregulate their brain GLUT3 transporter. Although T2D female rats might be already undergoing perimenopause, their slightly higher GLUT3 expression could arise from compensatory mechanisms mediated by estrogen, IGF-1 and insulin receptors that maintain the downstream signaling pathways (Candeias *et al.* 2017). Despite this, the higher GLUT3 density was only reflected by a slight increment in brain glucose uptake in T2D rat females, since in Wistar ones there was a massive drop in brain cortical glucose levels. The later could result from either a reduced transport activity and/or the immediate consumption of the glucose taken up by neurons. However, this metabolic stimulation may not to be the case herein, since glycolysis was attenuated in these females, as discussed below. Alternatively, since Hu *et al.* (2013) reported that brain glucose levels were lower in anterior regions of brain cortex in women and higher in the posterior ones compared to male subjects, we hypothesize that the drop of brain glucose content in control female rats may represent the net result of the different glucose uptake rates across the various regions of brain cortex. Conversely, the tendentiously higher brain cortical glucose levels in T2D female rats were in agreement with the increased brain glucose uptake observed by $^{18}$F-2-fluor-2-deoxiglucose positron emission tomography ($^{18}$F-FDG PET) in aged GK female rats (Díaz *et al.* 2019), as well as with previous evidence that estrogen stimulates brain glucose uptake (Ding *et al.* 2013a,b). Since perimenopause also inhibited brain glucose uptake (Mosconi *et al.* 2017), one cannot exclude that, despite the marked peripheral hyperglycemia, brain glucose levels in T2D female were only slightly increased. This appears to be further supported by the tendentiously lower brain glucose levels in T2D female rats compared with the non-diabetic ones, suggesting an interaction between estrogen and T2D-associated mechanisms to protect brain against the damaging effects of hyperglycemia.

As previously mentioned, the lower brain glucose levels in Wistar female rats than the respective males may not arise from its higher metabolism to glucose-6-phosphate and the subsequent catabolism along glycolysis, since all the main glycolytic metabolites measured (including the final product, pyruvate) were decreased in these female rat brains, thus

suggesting an attenuation of glycolysis. The slight stimulation of hexokinase in Wistar female rat brains was in line with the its stimulation in brain cortices from young adult female Wistar rats, together with hexokinase upregulation by estrogen (Leong *et al.* 1981; Rettberg *et al.* 2014). Although this could constitute a compensatory mechanism, it may not be sufficient to maintain the overall brain glycolysis in control female rats. A similar brain glycolytic inhibition appears to occur in GK female rats compared to their males. However, the slight increments in their brain glucose-6-phosphate and fructose-6-phosphate levels may result from the inhibition of downstream enzymes and subsequent accumulation of substrates.

Interestingly, the slowdown of brain glycolysis in both control and T2D female rats may not involve the shift of glucose-6-phosphate towards the oxidative branch of pentose phosphate pathway, since glucose-6-phosphate dehydrogenase was inhibited in the brains from both female rat cohorts. Since glucose-6-phosphate dehydrogenase can be upregulated under oxidative stress to produce reducing equivalents (*e.g.,* NADPH) for antioxidant defense mechanisms (Baxter and Hardingham 2016; Godon *et al*. 1998; Mamelak 2012; Thimmulappa *et al.* 2002), its inhibition in both control and T2D female rat brains may further indicate that they are still protected against oxidative stress when compared to male rats (Candeias *et al.* 2017). Since the transition to perimenopause leads to a progressive oxidative imbalance (Brinton *et al.* 2015), it is not surprising that GK rat females brains showed a slight stimulation of glucose-6-phosphate dehydrogenase compared to their healthy counterparts, possibly to increase the production of NADPH, as further supported by their nitrite levels (as discussed later).

Aiming to further confirm if the decreased brain pyruvate levels in both female groups resulted from the inhibition of glycolysis, instead of its higher consumption via fermentation and/or Krebs cycle, we analyzed its conversion into lactate by lactate dehydrogenase and to acetyl-CoA by pyruvate dehydrogenase, respectively. Despite the higher levels of lactate in brains from control female rats, their lactate dehydrogenase was downregulated, suggesting that lactate did not result from pyruvate catabolism, but rather from its uptake from the periphery and subsequent accumulation within the CNS. Indeed, a reasonable pool of the peripherally synthesized lactate can cross the blood-brain barrier to be used as energy source within the brain (van Hall *et al.* 2009; Proia *et al.* 2016), most likely as an alternative fuel for glycolysis or oxidative metabolism (the later via Krebs cycle), depending on the reaction that is favored (McKenna *et al.* 2012; Proia *et al.* 2016). Of note, conversion of pyruvate into lactate yields $NAD^+$ (an essential cofactor for glycolytic enzymes), whereas the reverse

reaction generates pyruvate and then acetyl-CoA to fuel Krebs cycle (McKenna *et al*. 2012; Proia *et al.* 2016). The inhibition of lactate dehydrogenase and of the initial steps of Krebs cycle in control female rat brains suggest that none of the reactions was favored under these conditions. Although no sex differences were initially reported in blood lactate levels among healthy individuals (Korhonen *et al.* 2005), in a more recent study Shen *et al.* (2012) observed that the higher plasma lactate levels in middle-aged T2D women were correlated with their estrogen levels in comparison with age-matched male patients and postmenopausal women. Additionally, treatment with the anti-T2D drug metformin further elevated their lactate content, suggesting that T2D and estrogen signaling may modulate peripheral lactate content (Shen *et al.* 2012). Surprisingly, we observed that T2D female rats had lower brain lactate levels than males, in agreement with their reduced lactate dehydrogenase activity. This suggests that less lactate may be taken up from the periphery in GK rat females and, therefore, lactate seems unlikely to constitute a crucial alternative fuel in our experimental conditions, as previously reported in brains from perimenopausal rats (Ding *et al.* 2013a). This further demonstrated the involvement of perimenopause in the metabolic alterations occurring in T2D female rat brain cortices.

The stimulation of pyruvate dehydrogenase in Wistar female rat brains suggested that their lower pyruvate levels could arise from its catabolism via the Krebs cycle. However, the subsequent inhibition of citrate synthase in both female rat cohorts (especially in GK rat ones) was in line with the inhibition of brain cortical citrate synthase from adult female Wistar rats, which further decreased with aging (Leong *et al*. 1981). This suggests that Krebs cycle may undergo an initial slowdown at this enzyme level, probably due to a lower availability of substrate if, *e. g.*, oxaloacetate is shifted towards gluconeogenesis. Indeed, estrogen may suppress gluconeogenesis in liver, namely upon T2D (Gao *et al.* 2012; Yan *et al.* 2019). However, further studies are needed to analyze the effect of sex on T2D brain gluconeogenesis.

Regarding the remaining enzymes of Krebs cycle, the stimulation of α-ketoglutarate dehydrogenase and succinate dehydrogenase in middle-aged Wistar female rats suggests that brain cortex may try to overcome the initial inhibition of citrate synthase possibly by promoting the flux of amino acids into the cycle (as providers of α-ketoglutarate and succinyl CoA) (McKenna *et al.* 2012; Owen *et al.* 2002). Indeed, α-ketoglutarate (the substrate for α-ketoglutarate dehydrogenase) may be alternatively formed from glutamate, glutamine, proline, histidine and arginine, whereas succinyl CoA (which is converted into succinate, the

substrate of succinate dehydrogenase) can be alternatively derived from methionine, valine and isoleucine (McKenna *et al.* 2012; Owen *et al.* 2002). The inhibition of brain malate dehydrogenase in our Wistar female rat brains appears to be in contrast with its tendentious stimulation in adult Wistar female rats upon aging (Leong *et al*. 1981). However, our results suggest that malate dehydrogenase may not be able to cope with the above-mentioned compensatory mechanisms. Importantly, similar compensatory responses in enzymes from the Krebs cycle occurred in middle-aged GK female rat brain cortices, that appear to maintain its function in later stages, particularly at the level of α-ketoglutarate dehydrogenase and malate dehydrogenase. Although pyruvate dehydrogenase and α-ketoglutarate dehydrogenase are inhibited by numerous pro-oxidant molecules, including 4-hydroxy-2-nonenal (a product from lipid peroxidation) (Gibson *et al.* 2008; Tretter and Adam-Vizi 2005), since both non-T2D and T2D female rat brains were more resistant to oxidative stress (Candeias *et al.* 2017), this may explain their partial stimulation in both female rat cohorts, but especially in Wistar ones. As such, female sex may promote compensatory mechanisms at the level of brain cortical Krebs cycle during midlife to surpass its initial inhibition compared to their male rat counterparts, being such response also conditioned by T2D.

Although female sex is known to impact brain mitochondrial function, the nature of such differences remains poorly understood. Indeed, Gaignard *et al.* (2015) failed to report alterations in the activities of mitochondrial respiratory chain complexes I-IV between young adult male and female C57BL6J mice brains, despite an increased NADH-linked respiration rate in female ones. Conversely, this difference in NADH-linked or $FADH_2$-linked respiration rates between sexes was lost upon aging (Gaignard *et al.* 2015). Our results in brain cortices from middle-aged rats showed that the tendentiously decreased NADH levels in both female rat cohorts appeared to correlate with the inhibition of mitochondrial respiratory chain complexes I and IV in Wistar female rats and with the overall inhibition of mitochondrial respiratory chain complexes I-IV in T2D ones. Surprisingly, control female rats appeared to compensate such inhibition with an upregulation of complexes II and III that, nonetheless, was not sufficient to replenish the intracellular pools of adenine nucleotides (ATP, ADP and AMP). On the other hand, the tendentiously higher brain cortical ATP and ADP pools in GK rat females suggest the involvement of alternative mechanisms to overcome their impaired mitochondrial energy metabolism and thus provide the energy for their brain function. One such alternative source of ATP in brain is the phosphocreatine/creatine kinase system, whose impairment may be involved in the pathophysiology of neurodegenerative diseases, including

AD (Andres *et al.* 2008). Moreover, recent studies demonstrated that T2D and sex also affect brain phosphocreatine/creatine kinase system in a region-specific manner, as observed in T2D *db/db* mice and in healthy adults (Hädel *et al.* 2013; Zheng *et al.* 2017). Hippocampal creatine kinase was also inhibited in ovariectomized adult Wistar female rats (Siebert *et al.* 2014). In this perspective, the phosphocreatine/creatine kinase system may account for the slight increment in brain ATP levels observed in T2D female rats compared to the respective males. Conversely, the increased pool of brain cortical phosphocreatine in middle-aged control female rats was not enough to restore their ATP levels. This, alongside their decreased ATP and AMP levels compared to males, reinforces the hypothesis of a general downregulation in the synthesis of adenine nucleotides. However, we cannot exclude that an increased downstream metabolism of adenine nucleotides towards the formation of purines (hypoxanthine, inosine and adenosine) might be occurring in Wistar female rats. This is supported by a decrement in adenosine and increased inosine levels in brain cortices from middle-aged women relatively to men (Kovács *et al.* 2010). A similar increment in hypoxanthine levels was obtained in *postmortem* brains from aged women compared to men (McFarland *et al.* 2013). Importantly, if AMP is metabolized to purines in Wistar female rats, then it would contribute to replenish their brain uric acid pools (an important antioxidant within the CNS) (McFarland *et al.* 2013) and, thereby, reinforce their protection against oxidative damage (Candeias *et al.* 2017).

Concerning brain ketone bodies, both female rat cohorts had slightly different profiles of 3-β-hydroxybutyrate and acetoacetate than their males, suggesting a dissimilar metabolism of these alternative fuels. In addition, sex exerted different effects between control and T2D animals. More specifically, the tendentiously lower levels of 3-β-hydroxybutyrate in brain cortices from Wistar female rats may arise from its lower uptake from the circulation and/or from a higher conversion into acetoacetate (Camandola and Mattson 2017), thereby explaining their slightly increased levels of the later. Alternatively, the increment in acetoacetate levels under these conditions may result from its lower metabolism towards acetyl-CoA, sterols and/or fatty acids (Camandola and Mattson 2017). However, we cannot exclude that the increased pool of acetoacetate in Wistar female rat brains may also promote the generation of those metabolites and, therefore, further studies are needed to clarify this issue. Regarding GK female rat brains, 3-β-hydroxybutyrate levels remained unaltered, whereas their acetoacetate levels were lower than males. This could result from a decreased uptake of acetoacetate from the circulation, and/or from its higher metabolism into acetyl-

CoA and/or fatty acids (Camandola and Mattson 2017). If so, then female rats (and especially T2D ones) could use acetoacetate to produce acetyl-CoA to feed citrate synthase and, ultimately, the Krebs cycle to overcome their hampered upstream metabolism, in agreement with evidence that perimenopause shifts the estrogen-controlled brain glucose metabolism towards the alternative ketogenic system (Ding *et al.* 2013a; Yao *et al.* 2010). However, in our case, this would still be redundant in terms of the energy generated, since the stage at the Krebs cycle whereby acetyl-CoA enters (citrate synthase) is attenuated in both female rat groups. This reinforces the need for further studies on brain ketone bodies' metabolism under these conditions.

Our previous study demonstrated that middle-aged Wistar and T2D female rat brains were more protected against the accumulation of AD-like and oxidative stress hallmarks (Candeias *et al.* 2017). The later could be due either to a lower production of reactive oxygen/nitrogen species and/or to more effective antioxidant defenses. In the present study, we aimed to clarify the impact of sex on the brain levels of a nitrosative stress markers (the reactive nitrogen species nitrites) upon T2D at midlife. Strikingly, Wistar and GK female rats exhibited inverse profiles of brain nitrites when compared to the respective males, in an apparent contrast with the previously measured intracellular oxidative/nitrosative stress markers (Candeias *et al.* 2017). The slightly lower levels of nitrites in brain cortices from Wistar female rats was in accordance with those from brain cortices of adult female Sprague-Dawley rats (Taskiran *et al.* 1997). Conversely, the tendentiously higher brain nitrites in T2D female rats, suggests an increased vulnerability to nitrosative stress that, nonetheless, may still not be harmful. Indeed, detrimental effects of nitric oxide and nitrites are often dependent of the presence of reactive oxygen species to initiate oxidative/nitrosative stress injury (Dalle-donne *et al.* 2005), against which these female rats were still protected (Candeias *et al.* 2017). However, we must emphasize that the progression of perimenopause may blunt this protection and, therefore, T2D female rat brains appear to be more prone to nitrosative damage than their age-matched male and non-diabetic cohorts. Interestingly, several authors showed that estrogen increases brain cortical nitric oxide/nitrite levels (Kant *et al.* 2000; Öge *et al.* 2003; Pratap *et al*. 2016). Thus, the decreased brain estrogen levels and higher nitrite levels found in T2D female rats would appear contradictory. However, we must bear in mind that the culprit of perimenopause-related alterations may be the selective expression of spliced variants of estrogen receptors, especially the estrogen receptor-α spliced variant MB1 (Brinton *et al.* 2015), whose increased expression in brains from perimenopausal women was associated with

the estrogen-induced activation of endothelial nitric oxide synthase (Brinton *et al.* 2015; Ishunina and Swaab 2008). However, we cannot exclude a role for the interaction between T2D and the estrogen receptor signaling herein.

Collectively, our results suggest a showdown of the glycolytic pathway and initial phases of Krebs cycle in the brain cortices from middle-aged Wistar and GK female rats compared to the respective male groups. Additionally, T2D female rats showed an overall attenuation of brain mitochondrial energetic function. However, both female rat cohorts appeared to develop compensatory mechanisms at different stages of metabolism. It is plausible that the decline in brain glucose metabolism in Wistar female rats compared to Wistar male ones results from their previously described brain sexual hormones' dysmetabolism (Candeias *et al.* 2017). However, we cannot exclude a role for a sexually dimorphic expression of estrogen receptors within their brains. Alternatively, since neuroendocrine aging precedes perimenopause and is regulated by DNA methylation of genes, *e. g.*, involved in hormone signaling (Bacon *et al.* 2019), it is possible that the downregulation of brain metabolism in female rats starts before perimenopause and precedes oxidative damage, being probably responsible for their resistance to oxidative stress and AD-like hallmarks (Candeias *et al.* 2017). This is reinforced by evidence that mitochondria are the major site for reactive oxygen species production in mammals (Apel and Hirt 2004). Hence, by slowing down brain mitochondrial energetic function, middle-aged female rats may be attenuating the generation of those species and defend their brains against oxidative stress compared to age-matched male rats (Chen *et al.* 2018). However, in the long-run, the progression of perimenopause may lead to a failure in compensatory mechanisms (especially in T2D) and, *e. g.*, if mitochondria become uncoupled, female rats brains may become more vulnerable to energy depletion and oxidative stress (Berry *et al.* 2018). This hypometabolism may in turn contribute to neurodegenerative processes (Brinton *et al*. 2015; Zilberter and Zilberter 2017), as postulated for the higher risk of menopausal women to AD (Riedel *et al.* 2016).

In sum, further studies are urgently needed to better understand the sex differences in intracellular stress mechanisms involved in T2D-related neurodegeneration (*e. g.,* endoplasmic reticulum stress, apoptosis), and their impact along reproductive aging.

# Conclusions

We provided evidence that sex differently affects brain cortical (energy) metabolism and nitrosative stress in middle-aged Wistar control and T2D GK rats, and in a disease-dependent manner.

### 6.1. Middle-aged control female *vs.* male rats

**a)** Despite their slightly increased brain GLUT3 density, glucose uptake from periphery may be already compromised;

**b)** This may slowdown their brain cortical glycolysis that did not involve the shift of glucose-6-phosphate towards the oxidative branch of the pentose phosphate pathway;

**c)** Their higher brain lactate levels may arise from an increased uptake from the periphery. However, lactate may not constitute an alternative fuel, since lactate dehydrogenase and citrate synthase are inhibited;

**d)** Their initial stage of Krebs cycle is inhibited, possibly due to the oxaloacetate shift towards gluconeogenesis. However, they developed compensatory responses later on, possible by metabolizing amino acids within the cycle;

**e)** Despite their slight increment in acetoacetic acid levels, this may not be as alternative fuel through Krebs cycle also due to its initial inhibition;

**f)** Their tendentiously higher mitochondrial respiratory complexes II/III and III activities, and phosphocreatine/creative kinase system were not sufficient to replenish the intracellular pools of adenine nucleotides;

**g)** However, their lower adenine nucleotides levels may result from a higher conversion into purines;

**h)** These females may be also protected from nitrosative stress.

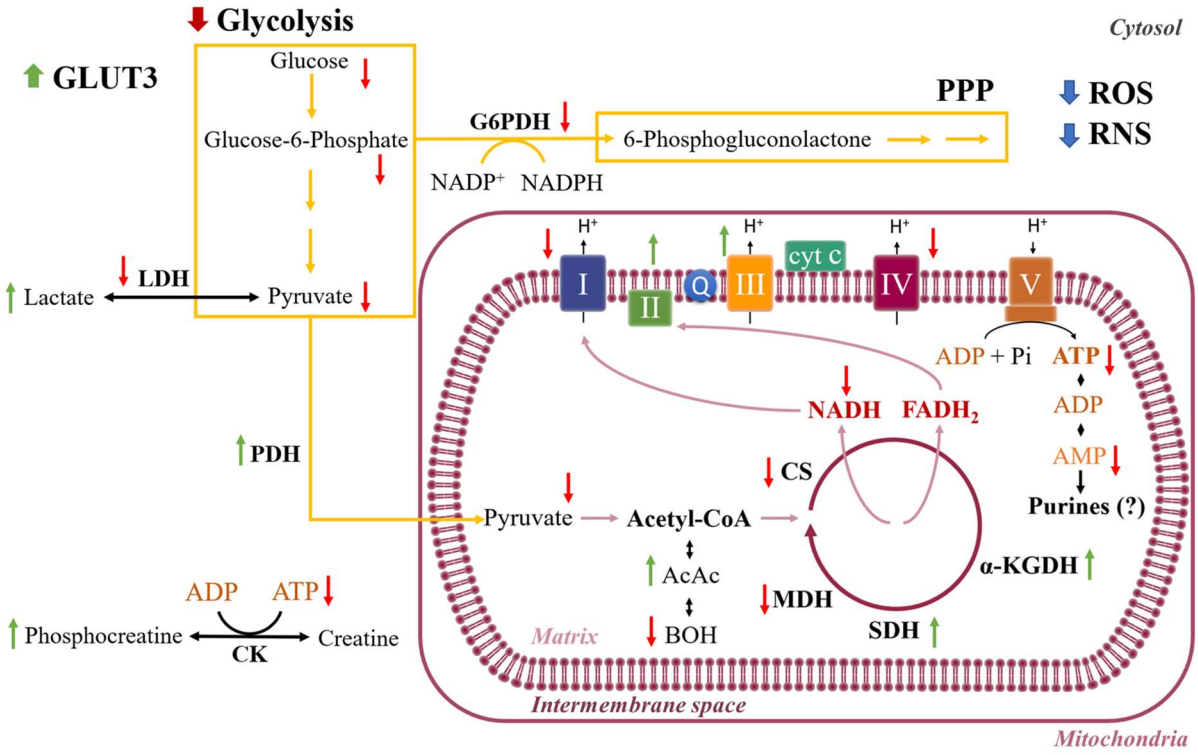



**Figure 6.1: Possible mechanisms underlying sex-mediated alterations in brain cortical glucose (mitochondrial) metabolism and oxidative/nitrosative stress in middle-aged Wistar female rats.** Abbreviations: α-KGDH, α-ketoglutarate dehydrogenase; AcAc, acetoacetate; BOH, 3-β-hydroxybutyrate; CK, creatine kinase; GLUT3, glucose transporter isoform-3; G6PDH, glucose-6-phosphate dehydrogenase; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; PDH, pyruvate, dehydrogenase; PPP, pentose phosphate dehydrogenase; SDH, succinate dehydrogenase; RNS, reactive nitrogen species; ROS, reactive oxygen species.

## 6.2. Middle-aged T2D female *vs.* male rats

**a)** They may be undergoing an early T2D stage, whereas males were still pre-diabetic;

**b)** Despite their slightly increased brain GLUT3 density, brain glucose content was only slightly higher than males;

**c)** Inhibition of their glycolysis did not involve a shift of glucose-6-phosphate towards the oxidative branch of the pentose phosphate pathway;

**d)** Despite their initial inhibition of Krebs cycle, the remaining cycle function was maintained;

**e)** Their lower brain acetoacetic acid levels was not used by Krebs cycle to produce energy due to its initial inhibition;

**f)** They had an overall inhibition of mitochondrial respiratory chain function (complexes I-IV);

**g)** However, phosphocreatine/creative kinase system may be replenishing their intracellular pools of ATP;

**h)** They were more vulnerable to nitrosative (but not to oxidative) stress, most likely due to the increased ERα splice variant MB1-related stimulation of nitric oxide synthase;

**i)** Such increased RNS may still not be harmful due to their decreased oxidative stress.

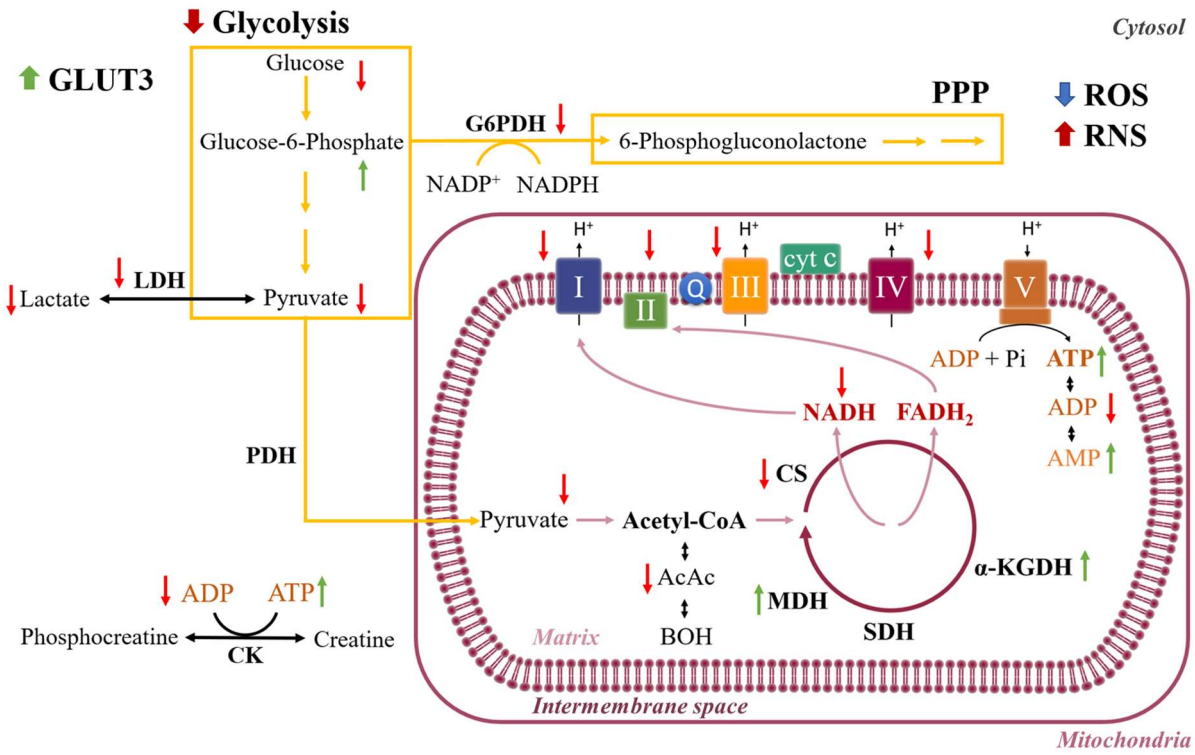



**Figure 6.2: Possible mechanisms underlying sex-mediated alterations of brain cortical glucose (mitochondrial) metabolism and oxidative/nitrosative stress in middle-aged GK female rats.** Abbreviations: α-KGDH, α-ketoglutarate dehydrogenase; AcAc, acetoacetate; BOH, 3-β-hydroxybutyrate; CK, creatine kinase; GLUT3, glucose transporter isoform-3; G6PDH, glucose-6-phosphate dehydrogenase; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; PDH, pyruvate, dehydrogenase; PPP, pentose phosphate dehydrogenase; SDH, succinate dehydrogenase; RNS, reactive nitrogen species; ROS, reactive oxygen species.

In sum, the slowdown of brain energy metabolism in both control and T2D middle-aged female rats may contribute for their reduced susceptibility to oxidative damage and AD-like neuropathology.

# Bibliography

Abdel-Rahim, E. A., El-Saadany, S. S., Abo-Eytta, A. M., & Wasi, M. M. (1992). The effect of Sammo administration on some fundamental enzymes of pentose phosphat pathway and energy metabolites of alloxanized rats. *Food/Nahrung*, *36*(1), 8-14.

Abdul-Rahman, O., Sasvari-Szekely, M., Ver, A., Rosta, K., Szasz, B. K., Kereszturi, E., & Keszler, G. (2012). Altered gene expression profiles in the hippocampus and prefrontal cortex of type 2 diabetic rats. *BMC genomics*, *13*(1), 81.

Ahmad, W., Ijaz, B., Shabbiri, K., Ahmed, F., & Rehman, S. (2017). Oxidative toxicity in diabetes and Alzheimer's disease: mechanisms behind ROS/RNS generation. *Journal of biomedical science*, *24*(1), 76.

Akash, M. S., Rehman, K., & Chen, S. (2013). Goto-Kakizaki rats: its suitability as non-obese diabetic animal model for spontaneous type 2 diabetes mellitus. *Current diabetes reviews*, *9*(5), 387-396.

Akhtar, M. W., Sanz-Blasco, S., Dolatabadi, N., Parker, J., Chon, K., Lee, M. S., ... & Lipton, S. A. (2016). Elevated glucose and oligomeric β-amyloid disrupt synapses via a common pathway of aberrant protein S-nitrosylation. *Nature communications*, *7*, 10242.

Alberti, G., Zimmet, P., Shaw, J., Bloomgarden, Z., Kaufman, F., & Silink, M. (2004). Type 2 diabetes in the young: the evolving epidemic: the international diabetes federation consensus workshop. *Diabetes care*, *27*(7), 1798-1811.

Alzheimer's Association. (2019). 2019 Alzheimer's disease facts and figures. *Alzheimer's & Dementia*, *15*(3), 321-387.

American Diabetes Association. (2014). Diagnosis and classification of diabetes mellitus. *Diabetes care*, *37*(Supplement 1), S81-S90.

American Diabetes Association. (2019)a. 2. Classification and diagnosis of diabetes: standards of medical care in diabetes—2019. *Diabetes Care*, *42*(Supplement 1), S13-S28.

American Diabetes Association. (2019)b. 6. Glycemic targets: standards of medical care in diabetes—2019. *Diabetes Care*, *42*(Supplement 1), S61-S70.

Andersen, J. V., Christensen, S. K., Nissen, J. D., & Waagepetersen, H. S. (2017)a. Improved cerebral energetics and ketone body metabolism in db/db mice. *Journal of Cerebral Blood Flow & Metabolism*, *37*(3), 1137-1147.

Andersen, J. V., Nissen, J. D., Christensen, S. K., Markussen, K. H., & Waagepetersen, H. S. (2017)b. Impaired Hippocampal Glutamate and Glutamine Metabolism in the db/db Mouse Model of Type 2 Diabetes Mellitus. *Neural plasticity*, *2017*.

Andres, R. H., Ducray, A. D., Schlattner, U., Wallimann, T., & Widmer, H. R. (2008). Functions and effects of creatine in the central nervous system. *Brain research bulletin*, *76*(4), 329-343.

Andrieu, S., Coley, N., Lovestone, S., Aisen, P. S., & Vellas, B. (2015). Prevention of sporadic Alzheimer's disease: lessons learned from clinical trials and future directions. *The Lancet Neurology*, *14*(9), 926-944.

Aouacheri, O., Saka, S., Krim, M., Messaadia, A., & Maidi, I. (2015). The investigation of the oxidative stress-related parameters in type 2 diabetes mellitus. *Canadian journal of diabetes*, *39*(1), 44-49.

Apel, K., & Hirt, H. (2004). Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Biol.*, *55*, 373-399.

Arevalo, M. A., Azcoitia, I., & Garcia-Segura, L. M. (2015). The neuroprotective actions of oestradiol and oestrogen receptors. *Nature Reviews Neuroscience*, *16*(1), 17.

Armand-Ugon, M., Ansoleaga, B., Berjaoui, S., & Ferrer, I. (2017). Reduced mitochondrial activity is early and steady in the entorhinal cortex but it is mainly unmodified in the frontal cortex in Alzheimer's disease. *Current Alzheimer Research*, *14*(12), 1327-1334.

Bacon, E. R., Mishra, A., Wang, Y., Desai, M. K., Yin, F., & Brinton, R. D. (2019). Neuroendocrine aging precedes perimenopause and is regulated by DNA methylation. *Neurobiology of aging*, *74*, 213-224.

Baker, L. D., Cross, D. J., Minoshima, S., Belongia, D., Watson, G. S., & Craft, S. (2011). Insulin resistance and Alzheimer-like reductions in regional cerebral glucose metabolism for cognitively normal adults with prediabetes or early type 2 diabetes. *Archives of neurology*, *68*(1), 51-57.

Balazs, L., & Leon, M. (1994). Evidence of an oxidative challenge in the Alzheimer's brain. *Neurochemical research*, *19*(9), 1131-1137.

Baquer, N. Z., Hothersall, J. S., & McLean, P. (1988). Function and regulation of the pentose phosphate pathway in brain. In *Current topics in cellular regulation* (Vol. 29, pp. 265-289). Academic Press.

Batista, A. F., Forny-Germano, L., Clarke, J. R., Lyra e Silva, N. M., Brito-Moreira, J., Boehnke, S. E., ... & Marques, S. A. (2018). The diabetes drug liraglutide reverses cognitive impairment in mice and attenuates insulin receptor and synaptic pathology in a non-human primate model of Alzheimer's disease. *The Journal of pathology*, *245*(1), 85-100.

Baxter, P. S., & Hardingham, G. E. (2016). Adaptive regulation of the brain's antioxidant defences by neurons and astrocytes. *Free Radical Biology and Medicine*, *100*, 147-152.

Bélanger, M., Allaman, I., & Magistretti, P. J. (2011). Brain energy metabolism: focus on astrocyte-neuron metabolic cooperation. *Cell metabolism*, *14*(6), 724-738.

Benedict, C., Kern, W., Schultes, B., Born, J., & Hallschmid, M. (2008). Differential sensitivity of men and women to anorexigenic and memory-improving effects of intranasal insulin. *The Journal of Clinical Endocrinology & Metabolism*, *93*(4), 1339-1344.

Bénit, P., Slama, A., & Rustin, P. (2008). Decylubiquinol impedes mitochondrial respiratory chain complex I activity. *Molecular and cellular biochemistry*, *314*(1-2), 45.

Bermeyer, H. & Bernt, E. (1974). *Methods of Enzymatic Analysis.* (Second Edition)

Berry, B. J., Trewin, A. J., Amitrano, A. M., Kim, M., & Wojtovich, A. P. (2018). Use the protonmotive force: mitochondrial uncoupling and reactive oxygen species. *Journal of molecular biology*, *430*(21), 3873-3891.

Biessels, G. J., Strachan, M. W., Visseren, F. L., Kappelle, L. J., & Whitmer, R. A. (2014). Dementia and cognitive decline in type 2 diabetes and prediabetic stages: towards targeted interventions. *The lancet Diabetes & endocrinology*, *2*(3), 246-255.

Birnbaum, J. H., Wanner, D., Gietl, A. F., Saake, A., Kündig, T. M., Hock, C., ... & Tackenberg, C. (2018). Oxidative stress and altered mitochondrial protein expression in the absence of amyloid-β and tau pathology in iPSC-derived neurons from sporadic Alzheimer's disease patients. *Stem cell research*, *27*, 121-130.

Blonz, E. R. (2017). Alzheimer's disease as the product of a progressive energy deficiency syndrome in the central nervous system: The neuroenergetic hypothesis. *Journal of Alzheimer's Disease*, *60*(4), 1223-1229.

Bolaños, J. P., & Almeida, A. (2010). The pentose-phosphate pathway in neuronal survival against nitrosative stress. *IUBMB life*, *62*(1), 14-18.

Bomfim, T. R., Forny-Germano, L., Sathler, L. B., Brito-Moreira, J., Houzel, J. C., Decker, H., ... & Holscher, C. (2012). An anti-diabetes agent protects the mouse brain from defective insulin signaling caused by Alzheimer's disease–associated Aβ oligomers. *The Journal of clinical investigation*, *122*(4), 1339-1353.

Bonds, D. E., Lasser, N., Qi, L., Brzyski, R., Caan, B., Heiss, G., ... & O'sullivan, M. J. (2006). The effect of conjugated equine oestrogen on diabetes incidence: the Women's Health Initiative randomised trial. *Diabetologia*, *49*(3), 459-468.

Bouche, C., Serdy, S., Kahn, C. R., & Goldfine, A. B. (2004). The cellular fate of glucose and its relevance in type 2 diabetes. *Endocrine reviews*, *25*(5), 807-830.

Brautigan, D. L., Ferguson-Miller, S., & Margoliash, E. (1978). [18] Mitochondrial cytochrome c: Preparation and activity of native and chemically modified cytochromes c. In *Methods in enzymology* (Vol. 53, pp. 128-164). Academic Press.

Brinton, R. D., Yao, J., Yin, F., Mack, W. J., & Cadenas, E. (2015). Perimenopause as a neurological transition state. *Nature reviews endocrinology*, *11*(7), 393.

Brooks, W. M., Lynch, P. J., Ingle, C. C., Hatton, A., Emson, P. C., Faull, R. L., & Starkey, M. P. (2007). Gene expression profiles of metabolic enzyme transcripts in Alzheimer's disease. *Brain research*, *1127*, 127-135.

Bubber, P., Haroutunian, V., Fisch, G., Blass, J. P., & Gibson, G. E. (2005). Mitochondrial abnormalities in Alzheimer brain: mechanistic implications. *Annals of Neurology: Official Journal of the American Neurological Association and the Child Neurology Society*, *57*(5), 695-703.

Butterfield, D. A., Di Domenico, F., & Barone, E. (2014). Elevated risk of type 2 diabetes for development of Alzheimer disease: a key role for oxidative stress in brain. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, *1842*(9), 1693-1706.

Butterfield, D. A., & Halliwell, B. (2019). Oxidative stress, dysfunctional glucose metabolism and Alzheimer disease. *Nature Reviews Neuroscience*, 1.

Butterfield, D. A., Reed, T., Perluigi, M., De Marco, C., Coccia, R., Cini, C., & Sultana, R. (2006). Elevated protein-bound levels of the lipid peroxidation product, 4-hydroxy-2-nonenal, in brain from persons with mild cognitive impairment. *Neuroscience letters*, *397*(3), 170-173.

Camandola, S., & Mattson, M. P. (2017). Brain metabolism in health, aging, and neurodegeneration. *The EMBO journal*, *36*(11), 1474-1492.

Candeias, E., Duarte, A. I., Sebastião, I., Fernandes, M. A., Plácido, A. I., Carvalho, C., ... & Oliveira, C. R. (2017). Middle-aged diabetic females and males present distinct susceptibility to Alzheimer disease-like pathology. *Molecular neurobiology*, *54*(8), 6471-6489.

Candeias, E., Duarte, A. I., Carvalho, C., Correia, S. C., Cardoso, S., Santos, R. X., ... & Moreira, P. I. (2012). The impairment of insulin signaling in Alzheimer's disease. *IUBMB life*, *64*(12), 951-957.

Carvalho, C., Catarina Correia, S., Sancha Santos, M., Baldeiras, I., Resende Oliveira, C., Seica, R., & Isabel Moreira, P. (2014). Vascular, oxidative, and synaptosomal abnormalities during aging and the progression of type 2 diabetes. *Current neurovascular research*, *11*(4), 330-339.

Carvalho, C., Cardoso, S., Correia, S. C., Santos, R. X., Santos, M. S., Baldeiras, I., ... & Moreira, P. I. (2012). Metabolic alterations induced by sucrose intake and Alzheimer's disease promote similar brain mitochondrial abnormalities. *Diabetes*, *61*(5), 1234-1242.

Carvalho, C., Machado, N., Mota, P. C., Correia, S. C., Cardoso, S., Santos, R. X., ... & Moreira, P. I. (2013). Type 2 diabetic and Alzheimer's disease mice present similar behavioral, cognitive, and vascular anomalies. *Journal of Alzheimer's Disease*, *35*(3), 623-635.

Carvalho, C., Santos, M. S., Oliveira, C. R., & Moreira, P. I. (2015). Alzheimer's disease and type 2 diabetes-related alterations in brain mitochondria, autophagy and synaptic markers. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, *1852*(8), 1665-1675.

Chai, Y. L., Xing, H., Chong, J. R., Francis, P. T., Ballard, C. G., Chen, C. P., & Lai, M. K. (2018). Mitochondrial Translocase of the Outer Membrane Alterations May Underlie Dysfunctional Oxidative Phosphorylation in Alzheimer's Disease. *Journal of Alzheimer's Disease*, (Preprint), 1-9.

Chakrabarty, M., Bhat, P., Kumari, S., D'Souza, A., Bairy, K. L., Chaturvedi, A., ... & Kamath, S. (2012). Cortico-hippocampal salvage in chronic aluminium induced neurodegeneration by Celastrus paniculatus seed oil: Neurobehavioural, biochemical, histological study. *Journal of pharmacology &*

*pharmacotherapeutics*, *3*(2), 161.

Chandel, N. S. (2015). Evolution of mitochondria as signaling organelles. *Cell metabolism*, *22*(2), 204-206.

Chen, R., Lai, U. H., Zhu, L., Singh, A., Ahmed, M., & Forsyth, N. R. (2018). Reactive oxygen species formation in the brain at different oxygen levels: the role of hypoxia inducible factors. *Frontiers in cell and developmental biology*, *6*, 132.

Chen, Z., & Zhong, C. (2013). Decoding Alzheimer's disease from perturbed cerebral glucose metabolism: implications for diagnostic and therapeutic strategies. *Progress in neurobiology*, *108*, 21-43.

Cheng, C. M., Cohen, M., Wang, J. I. E., & Bondy, C. A. (2001). Estrogen augments glucose transporter and IGF1 expression in primate cerebral cortex. *The FASEB Journal*, *15*(6), 907-915.

Cholerton, B., Baker, L. D., & Craft, S. (2013). Insulin, cognition, and dementia. *European journal of pharmacology*, *719*(1-3), 170-179.

Cisternas, P., Zolezzi, J. M., Martinez, M., Torres, V. I., Wong, G. W., & Inestrosa, N. C. (2018). Wnt-induced activation of glucose metabolism mediates the in vivo neuroprotective roles of Wnt signaling in Alzheimer disease. *Journal of neurochemistry*, *149*(1), 54-72.

Coore, H. G., Denton, R. M., Martin, B. R., & Randle, P. J. (1971). Regulation of adipose tissue pyruvate dehydrogenase by insulin and other hormones. *Biochemical Journal*, *125*(1), 115-127.

Correia, S., Carvalho, C., Santos, M. S., Proenca, T., Nunes, E., Duarte, A. I., ... & Moreira, P. I. (2008). Metformin protects the brain against the oxidative imbalance promoted by type 2 diabetes. *Medicinal chemistry*, *4*(4), 358-364.

Crabtree, B., Leech, A. R., Newsholme, E. A., & Kornberg, H. L. (1979). *Techniques in Metabolic Research: Measurement of Enzyme Activities in Crude Extracts of Tissues*. Elsevier/North-Holland.

Craft, S. (2012). Alzheimer disease: insulin resistance and AD—extending the translational path. *Nature Reviews Neurology*, *8*(7), 360.

Dalle-Donne, I., Scaloni, A., Giustarini, D., Cavarra, E., Tell, G., Lungarella, G., ... & Milzani, A. (2005). Proteins as biomarkers of oxidative/nitrosative stress in diseases: the contribution of redox proteomics. *Mass spectrometry reviews*, *24*(1), 55-99.

de la Monte, S. M. (2017). Insulin resistance and neurodegeneration: progress towards the development of new therapeutics for Alzheimer's disease. *Drugs*, *77*(1), 47-65.

de Matos, A. M., de Macedo, M. P., & Rauter, A. P. (2018). Bridging type 2 diabetes and Alzheimer's disease: assembling the puzzle pieces in the quest for the molecules with therapeutic and preventive potential. *Medicinal research reviews*, *38*(1), 261-324.

Díaz, A., López-Grueso, R., Gambini, J., Monleón, D., Mas-Bargues, C., Abdelaziz, K. M., ... & Borrás, C. (2019). Sex Differences in Age-Associated Type 2 Diabetes in Rats—Role of Estrogens and Oxidative Stress. *Oxidative Medicine and Cellular Longevity*, *2019*.

Ding, E. L., Song, Y., Manson, J. E., Rifai, N., Buring, J. E., & Liu, S. (2007). Plasma sex steroid hormones and risk of developing type 2 diabetes in women: a prospective study. *Diabetologia*, *50*(10), 2076-2084.

Ding, F., Yao, J., Rettberg, J. R., Chen, S., & Brinton, R. D. (2013)a. Early decline in glucose transport and metabolism precedes shift to ketogenic system in female aging and Alzheimer's mouse brain: implication for bioenergetic intervention. *PloS one*, *8*(11), e79977.

Ding, F., Yao, J., Zhao, L., Mao, Z., Chen, S., & Brinton, R. D. (2013)b. Ovariectomy induces a shift in fuel availability and metabolism in the hippocampus of the female transgenic model of familial Alzheimer's. *PloS one*, *8*(3), e59825.

Drummond, E., & Wisniewski, T. (2017). Alzheimer's disease: experimental models and reality. *Acta neuropathologica*, *133*(2), 155-175.

Duarte, A. I., Candeias, E., Correia, S. C., Santos, R. X., Carvalho, C., Cardoso, S., ... & Moreira, P. I. (2013). Crosstalk between diabetes and brain: glucagon-like peptide-1 mimetics as a promising therapy against neurodegeneration. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, *1832*(4), 527-541.

Duarte, A. I., Moreira, P. I., & Oliveira, C. R. (2012). Insulin in central nervous system: more than just a peripheral hormone. *Journal of aging research*, *2012*.

Duarte, A. I., Santos, M. S., Oliveira, C. R., & Moreira, P. I. (2018). Brain insulin signalling, glucose metabolism and females' reproductive aging: a dangerous triad in Alzheimer's disease. *Neuropharmacology*, *136*, 223-242.

Duarte, A. I., Santos, P., Oliveira, C. R., Santos, M. S., & Rego, A. C. (2008). Insulin neuroprotection against oxidative stress is mediated by Akt and GSK-3β signaling pathways and changes in protein expression. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, *1783*(6), 994-1002.

Duarte, A., Santos, M., Seiça, R., & de Oliveira, C. R. (2000). Effect of oxidative stress on the uptake of GABA and glutamate in synaptosomes isolated from diabetic rat brain. *Neuroendocrinology*, *72*(3), 179-186.

Espeland, M. A., Brinton, R. D., Hugenschmidt, C., Manson, J. E., Craft, S., Yaffe, K., ... & Jackson, R. D. (2015). Impact of type 2 diabetes and postmenopausal hormone therapy on incidence of cognitive impairment in older women. *Diabetes care*, *38*(12), 2316-2324.

Espeland, M. A., Miller, M. E., Goveas, J. S., Hogan, P. E., Coker, L. H., Williamson, J., ... & Resnick, for the WHISCA Study Group, S. M. (2011). Cognitive function and fine motor speed in older women with diabetes mellitus: results from the women's health initiative study of cognitive aging. *Journal of Women's Health*, *20*(10), 1435-1443.

Falkowska, A., Gutowska, I., Goschorska, M., Nowacki, P., Chlubek, D., & Baranowska-Bosiacka, I. (2015). Energy metabolism of the brain, including the cooperation between astrocytes and neurons, especially in the context of glycogen metabolism. *International journal of molecular sciences*, *16*(11), 25959-25981.

Faul, F., Erdfelder, E., Lang, A. G., & Buchner, A. (2007). G* Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behavior research methods*, *39*(2), 175-191.

Fernandez-Marcos, P. J., & Nóbrega-Pereira, S. (2016). NADPH: new oxygen for the ROS theory of aging. *Oncotarget*, *7*(32), 50814.

Gaignard, P., Savouroux, S., Liere, P., Pianos, A., Thérond, P., Schumacher, M., ... & Guennoun, R. (2015). Effect of sex differences on brain mitochondrial function and its suppression by ovariectomy and in aged mice. *Endocrinology*, *156*(8), 2893-2904.

Gao, J., He, J., Shi, X., Stefanovic-Racic, M., Xu, M., O'Doherty, R. M., ... & Xie, W. (2012). Sex-specific effect of estrogen sulfotransferase on mouse models of type 2 diabetes. *Diabetes*, *61*(6), 1543-1551.

Garcia-Casares, N., Jorge, R. E., Garcia-Arnes, J. A., Acion, L., Berthier, M. L., Gonzalez-Alegre, P., ... & Gonzalez-Santos, P. (2014). Cognitive dysfunctions in middle-aged type 2 diabetic patients and neuroimaging correlations: a cross-sectional study. *Journal of Alzheimer's Disease*, *42*(4), 1337-1346.

García-Nogales, P., Almeida, A., Fernández, E., Medina, J. M., & Bolaños, J. P. (1999). Induction of glucose-6-phosphate dehydrogenase by lipopolysaccharide contributes to preventing nitric oxide-mediated glutathione depletion in cultured rat astrocytes. *Journal of neurochemistry*, *72*(4), 1750-1758.

Gibson, G. E., Karuppagounder, S. S., & Shi, Q. (2008). Oxidant-induced changes in mitochondria and

calcium dynamics in the pathophysiology of Alzheimer's disease. *Annals of the New York Academy of Sciences*, *1147*(1), 221-232.

Girault, F. M., Sonnay, S., Gruetter, R., & Duarte, J. M. (2017). Alterations of brain energy metabolism in type 2 diabetic Goto-Kakizaki rats measured in vivo by 13 C magnetic resonance spectroscopy. *Neurotoxicity research*, 1-11.

Glancy, B., Willis, W. T., Chess, D. J., & Balaban, R. S. (2013). Effect of calcium on the oxidative phosphorylation cascade in skeletal muscle mitochondria. *Biochemistry*, *52*(16), 2793-2809.

Godon, C., Lagniel, G., Lee, J., Buhler, J. M., Kieffer, S., Perrot, M., ... & Labarre, J. (1998). The H2O2 stimulon in Saccharomyces cerevisiae. *Journal of Biological Chemistry*, *273*(35), 22480-22489.

Gök, M., Ulusu, N. N., Tarhan, N., Tufan, C., Ozansoy, G., Arı, N., & Karasu, Ç. (2016). Flaxseed protects against diabetes-induced glucotoxicity by modulating pentose phosphate pathway and glutathione-dependent enzyme activities in rats. *Journal of dietary supplements*, *13*(3), 339-351.

Gonzalez-Franquesa, A., & Patti, M. E. (2017). Insulin resistance and mitochondrial dysfunction. In *Mitochondrial Dynamics in Cardiovascular Medicine* (pp. 465-520). Springer, Cham.

Goto, Y., Kakizaki, M., & Masaki, N. (1975). Spontaneous diabetes produced by selective breeding of normal Wistar rats. *Proceedings of the Japan Academy*, *51*(1), 80-85.

Green, L. C., De Luzuriaga, K. R., Wagner, D. A., Rand, W., Istfan, N., Young, V. R., & Tannenbaum, S. R. (1981). Nitrate biosynthesis in man. *Proceedings of the National Academy of Sciences*, *78*(12), 7764-7768.

Gurtubay, J. I. G., Goni, F. M., Gomez-Fernandez, J. C., Otamendi, J. J., & Macarulla, J. M. (1980). Triton X-100 solubilization of mitochondrial inner and outer membranes. *Journal of bioenergetics and biomembranes*, *12*(1-2), 47-70.

Guzmán, M., & Blázquez, C. (2004). Ketone body synthesis in the brain: possible neuroprotective effects. *Prostaglandins, leukotrienes and essential fatty acids*, *70*(3), 287-292.

Hädel, S., Wirth, C., Rapp, M., Gallinat, J., & Schubert, F. (2013). Effects of age and sex on the concentrations of glutamate and glutamine in the human brain. *Journal of Magnetic Resonance Imaging*, *38*(6), 1480-1487.

Hamed, S. A. (2017). Brain injury with diabetes mellitus: evidence, mechanisms and treatment implications. *Expert review of clinical pharmacology*, *10*(4), 409-428.

Hara, Y., Waters, E. M., McEwen, B. S., & Morrison, J. H. (2015). Estrogen effects on cognitive and synaptic health over the lifecourse. *Physiological reviews*, *95*(3), 785-807.

Harr, S. D., Simonian, N. A., & Hyman, B. T. (1995). Functional alterations in Alzheimer's disease: decreased glucose transporter 3 immunoreactivity in the perforant pathway terminal zone. *Journal of Neuropathology & Experimental Neurology*, *54*(1), 38-41.

Harris, F., & Pierpoint, L. (2012). Photodynamic therapy based on 5-aminolevulinic acid and its use as an antimicrobial Agent. *Medicinal research reviews*, *32*(6), 1292-1327.

Herman, W. H., & Zimmet, P. (2012). Type 2 diabetes: an epidemic requiring global attention and urgent action. *Diabetes care*, *35*(5), 943-944.

Herst, P. M., Rowe, M. R., Carson, G. M., & Berridge, M. V. (2017). Functional mitochondria in health and disease. *Frontiers in endocrinology*, *8*, 296.

Hishikawa, N., Fukui, Y., Sato, K., Kono, S., Yamashita, T., Ohta, Y., ... & Abe, K. (2016). Cognitive and affective functions in Alzheimer's disease patients with metabolic syndrome. *European journal of neurology*, *23*(2), 339-345.

Hu, Y., Xu, Q., Li, K., Zhu, H., Qi, R., Zhang, Z., & Lu, G. (2013). Gender differences of brain glucose metabolic networks revealed by FDG-PET: evidence from a large cohort of 400 young adults. *PLoS*

*One*, *8*(12), e83821.

Huang, Q., Aluise, C. D., Joshi, G., Sultana, R., St. Clair, D. K., Markesbery, W. R., & Butterfield, D. A. (2010). Potential in vivo amelioration by N-acetyl-L-cysteine of oxidative stress in brain in human double mutant APP/PS-1 knock-in mice: toward therapeutic modulation of mild cognitive impairment. *Journal of neuroscience research*, *88*(12), 2618-2629.

Huang, S., Wang, Y., Gan, X., Fang, D., Zhong, C., Wu, L., ... & Yan, S. S. (2015). Drp1-mediated mitochondrial abnormalities link to synaptic injury in diabetes model. *Diabetes*, *64*(5), 1728-1742.

Humphries, K. M., & Szweda, L. I. (1998). Selective inactivation of α-ketoglutarate dehydrogenase and pyruvate dehydrogenase: reaction of lipoic acid with 4-hydroxy-2-nonenal. *Biochemistry*, *37*(45), 15835-15841.

Hussain, S., Mansouri, S., Sjöholm, Å., Patrone, C., & Darsalia, V. (2014). Evidence for cortical neuronal loss in male type 2 diabetic Goto-Kakizaki rats. *Journal of Alzheimer's disease*, *41*(2), 551-560.

International Diabetes Federation. (2017). *IDF Diabetes Atlas, 8th edn.* Brussels, Belgium: International Diabetes Federation. Retrieved from: http://www.diabetesatlas.org

Ishunina, T. A., & Swaab, D. F. (2008). Age-dependent ERα MB1 splice variant expression in discrete areas of the human brain. *Neurobiology of aging*, *29*(8), 1177-1189.

Islam, M. T. (2017). Oxidative stress and mitochondrial dysfunction-linked neurodegenerative disorders. *Neurological research*, *39*(1), 73-82.

Iwangoff, P., Armbruster, R., Enz, A., & Meier-Ruge, W. (1980). Glycolytic enzymes from human autoptic brain cortex: normal aged and demented cases. *Mechanisms of ageing and development*, *14*(1-2), 203-209.

Janson, J., Laedtke, T., Parisi, J. E., O'Brien, P., Petersen, R. C., & Butler, P. C. (2004). Increased risk of type 2 diabetes in Alzheimer disease. *Diabetes*, *53*(2), 474-481.

Janssen, A. J., Trijbels, F. J., Sengers, R. C., Smeitink, J. A., Van den Heuvel, L. P., Wintjes, L. T., ... & Rodenburg, R. J. (2007). Spectrophotometric assay for complex I of the respiratory chain in tissue samples and cultured fibroblasts. *Clinical chemistry*, *53*(4), 729-734.

Jha, M. K., Jeon, S., & Suk, K. (2012). Pyruvate dehydrogenase kinases in the nervous system: their principal functions in neuronal-glial metabolic interaction and neuro-metabolic disorders. *Current neuropharmacology*, *10*(4), 393-403.

Kalish, G. M., Barrett-Connor, E., Laughlin, G. A., & Gulanski, B. I. (2003). Association of endogenous sex hormones and insulin resistance among postmenopausal women: results from the Postmenopausal Estrogen/Progestin Intervention Trial. *The Journal of Clinical Endocrinology & Metabolism*, *88*(4), 1646-1652.

Kametani, F., & Hasegawa, M. (2018). Reconsideration of amyloid hypothesis and tau hypothesis in Alzheimer's disease. *Frontiers in neuroscience*, *12*, 25.

Kandimalla, R., Thirumala, V., & Reddy, P. H. (2017). Is Alzheimer's disease a type 3 diabetes? A critical appraisal. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, *1863*(5), 1078-1089.

Kant, L., Yilmaz, O., Taskiran, D., Kulali, B., Furedy, J. J., Demirgören, S., & Pögün, S. (2000). Sexually dimorphic cognitive style, female sex hormones, and cortical nitric oxide. *Physiology & behavior*, *71*(3-4), 277-287.

Kashiwaya, Y., Takeshima, T., Mori, N., Nakashima, K., Clarke, K., & Veech, R. L. (2000). d-β-Hydroxybutyrate protects neurons in models of Alzheimer's and Parkinson's disease. *Proceedings of the National Academy of Sciences*, *97*(10), 5440-5444.

Kashiwaya, Y., Bergman, C., Lee, J. H., Wan, R., King, M. T., Mughal, M. R., ... & Veech, R. L. (2013).

A ketone ester diet exhibits anxiolytic and cognition-sparing properties, and lessens amyloid and tau pathologies in a mouse model of Alzheimer's disease. *Neurobiology of aging*, *34*(6), 1530-1539.

Keller, J. N., Schmitt, F. A., Scheff, S. W., Ding, Q., Chen, Q., Butterfield, D. A., & Markesbery, W. R. (2005). Evidence of increased oxidative damage in subjects with mild cognitive impairment. *Neurology*, *64*(7), 1152-1156.

Kevil, C. G. & Lefer D. J. (2010). Nitrite Therapy for Ischemic Syndromes. In Ignarro, L. J. (Ed. 2), *Nitric Oxide: Biology and Pathology, 587-603.* Academic Press

Kim, J. A., Wei, Y., & Sowers, J. R. (2008). Role of mitochondrial dysfunction in insulin resistance. *Circulation research*, *102*(4), 401-414.

Korhonen, M. T., Suominen, H., & Mero, A. (2005). Age and sex differences in blood lactate response to sprint running in elite master athletes. *Canadian journal of applied physiology*, *30*(6), 647-665.

Kovács, Z., Juhász, G., Dobolyi, Á., Bobest, M., Papp, V., Takáts, L., & Kékesi, K. A. (2010). Gender- and age-dependent changes in nucleoside levels in the cerebral cortex and white matter of the human brain. *Brain research bulletin*, *81*(6), 579-584.

Kumagai, A. K. (1999). Glucose transport in brain and retina: implications in the management and complications of diabetes. *Diabetes/metabolism research and reviews*, *15*(4), 261-273.

Lai, Y. K., Lai, N. M., & Lee, S. W. H. (2017). Glucose-6-phosphate dehydrogenase deficiency and risk of diabetes: a systematic review and meta-analysis. *Annals of hematology*, *96*(5), 839-845.

Lakhman, S. S., Sharma, P., Kaur, G., & Kaur, G. (1994). Changes in glucose metabolism from discrete regions of rat brain and its relationship to reproductive failure during experimental diabetes. *Molecular and cellular biochemistry*, *141*(2), 97-102.

Lamprecht, W., Stein, P., Heinz, F., & Weisser, H. (1974). Creatine phosphate. In *Methods of enzymatic analysis* (pp. 1777-1785). Academic Press.

Lange, K. W., Lange, K. M., Makulska-Gertruda, E., Nakamura, Y., Reissmann, A., Kanaya, S., & Hauser, J. (2017). Ketogenic diets and Alzheimer's disease. *Food Science and Human Wellness*, *6*(1), 1-9.

Leffa, D. D., Rezin, G. T., Daumann, F., Longaretti, L. M., Dajori, A. L. F., Gomes, L. M., ... & de Andrade, V. M. (2017). Effects of Acerola (Malpighia emarginata DC.) juice intake on brain energy metabolism of mice fed a cafeteria diet. *Molecular neurobiology*, *54*(2), 954-963.

Lejri, I., & Eckert, A. (2018). Mitochondria, estrogen and female brain aging. *Frontiers in aging neuroscience*, *10*, 124.

Leong, S. F., Lai, J. C. K., Lim, L., & Clark, J. B. (1981). Energy-metabolising enzymes in brain regions of adult and aging rats. *Journal of neurochemistry*, *37*(6), 1548-1556.

Liang, W. S., Reiman, E. M., Valla, J., Dunckley, T., Beach, T. G., Grover, A., ... & Kukull, W. (2008). Alzheimer's disease is associated with reduced expression of energy metabolism genes in posterior cingulate neurons. *Proceedings of the National Academy of Sciences*, *105*(11), 4441-4446.

Liu, Y., Liu, F., Iqbal, K., Grundke-Iqbal, I., & Gong, C. X. (2008). Decreased glucose transporters correlate to abnormal hyperphosphorylation of tau in Alzheimer disease. *FEBS letters*, *582*(2), 359-364.

Long, J., Ma, J., Luo, C., Mo, X., Sun, L., Zang, W., & Liu, J. (2009). Comparison of two methods for assaying complex I activity in mitochondria isolated from rat liver, brain and heart. *Life sciences*, *85*(7-8), 276-280.

López-Grueso, R., Gambini, J., Abdelaziz, K. M., Monleón, D., Díaz, A., El Alami, M., ... & Viña, J. (2014). Early, but not late onset estrogen replacement therapy prevents oxidative stress and metabolic alterations caused by ovariectomy. *Antioxidants & redox signaling*, *20*(2), 236-246.

Lunenfeld, B., & Stratton, P. (2013). The clinical consequences of an ageing world and preventive

strategies. *Best Practice & Research Clinical Obstetrics & Gynaecology*, *27*(5), 643-659.

Luo, C., Long, J., & Liu, J. (2008). An improved spectrophotometric method for a more specific and accurate assay of mitochondrial complex III activity. *Clinica Chimica Acta*, *395*(1-2), 38-41.

Maffucci, J. A., & Gore, A. C. (2006). Age-related changes in hormones and their receptors in animal models of female reproductive senescence. In *Handbook of models for human aging* (pp. 533-552). Elsevier Inc..

Mamelak, M. (2012). Sporadic Alzheimer's disease: the starving brain. *Journal of Alzheimer's Disease*, *31*(3), 459-474.

Mamelak, M. (2017). Energy and the Alzheimer brain. *Neuroscience & Biobehavioral Reviews*, *75*, 297-313.

Margolis, K. L., Bonds, D. E., Rodabough, R. J., Tinker, L., Phillips, L. S., Allen, C., ... & Howard, B. V. (2004). Effect of oestrogen plus progestin on the incidence of diabetes in postmenopausal women: results from the Women's Health Initiative Hormone Trial. *Diabetologia*, *47*(7), 1175-1187.

Martins, R. N., Harper, C. G., Stokes, G. B., & Masters, C. L. (1986)a. Increased cerebral glucose-6-phosphate dehydrogenase activity in Alzheimer's disease may reflect oxidative stress. *Journal of neurochemistry*, *46*(4), 1042-1045.

Martins, R. N., Stokes, G. B., & Masters, C. L. (1986)b. Regulation of liver and brain hexose monophosphate dehydrogenases by insulin and dietary intake in the female rat. *Molecular and cellular biochemistry*, *70*(2), 169-175.

Masters, C. L., Bateman, R., Blennow, K., Rowe, C. C., Sperling, R. A., Cummings, J. L. (2015). Alzheimer's disease. *Nature Reviews Disease Primers,* 1:15056

Mastroeni, D., Khdour, O. M., Delvaux, E., Nolz, J., Olsen, G., Berchtold, N., ... & Coleman, P. D. (2017). Nuclear but not mitochondrial-encoded oxidative phosphorylation genes are altered in aging, mild cognitive impairment, and Alzheimer's disease. *Alzheimer's & Dementia*, *13*(5), 510-519.

Mastrogiacomo, F., Lindsay, J. G., Bettendorff, L., Rice, J., & Kish, S. J. (1996). Brain protein and α-ketoglutarate dehydrogenase complex activity in alzheimer-s disease. *Annals of Neurology: Official Journal of the American Neurological Association and the Child Neurology Society*, *39*(5), 592-598.

Mastrogiacomo, F., Bergeron, C., & Kish, S. J. (1993). Brain α-Ketoglutarate Dehydrotenase Complex Activity in Alzheimer's Disease. *Journal of neurochemistry*, *61*(6), 2007-2014.

Matafome, P., Louro, T., Rodrigues, L., Crisostomo, J., Nunes, E., Amaral, C., ... & Seica, R. (2011). Metformin and atorvastatin combination further protect the liver in type 2 diabetes with hyperlipidaemia. *Diabetes/metabolism research and reviews*, *27*(1), 54-62.

McFarland, N. R., Burdett, T., Desjardins, C. A., Frosch, M. P., & Schwarzschild, M. A. (2013). Postmortem brain levels of urate and precursors in Parkinson's disease and related disorders. *Neurodegenerative Diseases*, *12*(4), 189-198.

McKenna, M. C., Dienel, G. A., Sonnewald, U., Waagepetersen, H. S., & Schousboe, A. (2012). Energy metabolism of the brain. In *Basic neurochemistry* (pp. 200-231). Academic Press.

Mechlovich, D., Amit, T., Bar-Am, O., Weinreb, O., & Youdim, M. B. (2014). Molecular targets of the multifunctional iron-chelating drug, M30, in the brains of mouse models of type 2 diabetes mellitus. *British journal of pharmacology*, *171*(24), 5636-5649.

Mergenthaler, P., Lindauer, U., Dienel, G. A., & Meisel, A. (2013). Sugar for the brain: the role of glucose in physiological and pathological brain function. *Trends in neurosciences*, *36*(10), 587-597.

Mooradian, A. D., Chung, H. C., & Shah, G. N. (1997). GLUT-1 expression in the cerebra of patients with Alzheimer's disease. *Neurobiology of aging*, *18*(5), 469-474.

Moran, C., Beare, R., Wang, W., Callisaya, M., Srikanth, V., & Alzheimer's Disease Neuroimaging

Initiative (ADNI. (2019). Type 2 diabetes mellitus, brain atrophy, and cognitive decline. *Neurology*, *92*(8), e823-e830.

Moran, C., Phan, T. G., Chen, J., Blizzard, L., Beare, R., Venn, A., ... & Pearson, S. (2013). Brain atrophy in type 2 diabetes: regional distribution and influence on cognition. *Diabetes care*, *36*(12), 4036-4042.

Morán, M., Moreno-Lastres, D., Marín-Buera, L., Arenas, J., Martín, M. A., & Ugalde, C. (2012). Mitochondrial respiratory chain dysfunction: implications in neurodegeneration. *Free Radical Biology and Medicine*, *53*(3), 595-609.

Moreira, P. I. (2012). Alzheimer's disease and diabetes: an integrative view of the role of mitochondria, oxidative stress, and insulin. *Journal of Alzheimer's Disease*, *30*(s2), S199-S215.

Moreira, P. I. (2018). Sweet mitochondria: A shortcut to alzheimer's disease. *Journal of Alzheimer's Disease*, *62*(3), 1391-1401.

Moreira, P. I., Carvalho, C., Zhu, X., Smith, M. A., & Perry, G. (2010). Mitochondrial dysfunction is a trigger of Alzheimer's disease pathophysiology. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, *1802*(1), 2-10.

Moreira, P. I., Santos, M. S., Moreno, A. M., Seiça, R., & Oliveira, C. R. (2003). Increased vulnerability of brain mitochondria in diabetic (Goto-Kakizaki) rats with aging and amyloid-β exposure. *Diabetes*, *52*(6), 1449-1456.

Moreira, T., Malec, E., Östenson, C. G., Efendic, S., & Liljequist, S. (2007). Diabetic type II Goto-Kakizaki rats show progressively decreasing exploratory activity and learning impairments in fixed and progressive ratios of a lever-press task. *Behavioural brain research*, *180*(1), 28-41.

Morris, A. A. M. (2005). Cerebral ketone body metabolism. *Journal of inherited metabolic disease*, *28*(2), 109-121.

Mosconi, L. (2005). Brain glucose metabolism in the early and specific diagnosis of Alzheimer's disease. *European journal of nuclear medicine and molecular imaging*, *32*(4), 486-510.

Mosconi, L., Berti, V., Guyara-Quinn, C., McHugh, P., Petrongolo, G., Osorio, R. S., ... & Brinton, R.D. (2017). Perimenopause and emergence of an Alzheimer's bioenergetic phenotype in brain and periphery. *PloS one*, *12*(10), e0185926

Movassat, J., Bailbé, D., Lubrano-Berthelier, C., Picarel-Blanchot, F., Bertin, E., Mourot, J., & Portha, B. (2008). Follow-up of GK rats during prediabetes highlights increased insulin action and fat deposition despite low insulin secretion. *American Journal of Physiology-Endocrinology and Metabolism*, *294*(1), E168-E175.

Munujos, P., Collcanti, J., Gonzalezsastre, F., & Gella, F. J. (1993). Assay of succinate dehydrogenase activity by a colorimetric-continuous method using iodonitrotetrazolium chloride as electron acceptor. *Analytical biochemistry*, *212*(2), 506-509.

Nakayama, H., Tokubuchi, I., Wada, N., Tsuruta, M., Ohki, T., Oshige, T., ... & Matsuo, Y. (2015). Age-related changes in the diurnal variation of ketogenesis in patients with type 2 diabetes and relevance to hypoglycemic medications. *Endocrine journal*, *62*(3), 235-241.

Nakhaee, A., Bokaeian, M., Akbarzadeh, A., & Hashemi, M. (2010). Sodium tungstate attenuate oxidative stress in brain tissue of streptozotocin-induced diabetic rats. *Biological trace element research*, *136*(2), 221-231.

Nebel, R. A., Aggarwal, N. T., Barnes, L. L., Gallagher, A., Goldstein, J. M., Kantarci, K., ... & Maki, P. M. (2018). Understanding the impact of sex and gender in Alzheimer's disease: a call to action. *Alzheimer's & Dementia*, *14*(9), 1171-1183.

Neth, B. J., & Craft, S. (2017). Insulin resistance and Alzheimer's disease: bioenergetic linkages. *Frontiers in aging neuroscience*, *9*, 345.

Newman, J. C., & Verdin, E. (2014). Ketone bodies as signaling metabolites. *Trends in Endocrinology & Metabolism*, *25*(1), 42-52.

Newman, M., Kretzschmar, D., Khan, I., Chen, M., Verdile, G., & Lardelli, M. (2017). Animal Models of Alzheimer's Disease. In *Animal models for the study of human disease* (pp. 1031-1085). Academic Press.

Niatsetskaya, Z., Basso, M., Speer, R. E., McConoughey, S. J., Coppola, G., Ma, T. C., & Ratan, R. R. (2010). HIF prolyl hydroxylase inhibitors prevent neuronal death induced by mitochondrial toxins: therapeutic implications for Huntington's disease and Alzheimer's disease. *Antioxidants & redox signaling*, *12*(4), 435-443.

Nulton-Persson, A. C., & Szweda, L. I. (2001). Modulation of mitochondrial function by hydrogen peroxide. *Journal of Biological Chemistry*, *276*(26), 23357-23361.

Nunomura, A., Perry, G., Aliev, G., Hirai, K., Takeda, A., Balraj, E. K., ... & Chiba, S. (2001). Oxidative damage is the earliest event in Alzheimer disease. *Journal of Neuropathology & Experimental Neurology*, *60*(8), 759-767.

Observatório da Diabetes. (2016). *Diabetes: Factos e Números – O Ano de 2015 – Relatório Anual do Observatório Nacional da Diabetes.* Lisboa: Letra solúvel.

Öge, A., Sezer, E. D., Özgönül, M., Bayraktar, F., & Sözmen, E. Y. (2003). The effects of estrogen and raloxifene treatment on the antioxidant enzymes and nitrite-nitrate levels in brain cortex of ovariectomized rats. *Neuroscience letters*, *338*(3), 217-220.

Osellame, L. D., Blacker, T. S., & Duchen, M. R. (2012). Cellular and molecular mechanisms of mitochondrial function. *Best practice & research Clinical endocrinology & metabolism*, *26*(6), 711-723.

Ott, A., Stolk, R. P., Van Harskamp, F., Pols, H. A. P., Hofman, A., & Breteler, M. M. B. (1999). Diabetes mellitus and the risk of dementia: The Rotterdam Study. *Neurology*, *53*(9), 1937-1937.

Owen, O. E., Kalhan, S. C., & Hanson, R. W. (2002). The key role of anaplerosis and cataplerosis for citric acid cycle function. *Journal of Biological Chemistry*, *277*(34), 30409-30412.

Pal, K., Mukadam, N., Petersen, I., & Cooper, C. (2018). Mild cognitive impairment and progression to dementia in people with diabetes, prediabetes and metabolic syndrome: a systematic review and meta-analysis. *Social psychiatry and psychiatric epidemiology*, *53*(11), 1149-1160.

Palmer, A. M. (1999). The activity of the pentose phosphate pathway is increased in response to oxidative stress in Alzheimer's disease. *Journal of neural transmission*, *106*(3-4), 317-328.

Panigrahy, S. K., Bhatt, R., & Kumar, A. (2017). Reactive oxygen species: sources, consequences and targeted therapy in type 2 diabetes. *Journal of drug targeting*, *25*(2), 93-101.

Parker, W. D., Parks, J., Filley, C. M., & Kleinschmidt-DeMasters, B. K. (1994). Electron transport chain defects in Alzheimer's disease brain. *Neurology*, *44*(6), 1090-1090.

Pawlosky, R. J., Kemper, M. F., Kashiwaya, Y., King, M. T., Mattson, M. P., & Veech, R. L. (2017). Effects of a dietary ketone ester on hippocampal glycolytic and tricarboxylic acid cycle intermediates and amino acids in a 3xTg AD mouse model of Alzheimer's disease. *Journal of neurochemistry*, *141*(2), 195-207.

Pedrós, I., Petrov, D., Allgaier, M., Sureda, F., Barroso, E., Beas-Zarate, C., ... & Folch, J. (2014). Early alterations in energy metabolism in the hippocampus of APPswe/PS1dE9 mouse model of Alzheimer's disease. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, *1842*(9), 1556-1566.

Peng, Y., Liu, J., Shi, L., Tang, Y., Gao, D., Long, J., & Liu, J. (2016). Mitochondrial dysfunction precedes depression of AMPK/AKT signaling in insulin resistance induced by high glucose in primary cortical neurons. *Journal of neurochemistry*, *137*(5), 701-713.

Petrov, D., Pedrós, I., Artiach, G., Sureda, F. X., Barroso, E., Pallàs, M., ... & Vazquez-Carrera, M.

(2015). High-fat diet-induced deregulation of hippocampal insulin signaling and mitochondrial homeostasis deficiences contribute to Alzheimer disease pathology in rodents. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, *1852*(9), 1687-1699.

Pintana, H., Apaijai, N., Kerdphoo, S., Pratchayasakul, W., Sripetchwandee, J., Suntornsaratoon, P., ... & Chattipakorn, S. C. (2017). Hyperglycemia induced the Alzheimer's proteins and promoted loss of synaptic proteins in advanced-age female Goto-Kakizaki (GK) rats. *Neuroscience letters*, *655*, 41-45.

Portha, B. (2005). Programmed disorders of β-cell development and function as one cause for type 2 diabetes? The GK rat paradigm. *Diabetes/metabolism research and reviews*, *21*(6), 495-504.

Portha, B., Giroix, M. H., Tourrel-Cuzin, C., Le-Stunff, H., & Movassat, J. (2012). The GK rat: a prototype for the study of non-overweight type 2 diabetes. In *Animal Models in Diabetes Research* (pp. 125-159). Humana Press, Totowa, NJ.

Pratap, U. P., Patil, A., Sharma, H. R., Hima, L., Chockalingam, R., Hariharan, M. M., ... & ThyagaRajan, S. (2016). Estrogen-induced neuroprotective and anti-inflammatory effects are dependent on the brain areas of middle-aged female rats. *Brain research bulletin*, *124*, 238-253.

Proia, P., Di Liegro, C., Schiera, G., Fricano, A., & Di Liegro, I. (2016). Lactate as a metabolite and a regulator in the central nervous system. *International journal of molecular sciences*, *17*(9), 1450.

Pu, D., Zhao, Y., Chen, J., Lv, A., Zhu, S., Luo, C., ... & Xiao, Q. (2018). Protective effects of sulforaphane on cognitive impairments and ad-like lesions in diabetic mice are associated with the upregulation of Nrf2 transcription activity. *Neuroscience*, *381*, 35-45.

Puchalska, P., & Crawford, P. A. (2017). Multi-dimensional roles of ketone bodies in fuel metabolism, signaling, and therapeutics. *Cell metabolism*, *25*(2), 262-284.

Ralser, M., Wamelink, M. M., Kowald, A., Gerisch, B., Heeren, G., Struys, E. A., ... & Krobitsch, S. (2007). Dynamic rerouting of the carbohydrate flux is key to counteracting oxidative stress. *Journal of biology*, *6*(4), 10.

Ray, P. D., Huang, B. W., & Tsuji, Y. (2012). Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cellular signalling*, *24*(5), 981-990.

Raza, H., John, A., & Howarth, F. C. (2015). Increased oxidative stress and mitochondrial dysfunction in zucker diabetic rat liver and brain. *Cellular Physiology and Biochemistry*, *35*(3), 1241-1251.

Reagan, L. P., Magariños, A. M., Yee, D. K., Swzeda, L. I., Van Bueren, A., McCall, A. L., & McEwen, B. S. (2000). Oxidative stress and HNE conjugation of GLUT3 are increased in the hippocampus of diabetic rats subjected to stress. *Brain research*, *862*(1-2), 292-300.

Rettberg, J. R., Dang, H., Hodis, H. N., Henderson, V. W., John, J. A. S., Mack, W. J., & Brinton, R. D. (2016). Identifying postmenopausal women at risk for cognitive decline within a healthy cohort using a panel of clinical metabolic indicators: potential for detecting an at-Alzheimer's risk metabolic phenotype. *Neurobiology of aging*, *40*, 155-163.

Rettberg, J. R., Yao, J., & Brinton, R. D. (2014). Estrogen: a master regulator of bioenergetic systems in the brain and body. *Frontiers in neuroendocrinology*, *35*(1), 8-30.

Rex Sheu, K. F., Kim, Y. T., Blass, J. P., & Weksler, M. E. (1985). An immunochemical study of the pyruvate dehydrogenase deficit in Alzheimer's disease brain. *Annals of Neurology: Official Journal of the American Neurological Association and the Child Neurology Society*, *17*(5), 444-449.

Riedel, B. C., Thompson, P. M., & Brinton, R. D. (2016). Age, APOE and sex: triad of risk of Alzheimer's disease. *The Journal of steroid biochemistry and molecular biology*, *160*, 134-147.

Riske, L., Thomas, R. K., Baker, G. B., & Dursun, S. M. (2017). Lactate in the brain: an update on its relevance to brain energy, neurons, glia and panic disorder. *Therapeutic advances in psychopharmacology*, *7*(2), 85-89.

Roberts, R. O., Knopman, D. S., Cha, R. H., Mielke, M. M., Pankratz, V. S., Boeve, B. F., ... & Lowe,

V. J. (2014). Diabetes and elevated hemoglobin A1c levels are associated with brain hypometabolism but not amyloid accumulation. *Journal of Nuclear Medicine*, *55*(5), 759-764.

Russell, R. L., Siedlak, S. L., Raina, A. K., Bautista, J. M., Smith, M. A., & Perry, G. (1999). Increased neuronal glucose-6-phosphate dehydrogenase and sulfhydryl levels indicate reductive compensation to oxidative stress in Alzheimer disease. *Archives of Biochemistry and Biophysics*, *370*(2), 236-239.

Salminen, A., Haapasalo, A., Kauppinen, A., Kaarniranta, K., Soininen, H., & Hiltunen, M. (2015). Impaired mitochondrial energy metabolism in Alzheimer's disease: Impact on pathogenesis via disturbed epigenetic regulation of chromatin landscape. *Progress in neurobiology*, *131*, 1-20.

Santos, M. S., Duarte, A. I., Matos, M. J., Proença, T., Seiça, R., & Oliveira, C. R. (2000). Synaptosomes isolated from Goto-Kakizaki diabetic rat brain exhibit increased resistance to oxidative stress: role of vitamin E. *Life sciences*, *67*(25), 3061-3073.

Santos, M. S., Santos, D. L., Palmeira, C. M., Seiça, R., Moreno, A. J., & Oliveira, C. R. (2001). Brain and liver mitochondria isolated from diabetic Goto-Kakizaki rats show different susceptibility to induced oxidative stress. *Diabetes/metabolism research and reviews*, *17*(3), 223-230.

Santos, R. X., Correia, S. C., Alves, M. G., Oliveira, P. F., Cardoso, S., Carvalho, C., ... & Moreira, P. I. (2014). Mitochondrial quality control systems sustain brain mitochondrial bioenergetics in early stages of type 2 diabetes. *Molecular and cellular biochemistry*, *394*(1-2), 13-22.

Sebastião, I., Candeias, E., Santos, M. S., de Oliveira, C. R., Moreira, P. I., & Duarte, A. I. (2014). Insulin as a bridge between type 2 diabetes and Alzheimer disease–how anti-diabetics could be a solution for dementia. *Frontiers in endocrinology*, *5*, 110.

Sedmak, J. J., & Grossberg, S. E. (1977). A rapid, sensitive, and versatile assay for protein using Coomassie brilliant blue G250. *Analytical biochemistry*, *79*(1-2), 544-552.

Serrano-Pozo, A., Frosch, M. P., Masliah, E., & Hyman, B. T. (2011). Neuropathological alterations in Alzheimer disease. *Cold Spring Harbor perspectives in medicine*, *1*(1), a006189.

Shah, K., DeSilva, S., & Abbruscato, T. (2012). The role of glucose transporters in brain disease: diabetes and Alzheimer's disease. *International journal of molecular sciences*, *13*(10), 12629-12655.

Shen, Y., Liu, F., Li, Q., Tang, J., Zheng, T., Lu, F., ... & Jia, W. (2012). The gonadal hormone regulates the plasma lactate levels in type 2 diabetes treated with and without metformin. *Diabetes technology & therapeutics*, *14*(6), 469-474.

Shi, X., Lu, X. G., Zhan, L. B., Qi, X., Liang, L. N., Hu, S. Y., ... & Zhang, F. L. (2011). The effects of the Chinese medicine ZiBu PiYin recipe on the hippocampus in a rat model of diabetes-associated cognitive decline: a proteomic analysis. *Diabetologia*, *54*(7), 1888-1899.

Sickmann, H. M., Waagepetersen, H. S., Schousboe, A., Benie, A. J., & Bouman, S. D. (2010). Obesity and type 2 diabetes in rats are associated with altered brain glycogen and amino-acid homeostasis. *Journal of Cerebral Blood Flow & Metabolism*, *30*(8), 1527-1537.

Siebert, C., Kolling, J., Scherer, E. B., Schmitz, F., da Cunha, M. J., Mackedanz, V., ... & Wyse, A. T. (2014). Effect of physical exercise on changes in activities of creatine kinase, cytochrome c oxidase and ATP levels caused by ovariectomy. *Metabolic brain disease*, *29*(3), 825-835.

Silverman, D. H., Small, G. W., Chang, C. Y., Lu, C. S., de Aburto, M. A. K., Chen, W., ... & Schapiro, M. B. (2001). Positron emission tomography in evaluation of dementia: regional brain metabolism and long-term outcome. *Jama*, *286*(17), 2120-2127.

Simpson, I. A., Chundu, K. R., Davies-Hill, T., Honer, W. G., & Davies, P. (1994). Decreased concentrations of GLUT1 and GLUT3 glucose transporters in the brains of patients with Alzheimer's disease. *Annals of Neurology: Official Journal of the American Neurological Association and the Child Neurology Society*, *35*(5), 546-551.

Sims-Robinson, C., Kim, B., Rosko, A., & Feldman, E. L. (2010). How does diabetes accelerate

Alzheimer disease pathology?. *Nature Reviews Neurology*, *6*(10), 551.

Singh, A., Kukreti, R., Saso, L., & Kukreti, S. (2019). Oxidative stress: a key modulator in neurodegenerative diseases. *Molecules*, *24*(8), 1583.

Solfrizzi, V., Scafato, E., Capurso, C., D'Introno, A., Colacicco, A. M., Frisardi, V., ... & Galluzzo, L. (2011). Metabolic syndrome, mild cognitive impairment, and progression to dementia. The Italian Longitudinal Study on Aging. *Neurobiology of aging*, *32*(11), 1932-1941.

Sorbi, S., Bird, E. D., & Blass, J. P. (1983). Decreased pyruvate dehydrogenase complex activity in Huntington and Alzheimer brain. *Annals of neurology*, *13*(1), 72-78.

Soucek, T., Cumming, R., Dargusch, R., Maher, P., & Schubert, D. (2003). The regulation of glucose metabolism by HIF-1 mediates a neuroprotective response to amyloid beta peptide. *Neuron*, *39*(1), 43-56.

Spillane, J. A., Curzon, G., Meier-Ruge, W., White, P., Goodhardt, M. J., Iwangoff, P., ... & Bowen, D. M. (1979). Accelerated ageing or selective neuronal loss as an important cause of dementia?. *The Lancet*, *313*(8106), 11-14.

Stanton, R. C. (2012). Glucose-6-phosphate dehydrogenase, NADPH, and cell survival. *IUBMB life*, *64*(5), 362-369.

Starkov, A. A., Fiskum, G., Chinopoulos, C., Lorenzo, B. J., Browne, S. E., Patel, M. S., & Beal, M. F. (2004). Mitochondrial α-ketoglutarate dehydrogenase complex generates reactive oxygen species. *Journal of Neuroscience*, *24*(36), 7779-7788.

Stincone, A., Prigione, A., Cramer, T., Wamelink, M. M., Campbell, K., Cheung, E., ... & Keller, M. A. (2015). The return of metabolism: biochemistry and physiology of the pentose phosphate pathway. *Biological Reviews*, *90*(3), 927-963.

Stocchi, V., Magnani, M., Cucchiarini, L., Novelli, G., Dallapiccola, B., Opitz, J. M., & Reynolds, J. F. (1985). Red blood cell adenine nucleotides abnormalities in Down syndrome. *American journal of medical genetics*, *20*(1), 131-135.

Studzinski, C. M., MacKay, W. A., Beckett, T. L., Henderson, S. T., Murphy, M. P., Sullivan, P. G., & Burnham, W. M. (2008). Induction of ketosis may improve mitochondrial function and decrease steady-state amyloid-β precursor protein (APP) levels in the aged dog. *Brain research*, *1226*, 209-217.

Sultana, R., Piroddi, M., Galli, F., & Butterfield, D. A. (2008). Protein levels and activity of some antioxidant enzymes in hippocampus of subjects with amnestic mild cognitive impairment. *Neurochemical research*, *33*(12), 2540-2546.

Szablewski, L. (2017). Glucose transporters in brain: in health and in Alzheimer's disease. *Journal of Alzheimer's Disease*, *55*(4), 1307-1320.

Takahashi, S., Izawa, Y., & Suzuki, N. (2012). Astroglial pentose phosphate pathway rates in response to high-glucose environments. *ASN neuro*, *4*(2), AN20120002.

Taskiran, D., Kutay, F. Z., Sozmen, E., & Pogun, S. (1997). Sex differences in nitrite/nitrate levels and antioxidant defense in rat brain. *Neuroreport*, *8*(4), 881-884.

Thimmulappa, R. K., Mai, K. H., Srisuma, S., Kensler, T. W., Yamamoto, M., & Biswal, S. (2002). Identification of Nrf2-regulated genes induced by the chemopreventive agent sulforaphane by oligonucleotide microarray. *Cancer research*, *62*(18), 5196-5203.

Tisdale, H. D. (1967). [39] Preparation and properties of succinic—cytochrome c reductase (complex II–III). In *Methods in enzymology* (Vol. 10, pp. 213-215). Academic Press.

Tiwari, V., & Patel, A. B. (2014). Pyruvate Carboxylase and Pentose Phosphate Fluxes are Reduced in AβPP-PS1 Mouse Model of Alzheimer's Disease: A 13C NMR Study. *Journal of Alzheimer's Disease*, *41*(2), 387-399.

Tönnies, E., & Trushina, E. (2017). Oxidative stress, synaptic dysfunction, and Alzheimer's disease. *Journal of Alzheimer's Disease*, *57*(4), 1105-1121.

Tretter, L., & Adam-Vizi, V. (2005). Alpha-ketoglutarate dehydrogenase: a target and generator of oxidative stress. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *360*(1464), 2335-2345.

Ulusu, N. N., Sahilli, M., Avci, A., Canbolat, O., Ozansoy, G., Ari, N., ... & Karasu, Ç. (2003). Pentose phosphate pathway, glutathione-dependent enzymes and antioxidant defense during oxidative stress in diabetic rodent brain and peripheral organs: effects of stobadine and vitamin E. *Neurochemical Research*, *28*(6), 815-823.

Valotassiou, V., Malamitsi, J., Papatriantafyllou, J., Dardiotis, E., Tsougos, I., Psimadas, D., ... & Georgoulias, P. (2018). SPECT and pet imaging in Alzheimer's disease. *Annals of nuclear medicine*, *32*(9), 583-593.

van Gijsel-Bonnello, M., Baranger, K., Benech, P., Rivera, S., Khrestchatisky, M., de Reggi, M., & Gharib, B. (2017). Metabolic changes and inflammation in cultured astrocytes from the 5xFAD

mouse model of Alzheimer's disease: alleviation by pantethine. *PloS one*, *12*(4), e0175369.

van Hall, G., Strømstad, M., Rasmussen, P., Jans, Ø., Zaar, M., Gam, C., ... & Nielsen, H. B. (2009). Blood lactate is an important energy source for the human brain. *Journal of Cerebral Blood Flow & Metabolism*, *29*(6), 1121-1129.

Verdile, G., Fuller, S. J., & Martins, R. N. (2015). The role of type 2 diabetes in neurodegeneration. *Neurobiology of disease*, *84*, 22-38.

Viticchi, G., Falsetti, L., Buratti, L., Luzzi, S., Bartolini, M., Acciarri, M. C., ... & Silvestrini, M. (2015). Metabolic syndrome and cerebrovascular impairment in Alzheimer's disease. *International journal of geriatric psychiatry*, *30*(12), 1164-1170.

Wallace, D. C., Fan, W., & Procaccio, V. (2010). Mitochondrial energetics and therapeutics. *Annual Review of Pathological Mechanical Disease*, *5*, 297-348.

Wan, G. H., Tsai, S. C., & Chiu, D. T. Y. (2002). Decreased blood activity of glucose-6-phosphate dehydrogenase associates with increased risk for diabetes mellitus. *Endocrine*, *19*(2), 191-195.

Wang, J., Markesbery, W. R., & Lovell, M. A. (2006). Increased oxidative damage in nuclear and mitochondrial DNA in mild cognitive impairment. *Journal of neurochemistry*, *96*(3), 825-832.

Wang, Y. W., Sun, G. D., Sun, J., Liu, S. J., Wang, J., Xu, X. H., & Miao, L. N. (2013). Spontaneous type 2 diabetic rodent models. *Journal of diabetes research*, *2013*.

Willette, A. A., Bendlin, B. B., Starks, E. J., Birdsill, A. C., Johnson, S. C., Christian, B. T., ... & Jonaitis, E. M. (2015). Association of insulin resistance with cerebral glucose uptake in late middle–aged adults at risk for Alzheimer disease. *JAMA neurology*, *72*(9), 1013-1020.

Willette, A. A., Modanlo, N., & Kapogiannis, D. (2015). Insulin resistance predicts medial temporal hypermetabolism in mild cognitive impairment conversion to Alzheimer disease. *Diabetes*, *64*(6), 1933-1940.

Włodarek, D. (2019). Role of ketogenic diets in neurodegenerative diseases (Alzheimer's Disease and Parkinson's Disease). *Nutrients*, *11*(1), 169.

Wojsiat, J., Zoltowska, K. M., Laskowska-Kaszub, K., & Wojda, U. (2018). Oxidant/antioxidant imbalance in Alzheimer's disease: therapeutic and diagnostic prospects. *Oxidative medicine and cellular longevity*, *2018*.

Yan, H., Yang, W., Zhou, F., Li, X., Pan, Q., Shen, Z., ... & Liu, W. (2019). Estrogen improves insulin sensitivity and suppresses gluconeogenesis via the transcription factor Foxo1. *Diabetes*, *68*(2), 291-304.

Yao, J., Chen, S., Mao, Z., Cadenas, E., & Brinton, R. D. (2011). 2-Deoxy-D-glucose treatment induces ketogenesis, sustains mitochondrial function, and reduces pathology in female mouse model of Alzheimer's disease. *PloS one*, *6*(7), e21788.

Yao, J., Hamilton, R. T., Cadenas, E., & Brinton, R. D. (2010). Decline in mitochondrial bioenergetics and shift to ketogenic profile in brain during reproductive senescence. *Biochimica et Biophysica Acta (BBA)-General Subjects*, *1800*(10), 1121-1126.

Yao, J., Irwin, R. W., Zhao, L., Nilsen, J., Hamilton, R. T., & Brinton, R. D. (2009). Mitochondrial bioenergetic deficit precedes Alzheimer's pathology in female mouse model of Alzheimer's disease. *Proceedings of the National Academy of Sciences*, *106*(34), 14670-14675.

Yin, F., Yao, J., Sancheti, H., Feng, T., Melcangi, R. C., Morgan, T. E., ... & Brinton, R. D. (2015). The perimenopausal aging transition in the female rat brain: decline in bioenergetic systems and synaptic plasticity. *Neurobiology of aging*, *36*(7), 2282-2295.

Yin, J. X., Maalouf, M., Han, P., Zhao, M., Gao, M., Dharshaun, T., ... & Reiman, E. M. (2016). Ketones block amyloid entry and improve cognition in an Alzheimer's model. *Neurobiology of aging*, *39*, 25-37.

Zhang, S. Y., Ji, S. X., Bai, X. M., Yuan, F., Zhang, L. H., & Li, J. (2019). L-3-n-butylphthalide attenuates cognitive deficits in db/db diabetic mice. *Metabolic brain disease*, *34*(1), 309-318.

Zhao, L., Mao, Z., Woody, S. K., & Brinton, R. D. (2016). Sex differences in metabolic aging of the brain: insights into female susceptibility to Alzheimer's disease. *Neurobiology of aging*, *42*, 69-79.

Zheng, H., Zheng, Y., Zhao, L., Chen, M., Bai, G., Hu, Y., ... & Gao, H. (2017). Cognitive decline in type 2 diabetic db/db mice may be associated with brain region-specific metabolic disorders. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, *1863*(1), 266-273.

Zhu, H., Itoh, K., Yamamoto, M., Zweier, J. L., & Li, Y. (2005). Role of Nrf2 signaling in regulation of antioxidants and phase 2 enzymes in cardiac fibroblasts: protection against reactive oxygen and nitrogen species-induced cell injury. *FEBS letters*, *579*(14), 3029-3036.

Zilberter, Y., & Zilberter, M. (2017). The vicious circle of hypometabolism in neurodegenerative diseases: Ways and mechanisms of metabolic correction. *Journal of neuroscience research*, *95*(11), 2217-2235.

World Health Organization. (2016). Global report on diabetes. World Health Organization.

World Heath Organization. (2018). *Aging And Health.* Retrieved from: https://www.who.int/news-room/fact-sheets/detail/ageing-and-health (viewed: January 30, 2019).

World Heath Organization. (2019). *Dementia.* Retrieved from: https://www.who.int/news-room/fact-sheets/detail/dementia (viewed: May 17, 2019).