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The Amazing Anti-Type 2 Diabetic Drugs in Neurodegeneration: the impact of Exendin-4, Liraglutide and Linagliptin in Type 2 diabetes, Alzheimer disease and Parkinson disease

Tese no âmbito do Doutoramento em Biologia Experimental e Biomedicina (BEB)/área Neurociências e Doença, orientada pela Doutora Ana Isabel Marques Duarte e pela Professora Doutora Paula Isabel da Silva Moreira e apresentada ao Instituto de Investigação Interdisciplinar (IIIUC)/Centro de Neurociências e Biologia Celular (CNC), Universidade de Coimbra.

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THE AMAZING ANTI-TYPE 2 DIABETIC DRUGS IN NEURODEGENERATION: THE IMPACT OF EXENDIN-4, LIRAGLUTIDE AND LINAGLIPTIN IN TYPE 2 DIABETES, ALZHEIMER DISEASE AND PARKINSON DISEASE

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Index

Abbreviations

3xTg-AD - Triple transgenic mouse model of Alzheimer disease

6-OHDA - 6-hydroxydopamine

8-OHdG - 8-hydroxy-2-deoxyguanosine

AcAc - Acetoacetic acid

Ach – Acetylcholine

AChE – Acetylcholinesterase

AChEIs - Acetyl-cholinesterase inhibitors

AD - Alzheimer disease

ADP - Adenosine diphosphate

AEC - Adenylate energy charge

AGEs - Advanced glycation end products

Akt (or PKB) – Protein kinase B

ALP - Alkaline phosphatase

AMP - Adenosine monophosphate

AMPA - Glutamate α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AMPK - AMP-activated protein kinase

ANOVA - Analysis of variance

AP - Area postrema

APOE - Apolipoprotein E

APP - Amyloid precursor protein

Atg7 - Autophagy-related protein

ATP - Adenosine triphosphate

AUC - Area under the curve

Aβ – Amyloid beta

BACE - β-secretase

Bad - Bcl2-associated death promoter

Bax - Bcl-2-associated X protein; Bcl-2-like protein 4

BBB - Blood-brain barrier

BCAA - Brain branched-chain amino acid

Bcl2 - B-cell lymphoma 2

Bcl-xL - B-cell lymphoma-extra large

BDNF - Brain-derived neurotrophic factor

BHT - β-hydroxytoluene

BOH - 3-hydroxybutyric acid; β-Hydroxybutyric acid; conjugate base is β-hydroxybutyrate

BPM – Beats/min

BSA - Bovine serum albumin

Ca^{2+} - Calcium

cAMP - Cyclic adenosine monophosphate

Cdk5 - Cyclin-dependent kinase 5

cGMP - Cyclic guanosine monophosphate

CHC - α-cyano-4-hydroxycinnamate

CHCHD2 - Coiled-coil-helix-coiled-coil-helix domain containing 2

CNS – Central nervous system

CPCs - Cardiac progenitor cells

CREB - Cyclic AMP response element binding protein

CRP - C-Reactive Protein

CSF - Cerebrospinal fluid

Cu/Zn SOD - Copper/zinc superoxide dismutase

CVD - Cardiovascular disease

DA – Dopamine

DAB – Diaminobenzidine

DAT-SPECT - Dopamine transporter imaging by single photon emission tomography

DCPIP – Dichlorophenolindophenol

DHEA – Dehydroepiandrosterone

DJ-1 - Protein deglycase DJ-1; Parkinson disease protein 7

DNPH - 2,4-dinitrophenylhydrazine

DPP-4 - Dipeptidyl peptidase-4

DPP-4i - Dipeptidyl peptidase-4 inhibitor

Drp1 - Dynamin-1-like protein; Dlp1

DTNB - Ellman's reagent; 5,5'-dithiobis-(2-nitrobenzoic acid)

DTT - 1,4-dithiotreitol

ECF - Enhanced chemifluorescence

EIA - Enzyme immunoassay

EIF4G1 - Eukaryotic translation initiation factor 4 gamma 1

ELISA - Enzyme-Linked Immunosorbent Assay

eNOS - Endothelial nitric oxide synthase

Epac2 - Exchange protein activated by cAMP-2

ER - Endoplasmic reticulum

ERK - Extracellular signal-regulated kinases

ERs - Estrogen receptors

ET-1 - Endothelin-1

Ex-4 - Exendin-4; exenatide

FDG - Fluorodeoxyglucose (^{18}F)

FDOPA-PET - Fluorodopa positron emission tomography

FFA - Free fatty acid

Fis1 - Mitochondrial fission 1 protein

FOXO – Forkhead box transcription factors

G6P – Glucose-6-phosphate

G6PDH - Glucose-6-phosphate-dehydrogenase

GABA - γ-aminobutyric acid

GBA - Glucocerebrosidase

GDM - Gestational *diabetes mellitus*

GDNF - Glial derived neurotrophic factor

GFAP - Glial fibrillary acidic protein

GI – Gastrointestinal

GIP - Glucose-dependent insulinotrophic polypeptide

GK - Goto-Kakizaki rat

GLP-1 - Glucagon-like peptide-1

GLP-1R - Glucagon-like peptide-1 receptor

GLUT - Glucose transporter

GRP - Gastrin-releasing peptide

GSH – Reduced glutathione

GSK-3β - Glycogen synthase kinase-3β

GTT - Glucose tolerance test

HASMC - Human aortic smooth muscle cells

HbA1c - Hemoglobin A1c; glycated hemoglobin

HD - Huntington disease

HDL - High-density lipoprotein

HFD - High fat diet

HGP - Hepatic glucose production

HIF - Hypoxia-inducible factor

HOMA-IR - Homeostasis assessment model-insulin resistance

HOMA-β - Homeostasis model assessment of β-cell function

HPLC - High performance liquid chromatography

i.m - Intramuscular

i.p. - Intraperitoneal

Iba-1 - Ionized calcium-binding adaptor molecule-1

icv – Intracerebroventricular

IDE - Insulin-degrading enzyme

IDF - International Diabetes Federation

IGF-1 - Insulin-like growth factor-1

IGF-1R - Insulin-like growth factor-1 receptor

IHC - Immunohistochemistry

IL – Interleukin

IP-2 - Intervening peptide-2

ipGTT - Intraperitoneal glucose tolerance test

IR - Insulin receptor

IRS - Insulin receptor substrate

JNK - c-Jun N-terminal kinase

K^+ATP channels - ATP-sensitive potassium channels

LAMP - Lysosomal-associated membrane protein

LBs - Lewy bodies

LC3 - Microtubule-associated protein 1A/1B-light chain 3B

LDH - Lactate dehydrogenase

LDL - Low density lipoproteins

LEAD - Liraglutide Effect and Action in Diabetes

LRRK2 - Leucine-rich repeat kinase 2

LSD - Least Significant Difference

LTD - Long-term depression

LTP - Long-term potentiation

MAPK - Mitogen-activated protein kinase

MCAO - Middle cerebral artery occlusion

MCI - Mild cognitive impairment

MCTs - Monocarboxylate transporters

MDA – Malondialdehyde

MEKK - Mammalian mitogen-activated protein kinase kinase kinase

Mfn - Mitofusin

MMSE - Mini-mental state examination

MnSOD - Manganese superoxide dismutase

MOM - Mitochondrial outer membrane

MPTP - 1-methyl-4-phenyl-1,2,3,6-tetrahydropypridine

mPTP - Mitochondrial permeability transition pore

MRI - Magnetic resonance imaging

mTOR - Mechanistic target of rapamycin

mTORC1 - mTOR Complex 1

MWM - Morris water maze test

N2a - Neuro-2a cells; mouse neuroblastoma cell line

NAD^+ - Nicotinamide adenine dinucleotide

$NADP^+$ - Nicotinamide adenine dinucleotide phosphate

NADPH - reduced form of $NADP^+$

NCD-RisC – NCD Risk Factor Collaboration

NEP - Neutral endopeptidase

NF-kB - Nuclear factor-kB

Nfr2 - Nuclear factor erythroid 2-related factor

NFT - Neurofibrillary tangles

NHS - Natural horse serum

NMDA - N-methyl-D-aspartate

NO - Nitric oxide

NPY - Neuropeptide Y

NSC - Neural stem cell

NTS - Solitary tract nucleus

OCT - Optimal cutting temperature

OGTT - Oral glucose tolerance test

OPA1 - Mitochondrial dynamin-like 120 kDa protein

OVX – Ovariectomized

PACAP - Pituitary adenylate cyclase-activating polypeptide

PBS - Phosphate buffer saline

PD - Parkinson disease

PDK1 – 3-phosphoinositide-dependent protein kinase 1

PET - Positron emission tomography

PFA – Paraformaldehyde

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

PI3K - Phosphatidylinositol 3-kinase; phosphoinositide 3-kinase

PINK1 - PTEN-induced kinase 1

PKA - Protein kinase A

PKC - Protein kinase C

PMSF - Phenylmethanesulfonyl fluoride

PPAR-γ - Peroxisome proliferator-activated receptor gamma

PPP – Pentose phosphate pathway

PRKN - Parkin ligase

PSEN - Presenilin

PTEN - Phosphatase and tensin homolog

PV – Parvalbumin

PVDF - Polyvinyl difluoride

PYY - Peptide YY

RAB39B - Ras-related protein Rab-39B

RFU - Relative fluorescence units

RIP - Receptor-interacting serine/threonine-protein kinase

ROS - Reactive oxygen species

s.c. - Subcutaneous

SD – Standard diet

SDS - Sodium dodecyl sulfate

Ser – Serine

SGLT - Sodium-glucose co-transporter

Shc - Src homology collagen

SIRT – Sirtuin

SNCA - α-synuclein

SNpc - Substantia nigra pars compacta

SPD - Sociedade Portuguesa de Diabetologia

SS – Somatostatin

STZ – Streptozotocin

SUs – Sulfonylureas

T1D – Type 1 diabetes

T2D – Type 2 diabetes

TBA - Thiobarbituric acid

TBARS - Thiobarbituric acid reactive substances

TBS - Tris-buffered saline

TCA - Trichloroacetic acid; trichloroethanoic acid; TCAA

TCA cycle - Tricarboxylic acid cycle

TEA – Triethanolamine

TFAM - Mitochondrial transcription factor A; mtFA

TH - Tyrosine hydroxylase

Thr – Threonine

TLR - Toll-like receptor

TNFα - Tumor necrosis factor α

TPP - Thiamine pyrophosphate

TrkB - Tropomyosin receptor kinase B

TZDs – Thiazolidinediones

UPS - Ubiquitin–proteasome system

VDAC – Voltage-dependent anion channel

VEGF - Vascular endothelial growth factor

VMAT2 - Vesicular monoamine transporter 2

VPS35 - Vacuolar protein sorting-associated protein 35

WHO - World Health Organization

WT – Wild type mice

ZDF - Zucker diabetic fatty rats

Abstract

Type 2 diabetes (T2D) is a noisy “silent killer”, which has already attained the status of a global pandemic, which affects millions of people spread to every corner of the world, with the aggravating that millions more are currently living with this disease without even knowing. Even so, more efforts have to be done to the awareness, understanding and treatment of T2D in order to avoid an uncontrolled developing of long-term complications that influence on disease outcome, quality of life and mortality. Indeed, T2D menace and health burden continues to rise as consistent epidemiological evidence suggests an increased risk of neurodegenerative disorders associated to T2D. Though the precise mechanisms underlying the deleterious T2D-related effects to brain structure and function remain elusive, the most plausible players include impaired insulin signaling, impaired glucose distribution and utilization, hyperglycemia, pro-inflammatory state, as well as brain insulin resistance, metabolic impairments, oxidative stress and mitochondrial dysfunction.

The work presented in this Thesis is a mean of clarifying and solidifying notions of the impact of T2D in the brain and of it association with other prevalent age-related neurodegenerative diseases, such as Alzheimer disease (AD) and Parkinson disease (PD). In addition, we addressed the sex dimorphism upon T2D and how sex-associated alterations differentially affects the risk for cognitive decline and for dementia. Furthermore, given the pathophysiological mechanisms linking T2D and neurodegeneration, we evaluated the efficacy of incretin-based therapies in the improvement of central nervous system (CNS) function associated with impairments upon T2D, aging, AD and PD.

In Chapter 3, we used the non-obese, spontaneously T2D, Goto-Kakizaki (GK) rat to compare middle-aged males and females’ hormone-mediated intracellular signaling pathways (estrogen/insulin-like growth factor-1 (IGF-1)/insulin-related signaling) in T2D brain cortices, and consequent modulation of disease outcome (oxidative stress markers and AD-like hallmarks). Overall, observations showed a sex-specific time window for efficient approaches against T2D and AD pathologies. If on the one hand, perimenopause females’ brains exhibited lower brain cholesterol, dehydroepiandrosterone (DHEA), testosterone and IGF-1 levels, particularly upon T2D, on the other hand, compensatory mechanisms based on the upkeep of estrogen, IGF-1,

and insulin receptors function and downstream signaling were responsible for the decreased levels of oxidation (thiobarbituric acid reactive substances (TBARS) and 8-hydroxy-2-deoxyguanosine (8-OHdG) levels) and AD-related (β-secretase (BACE) activity and amyloid beta ($A\beta)_{1-42}$ levels) markers.

Regarding the impact of incretin-based anti-T2D drugs, using either glucagon-like peptide-1 (GLP-1) receptor (GLP-1R) agonists or dipeptidyl peptidase-4 (DPP-4) inhibitors (DPP-4i), in Chapters 4 and 5 we evaluated the effects of the GLP-1 mimetic exendin-4 (Ex-4) in middle-aged male T2D GK rats. Data derived from Chapter 4 elucidated the impact of a chronic continuous peripheral Ex-4 therapy in brain cortical GLP-1/insulin/IGF-1 signaling, and the subsequent autophagic and cell death mechanisms (apoptosis and necroptosis) upon T2D. In Chapter 5, we evaluated the effects of Ex-4 administration on the T2D brain energetic status through the evaluation of the brain glucose uptake, glucose levels and metabolism, of the content of essential aminoacids, levels of ketone bodies, energy production, mitochondrial respiratory chain activity and dynamics. Altogether, studies with Ex-4 demonstrated a promising therapy against T2D, overcoming T2D-associated peripheral pathological hallmarks, as well as chronic complications affecting the brain, namely by rescuing of brain cortical GLP-1 and IGF-1 levels, activating brain cortical protein kinase A (PKA) and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) signaling pathways. These may in turn, reverse the brain glucose dysmetabolism (by reduction of brain glucose levels and 3-hydroxybutyric acid (BOH) concentration, and increase of synaptic glucose uptake, enzymatic activities and energy charge (adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP) levels)), and promote autophagy (through the mechanistic target of rapamycin (mTOR) activation, PI3K class III, microtubule-associated protein 1A/1B-light chain 3B (LC3) II, autophagy-related protein (Atg)7, p62 and lysosomal-associated membrane protein (LAMP-1) expressions) and mitophagy (Parkin expression), ultimately exerting an anti-apoptotic effect (caspase-1, caspase-3 and caspase-8-like activities and mitochondrial cytochrome c and B-cell lymphoma 2 (Bcl2) levels).

Chapter 6 focused on the T2D- and/or aging-induced neural alterations in the brain areas of the dopaminergic system, modulating the neuronal degeneration that characterizes diseases such as PD, and on the potential of the DPP-4i, linagliptin, as a therapy to revert these pathological changes in middle-aged, high fat diet (HFD)-

induced T2D mice. Observations from these studies revealed an age-associated loss of parvalbumin (PV) interneurons (involved in the protection of the striatal pathway) and an increase in astrogliosis and neuroinflammation (by the higher presence of glial fibrillary acidic protein (GFAP) and ionized calcium-binding adaptor molecule-1 (Iba-1) positive cells), while T2D did not exacerbate these effects. Moreover, chronic oral administration of linagliptin demonstrated neuroprotective effects by mitigating the rise of astrocytes and microglial cells in middle-aged T2D mice.

In order to validate the GLP-1 analogous liraglutide as a treatment against AD, in Chapter 7 we evaluated the impact of a chronic peripheral administration of the anti-T2D drug in mature 3xTg-AD (triple transgenic mouse model of AD) female mice. Our results suggest that liraglutide therapy positively impacted peripheral and brain cortical inflammation (C-Reactive Protein (CRP), interleukin (IL)-1β and IL-10 levels), normalized GLP-1-related signaling (estradiol, GLP-1 and active PKA levels), promoted brain glucose metabolism (glucose-6-phosphate-dehydrogenase (G6PDH) activity), altered brain mitochondrial fission/fusion machinery (mitochondrial fission 1 protein (Fis1) and mitochondrial dynamin-like 120 kDa protein (OPA1) levels), attenuated oxidative/nitrosative stress (8-OHdG, TBARS, carbonyl groups and nitrites content) and reduced cortical $A\beta_{1-42}$ levels of female mice with early AD-like pathology.

Taken together, the findings obtained from this Thesis revealed that a differential sex steroid hormone profile/action plays a pivotal role in brain over T2D progression, affecting the risk to develop AD-like pathology. Furthermore, it also serves to reinforce the existing link between T2D pathophysiology and the exacerbation of neurodegenerative processes, contributing to the development of a number of brain disorders (*e.g.* AD and PD). Finally, the work presented herein represents the potential implications of anti-T2D incretin-based therapies (*e.g.* Ex-4, liraglutide and linagliptin) against CNS impairments associated with T2D, AD, PD and/or aging.

Resumo

A diabetes tipo 2 (DT2) é uma barulhenta "assassina silenciosa", sendo considerada uma pandemia que afeta milhões de pessoas espalhadas por todos os cantos do mundo, com a agravante de que milhões mais vivem atualmente com esta doença sem sequer o saberem. Mesmo assim, mais esforços devem ser feitos para a consciencialização, compreensão e tratamento da DT2, de modo a evitar o desenvolvimento descontrolado de complicações a longo prazo que influenciem o desfecho da doença, a qualidade de vida e a mortalidade. De fato, a ameaça da DT2 e o seu peso na saúde continuam a aumentar, enquanto evidências epidemiológicas consistentes sugerem um risco aumentado de doenças neurodegenerativas associadas à patologia. Embora os mecanismos subjacentes aos efeitos deletérios da DT2 na estrutura e função do cérebro permaneçam elusivos, os intervenientes mais plausíveis incluem o comprometimento da sinalização mediada pela insulina e da distribuição e utilização de glicose, a hiperglicemia, o estado pró-inflamatório e, a nível cerebral, uma eventual resistência à insulina, défices metabólicos, stress oxidativo e disfunção mitocondrial.

O trabalho apresentado nesta Tese serviu para esclarecer e solidificar noções sobre o impacto da DT2 no cérebro, e a associação desta com outras doenças neurodegenerativas relacionadas com a idade, tais como a doença de Alzheimer (DA) e a doença de Parkinson (DP). Para além do mais, também abordámos o dimorfismo sexual na DT2 e como as alterações associadas ao sexo afetam diferencialmente o risco para o declínio cognitivo e a demência. Além disso, dados os mecanismos fisiopatológicos que ligam a DT2 e a neurodegeneração, avaliámos a eficácia de terapias baseadas em incretinas na melhoria da função do sistema nervoso central (SNC) associada a deficiências na DT2, no envelhecimento, na DA e na DP.

No Capítulo 3, usámos o rato Goto-Kakizaki (GK), não obeso, espontaneamente DT2, para comparar as vias de sinalização intracelular mediadas por hormonas em animais do sexo masculino e feminino, de meia-idade (sinalização relacionada com o estrogénio/fator de crescimento semelhante à insulina-1 (IGF-1)/insulina) em córtices cerebrais DT2 e consequente modulação do resultado da doença (marcadores de stresse oxidativo e marcadores associados à DA). As observações gerais mostraram uma janela temporal específica relacionada com o sexo para abordagens eficientes contra a DT2 e

DA. Se por um lado, os cérebros de ratos do sexo feminino na perimenopausa exibiram níveis mais baixos de colesterol cerebral, dihidroepiandrosterona (DHEA), testosterona e IGF-1, particularmente em DT2. Por outro lado, mecanismos compensatórios baseados na manutenção da função do estrogénio, do IGF-1 e dos recetores de insulina e da sinalização a jusante foram responsáveis pela diminuição dos níveis de oxidação (substâncias reativas ao ácido tiobarbitúrico (TBARS, do inglês *Thiobarbituric Acid Reactive Substances*) e níveis de 8-hidroxi-2-desoxiguanosina (8-OHdG)) e dos marcadores relacionados com a DA (atividade da β-secretase (BACE) e níveis do peptídeo beta-amilóide $(\beta A)_{1-42}$).

Em relação ao impacto dos medicamentos anti-DT2 à base de incretina, nos Capitulos 4 e 5 utilizaram-se agonistas do recetor do peptídeo semelhante ao glucagão-1 (GLP-1) (GLP-1R) ou inibidores da dipeptidil peptidase-4 (DPP-4) (DPP-4i), para avaliar os efeitos do mimético do GLP-1, exendina-4 (Ex-4), em ratos do sexo masculino, DT2 GK de meia-idade. Os dados derivados do Capítulo 4 elucidaram sobre o impacto de uma terapia periférica, contínua e crónica com Ex-4 na sinalização cortical cerebral de GLP-1/insulina/IGF-1 e subsequentes mecanismos autofágicos e de morte celular (apoptose e necroptose) em DT2. No Capítulo 5, avaliámos os efeitos da administração de Ex-4 no estado energético do cérebro DT2 por meio da captação cerebral de glicose, dos níveis e metabolismo da glicose, do conteúdo de aminoácidos essenciais, dos níveis de corpos cetónicos, da produção de energia e da atividade da cadeia respiratória e da dinâmica mitocondrial. No conjunto, os estudos com Ex-4 demonstraram ser uma terapia promissora contra a DT2, superando marcas patológicas periféricas associadas à patologia, bem como complicações crónicas que afetam o cérebro, nomeadamente pelo resgate dos níveis cortical cerebrais de GLP-1 e IGF-1, ativando as vias de sinalização cortical cerebrais mediadas pela proteína da quinase A (PKA) e pelas fosfatidilinositol 3-quinase (PI3K)/proteína quinase B (Akt). Estas reverteram o dismetabolismo da glicose (por redução dos níveis de glicose no cérebro e da concentração de ácido beta-hidroxibutírico (BOH) e pelo aumento da captação sináptica de glicose, das atividades enzimáticas e da carga energética (níveis de adenosina trifosfato (ATP), adenosina difosfato (ADP) e adenosina monofosfato (AMP)) e promoveram a autofagia (ativação do alvo mecanístico de rapamicina (mTOR), expressões da PI3K classe III, da proteína 1A/1B- associada aos microtúbulos da cadeia leve 3B (LC3) II, da proteína relacionada à autofagia (Atg) 7, da p62 e da

proteína de membrana associada a lisossoma (LAMP-1)) e mitofagia (expressão da Parkina), e por fim, exerceram um efeito anti-apoptótico (atividades semelhantes à caspase-1, caspase-3 e caspase-8 e níveis de citocromo c mitocondrial e de linfoma 2 de células B (Bcl2)).

O Capítulo 6 focou-se nas alterações neurais induzidas pela DT2 e/ou envelhecimento nas áreas cerebrais do sistema dopaminérgico, modulando a degeneração neuronal que caracteriza doenças como a DP, e no potencial do DPP-4i, linagliptina, como uma terapia para reverter essas mudanças patológicas em murganhos DT2 de meia-idade induzidos por dieta rica em gordura (HFD, do inglês *High-Fat Diet*). As observações destes estudos revelaram uma perda de interneurónios de parvalbumina (PV) (envolvida na proteção da via estriatal) associada à idade e um aumento na astrogliose e neuroinflamação (pela maior presença de células positivas à proteína glial fibrilar ácida (GFAP) e à ligação de cálcio ionizado da molécula adaptadora 1 (Iba-1)), enquanto a DT2 não exacerbou esses efeitos. Além disso, a administração oral e crónica de linagliptina demonstrou efeitos neuroprotetores ao mitigar o aumento de astrócitos e células microgliais em murganhos DT2 de meia-idade.

A fim de validar o análogo do GLP-1 liraglutide como um tratamento contra a DA, no Capítulo 7 avaliámos o impacto de uma administração periférica e crónica do fármaco anti-DT2 em murganhos do sexo feminino, maduras, da estirpe 3xTg-AD (modelo de murganho triplo transgénico para a DA). Os nossos resultados sugerem que a terapia com liraglutide teve um impacto positivo contra a inflamação periférica e cortical cerebral (níveis da proteína C-reativa (CRP), da interleucina (IL)-1β e da IL-10), normalizou a sinalização relacionada ao GLP-1 (níveis de estradiol, de GLP-1 e da PKA ativa), promoveu o metabolismo da glicose cerebral (atividade da glicose-6-fosfato-desidrogenase (G6PDH)), alterou a maquinaria de fusão/fissão mitocondrial do cérebro (níveis da proteína de fissão mitocondrial 1 (Fis1) e da proteína mitocondrial tipo dinamina 120 kDa (OPA1)), atenuou o stress oxidativo/nitrosativo (8-OHdG, TBARS, grupos carbonil e conteúdo de nitritos) e reduziu os níveis corticais de βA_{1-42} de murganhos do sexo feminino com patologia semelhante à DA numa fase precoce.

No seu conjunto, os resultados obtidos ao longo desta Tese revelaram que um perfil/ação diferencial das hormonas esteroides sexuais desempenha um papel central no cérebro durante a progressão da DT2, afetando o risco de desenvolver patologia

semelhante à DA. Além disso, também serve como um reforço da ligação existente entre a fisiopatologia da DT2 e a exacerbação de processos neurodegenerativos, contribuindo para o desenvolvimento de uma série de distúrbios cerebrais (por exemplo, DA e DP). Finalmente, o trabalho aqui apresentado representa as implicações potenciais das terapias anti-DT2 baseadas em incretinas (por exemplo, Ex-4, liraglutide e linagliptina) contra deficiências do SNC associadas à DT2, DA, DD e/ou envelhecimento.

Chapter 1

Introduction

1.1 – Type 2 diabetes

Diabetes Mellitus is nowadays one of the leading causes of death worldwide. In about 30 years the number of diabetic people rose from 108 million to more than 420 million, corresponding to 9% of the total adult population (aged 20–79 years). Moreover, estimates suggest that by 2040 the number of diabetics will rise to 642 million (Zheng *et al.*, 2018; Chen *et al.*, 2011), rendering diabetes a major global societal concern. Portugal is no exception. In fact, it ranks among the European countries with highest prevalence rates of diabetes. In 2015, 13.3% of the Portuguese adults aged between 20 and 79 years old (more than 1 million people) was estimated to have diabetes (O Observatório Nacional da Diabetes, 2016).

According to the World Health Organization (WHO) and the International Diabetes Federation (IDF), the most recent criteria established for type 2 diabetes (T2D) diagnosis include: fasting blood glucose levels ≥126 mg/dL (7.0 mmol/L); a glycemia ≥200 mg/dL (11.1 mmol/L) after an oral glucose tolerance test (OGTT) (*i.e.,* the blood glucose levels measured during 2h after the ingestion of 5g glucose); an occasional blood glucose level ≥200 mg/dL (11.1 mmol/L) or a blood hemoglobin A1c (HbA1c) content ≥6.5% (48 mmol/mol) (American Diabetes, 2021a; World Health Organization (WHO), 2019; International Diabetes Federation (IDF), 2017). According to the WHO, the majority of diabetes cases may be attributed to either type 1 diabetes (T1D) or T2D (World Health Organization (WHO), 2019). T1D is a chronic autoimmune disease classically characterized by hyperglycemia resulting from a total or partial insulin deficiency (Atkinson *et al.*, 2014). The pathogenesis of T1D often includes a complex interplay between environmental factors and microbiome, genome, metabolism, and immune systems, resulting in the autoimmune idiopathic destruction of the pancreatic β-cells and the subsequent deficiency in insulin production (DiMeglio *et al.*, 2018; Li *et al.*, 2017). Although its onset can occur at any age, T1D has been traditionally associated with a juvenile onset, with the most common symptoms including polyuria, polydipsia, weight loss and diabetic ketoacidosis (DiMeglio *et al.*, 2018; Monaghan *et al.*, 2015). The clinical management of T1D is mainly focused on the optimal glycemic control through multiple-dose insulin regimens that mimic physiological insulin release (Brinkman, 2017; International Diabetes Federation (IDF), 2017). Conversely, T2D accounts for around 90% of all diabetes cases worldwide, largely resulting from the association of a genetic burden with the epidemic of obesity and physical inactivity

(Zheng *et al.*, 2018). Therefore, healthy diet, regular physical activity, maintenance of normal body weight and smoke cessation may play a pivotal role in the management of T2D onset and/or progression (American Diabetes, 2021b; International Diabetes Federation (IDF), 2017). This heterogeneous and complex multisystem disorder is generally characterized by abnormally high blood glucose levels due to an ineffective action of insulin (the so-called insulin resistance) (Brunton, 2016) that traditionally affects the peripheral tissues (namely the pancreas, liver and muscle tissue). At this respect, DeFronzo recognized more than a decade ago the involvement of an "ominous octet", composed by alterations in muscle, liver and β-cells that were accompanied by the effects on adipose tissue, gastrointestinal tract, pancreatic α-cells, kidneys and brain in T2D pathophysiology (Defronzo, 2009).

Physiologically, the increase in plasma glucose levels that occur after a meal leads to the compensatory increase in insulin production by the pancreatic β-cells to promote the glucose uptake by tissues and ultimately restore its normal levels in circulation (Roder *et al.*, 2016; Cersosimo *et al.*, 2000). However, under pathological conditions the insulin produced by β-cells may not be able to cope with the abnormally high blood glucose levels that may in turn remain in circulation, leading to T2D, whose onset has been related with normo- or even hyperinsulinemia that, with the progression of the disease may be accompanied by a decline in insulin levels and a poorer metabolic control (Ghasemi and Norouzirad, 2019; Blaslov *et al.*, 2018). Adding to this and despite the initially increased fasting plasma insulin levels in T2D patients, insulin resistance may stimulate hepatic gluconeogenesis, increasing their basal hepatic glucose production (HGP) (Roden and Shulman, 2019; Hatting *et al.*, 2018; Petersen *et al.*, 2017). The later may be further exacerbated by the T2D-related dysregulation of pancreatic α-cells and the consequent elevation of basal plasma glucagon concentration (the hormone that under physiological conditions counteracts the role of insulin to increase hepatic glucose production and ensure the glucose supply, *e.g.*, to the brain) (Moon and Won, 2015). Furthermore, the suppression of muscle glucose uptake after a carbohydrate meal may occur in T2D insulin resistant individuals, alongside the resistance of their fat cells to insulin and the consequent increase in lipolysis and in free fatty acid (FFA) concentration that may in turn cause lipotoxicity and a vicious cycle of more insulin resistance (Roden and Shulman, 2019; Alvim *et al.*, 2015; Corpeleijn *et al.*, 2009).

1.2 – The role of Glucagon-like peptide-1

To further intricate the above scenario, the regulation of the postprandial insulin response may also involve the secretion of incretin hormones by another major endocrine organ: the gut. More specifically, after a meal the incretin effect is activated and involves the secretion of the hormones glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotrophic polypeptide (GIP) from the gut into the bloodstream to modulate the pancreatic insulin secretion and normalize the blood glucose levels (Holst, 2019; Nauck and Meier, 2018). This incretin effect was discovered in the 1960s, but the exploration of the involvement of the gut-brain axis in metabolic regulation and on the neuroprotection against T2D only gained relevance in the more recent decades (Figure 1) (Candeias *et al.*, 2015; Elrick *et al.*, 1964). As further detailed in section 1.5.2, GLP-1 and GIP account for ~90% of the physiological incretin effect, by activating specific receptors in both α- and β-cells of the pancreas to reduce postprandial glucagon and promote insulin secretion, delaying gastric emptying and normalize plasma glucose levels (Campbell and Drucker, 2013; Deacon and Ahren, 2011).

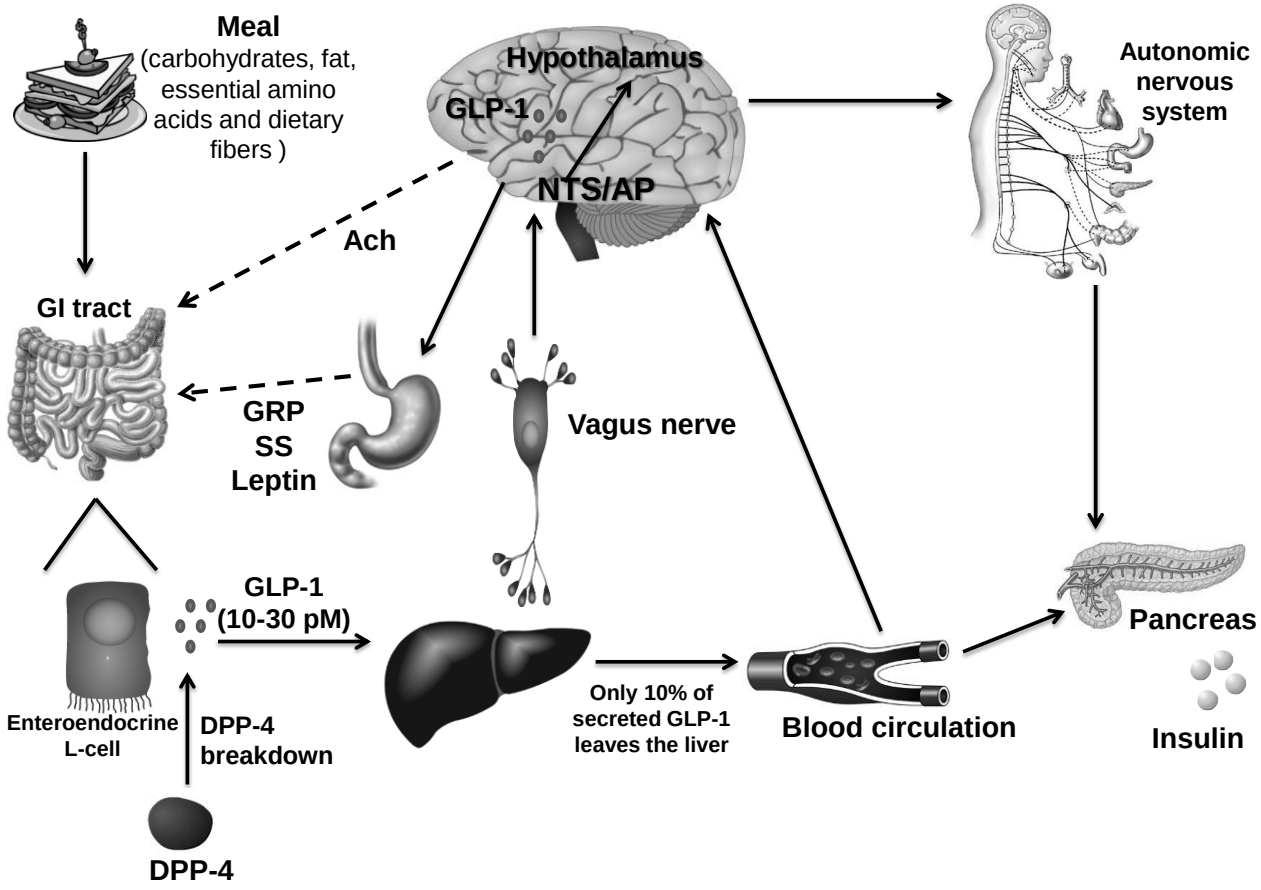


Figure 1.1 - The gut-brain axis for the actions of GLP-1. After the ingestion of a meal,

gastrointestinal (GI) tract is rapidly stimulated and glucagon-like peptide-1 (GLP-1) is secreted in the gut lumen by enteroendocrine L-cells. Besides the direct interaction of nutrients with L-cells, neural (acetylcholine) and endocrine (gastrin-releasing peptide, somatostatin and leptin) mechanisms are also involved in the control of GLP-1 secretion after food intake. Bioactive GLP-1 diffuses into the capillaries, immediately beginning to be degraded by dipeptidyl peptidase-4, so that more than 50% of the hormone is inactivated before reaching the portal circulation. In the liver, a further large amount is truncated, thus only 10% of the secreted GLP-1 leaves the liver and enters the systemic circulation and may reach the pancreas, the brain and other tissues via the endocrine pathway. However, the passage of GLP-1 through the hepatoportal vein activates vagal afferents nerves that initiate a neural signal towards the brain. In the central nervous system, the metabolic information is received by the solitary tract nucleus and the AP in the brainstem, which synthesize and project the GLP-1 to the hypothalamus. The GLP-1 receptor signaling is involved in the central control of energy homeostasis and food intake, and several autonomous functions, such as glucose-dependent stimulation of insulin secretion and inhibition of glucagon secretion in the pancreas, cardiovascular effects, regulation of gastric emptying and of endogenous glucose production in liver and glucose uptake and storage in muscle and adipose tissue. GRP: Gastrin-releasing peptide; Ach: Acetylcholine; SS: Somatostatin; DPP-4: Dipeptidyl peptidase-4; AP: Area postrema. *Adapted from (Candeias et al., 2015).*

GLP-1 is primarily synthesized as proglucagon, which can be post-translationally cleaved by the prohormone convertase to originate different products, depending on the tissue (Baggio and Drucker, 2007). For instance, in pancreas the major cleavage products are glucagon, glycentin related polypeptide and a major proglucagon fragment containing the GLP-1 and GLP-2 sequences, whilst in gut and brain the processing of proglucagon may liberate GLP-1, GLP-2 (which is not an incretin, since it is deprived from insulinotropic and glucose lowering properties), intervening peptide-2 (IP-2), glicentin, and oxyntomodulin (Cabou and Burcelin, 2011; Baggio and Drucker, 2007). Additionally, multiple forms of GLP-1 are secreted by humans, including GLP-1 (1-37) and GLP-1 (1-36) amides (synthesized as immature forms), or the bioactive forms glycine-extended form GLP-1 (7-37)-amide and the GLP-1 (7-36)-amide (the later being the predominant form in plasma and brain) (Gejl *et al.*, 2014; Cabou and Burcelin, 2011). Despite all these isoforms, most of the GLP-1 is secreted by the enteroendocrine L cells at the intestinal ileum and colon (Holst, 2007;

Rocca and Brubaker, 1999), mainly postprandially, particularly after fat- and carbohydrate-rich meals, and in a concentration proportional to the size of the meal, eventually reaching a plasma content of 10-30 pM (Kim and Egan, 2008; Combettes, 2006). However, individual nutrients (including glucose and other sugars, fatty acids, essential amino acids and dietary fibers) may also stimulate GLP-1 release (Herrmann *et al.*, 1995). Amongst these, the glucose and fructose mechanism of stimulation have been the more explored, with evidence demonstrating that oral (but not intravenous) glucose administration to healthy individuals stimulates GLP-1 secretion (Baggio and Drucker, 2007; Combettes, 2006). Indeed, the plasma levels of GLP-1 (7-36)-amide increased within just a few minutes after an oral glucose load, through a biphasic pattern of secretion and release that was composed by an early phase (in the first 10-15 min), followed by a prolonged second phase (30-60 min later) (Koole *et al.*, 2013; Herrmann *et al.*, 1995). All these may be possible thanks to the intimate contact of L-cells with different regions of the intestine and to their stimulation by a variety of mediators. For instance, L-cells can contact directly with nutrients at their luminal surface and with vascular tissue through their basolateral surface (Theodorakis *et al.*, 2006; Rocca and Brubaker, 1999). Moreover, these cells were described to contact with the enteric nervous system and the central nervous system (CNS) via the vagus nerve (Theodorakis *et al.*, 2006; Rocca and Brubaker, 1999). Hence, it is plausible that the early and late phases of GLP-1 secretion may occur through: (1) the direct nutrient stimuli to L-cells (particularly those located in more proximal regions of the small intestine), with the consequent (at least) partial induction of the first phase of GLP-1 secretion; or (2) via the coordinated indirect action of neural and endocrine factors, possibly involving the autonomic nervous system, neurotransmitters and peptides [*e.g.* gastrin releasing peptide, acetylcholine (Ach), γ-aminobutyric acid (GABA), calcitonin gene-related peptide and GIP] via the vagus nerve (Kim and Egan, 2008; Lim and Brubaker, 2006; Theodorakis *et al.*, 2006; Rocca and Brubaker, 1999). Additionally, others proposed that non-nutrient factors (such as leptin and insulin) could also contribute to the rapid release of GLP-1 (Lim and Brubaker, 2006; Bojanowska, 2005). Accordingly, the basal secretion of GLP-1 was also demonstrated to occur as a product of glucagon secretion under fasting, and may reach 5-10 pM in circulation, which is essential to maintain the glucose homeostasis (Tomas and Habener, 2010; Combettes, 2006). Finally, several authors reported that GLP-1 could be secreted, though to a lesser extent, by pancreatic α-cells and by neurons located at the nuclei of brainstem [solitary tract nucleus (NTS),

caudal brainstem and area postrema (AP)] (Cabou and Burcelin, 2011; Whalley *et al.*, 2011; Tomas and Habener, 2010) (as further detailed below).

Although the molecular mechanisms underlying GLP-1 secretion remain incompletely understood, several authors proposed that, upon a meal, the increase in blood glucose levels and its subsequent uptake into the cells (namely via sodium/glucose transporters) and metabolization may increase adenosine triphosphate (ATP) levels, thus closing the ATP-linked potassium channels and stimulating GLP-1 secretion (Lim and Brubaker, 2006; Reimann and Gribble, 2002). Notably, this may involve the activation of protein kinases A (PKA)-, C (PKC)- and mitogen-activated protein kinase (MAPK)-mediated signaling pathways, as well as an increase in intracellular calcium (Ca^{2+}) (Reimann and Gribble, 2002). Conversely, the inhibition of GLP-1 secretion in gut appears to involve a negative feedback, probably via GLP-1-mediated stimulation of somatostatin secretion (Bojanowska, 2005; Chisholm and Greenberg, 2002) and/or the neuropeptide galanin, as demonstrated in intestinal L-cells, both *in vitro* and *in vivo* (Baggio and Drucker, 2007; Bojanowska, 2005).

Importantly, GLP-1 is also expressed within the CNS, whereby it may exert neuroprotective effects (Orskov *et al.*, 1996). Although increasing evidence suggests that GLP-1 can be peripherally originated within the intestinal L-cells and, due to its relatively small size, the hormone may reach the CNS through the ready diffusion across the area postrema and subfornical organs (at the blood-brain barrier (BBB)) to regulate the activity of afferent vagal neurons (Orskov *et al.*, 1996), as referred above GLP-1 can be also locally synthesized within the brain. The later process appears to depend on the complex brainstem-hypothalamic-preproglucagon system (Larsen and Holst, 2005). More specifically, in the cell bodies of the preproglucagon neurons from the CNS, proglucagon can be processed to GLP-1 (Larsen and Holst, 2005). This is supported by evidence that preproglucagon neurons are primarily located in the lower brainstem (particularly in the caudal NTS and AP), with some cell bodies being also found in the dorsomedial part of the medullary reticular nucleus (Llewellyn-Smith *et al.*, 2011; Vrang *et al.*, 2007; Larsen *et al.*, 1997). Moreover, NTS and AP appear to receive visceral sensory inputs generated by the vagal nerves that innervate the gastroduodenal tract (Critchley and Harrison, 2013), *i.e.*, hepatoportal vein sensors may activate the vagus nerve, initiating a glucose neural signal to the NTS/AP in the brainstem, which in turn transmits the information through the axons until the

hypothalamic nuclei (Burcelin *et al.*, 2001). Adding to the above, the largest population of GLP-1 immunoreactive innervations occur in the dorsomedial and paraventricular nuclei of the hypothalamus and, to a lesser extent, in the cortex and hindbrain (Larsen *et al.*, 1997; Jin *et al.*, 1988). Therefore, it is not surprising that at least part of the GLP-1-associated endocrine effects (*e.g.*, the regulation of insulin secretion) may be indirectly mediated by neural mechanisms (Burcelin *et al.*, 2001). Indeed, it has been increasingly demonstrated that that the GLP-1-mediated activation of specific receptors (the GLP-1 receptors; GLP-1R) may generate new signals to guide the energetic flux towards tissues via the autonomic nervous system and thus regulate a diverse array of homeostatic functions (Cabou and Burcelin, 2011; Knauf *et al.*, 2008; Burcelin *et al.*, 2000).

Under physiological conditions, GLP-1 binds to its receptors, which belong to the class B family of 7-transmembrane heterotrimeric expressed G-protein-coupled receptors (a family that also includes receptors for glucagon, GLP-2, and GIP) (Doyle and Egan, 2007; Mayo *et al.*, 2003). GLP-1Rs are ubiquitously expressed throughout the organism, including the pancreas (α, β, and δ cells), lung, heart, kidney, stomach, intestine, pituitary, skin and ganglion neurons of the vagus nerve (Baggio and Drucker, 2007; Holst, 2007). Furthermore, its expression was also detected in mammalian brain neurons, astrocytes, microglia and endothelial cells from several regions of CNS (including the brainstem, hypothalamus, hippocampus and cortex) (Hou *et al.*, 2012). Strikingly, GLP-1Rs were further identified in lipid rafts, where they interact with caveolin-1, possibly to regulate the receptor subcellular localization, trafficking, and signaling (Baggio *et al.*, 2004). Rat and human GLP-1Rs are polypeptide chains with 463 amino acids that share 90% sequence homology (Kim and Egan, 2008). They comprise a long N-terminal extracellular region responsible for peptide recognition and binding, plus a cytoplasmic C-terminal comprising the components for specific G protein coupling, that has a major influence in signaling specificity and transmission (Coopman *et al.*, 2011; Al-Sabah and Donnelly, 2003). Once activated, GLP-1R may stimulate the adenylyl cyclase system, increasing intracellular cyclic adenosine monophosphate (cAMP) levels that may in turn activate the downstream PKA and exchange protein activated by cAMP-2 (Epac2) pathways (Koole *et al.*, 2013; Combettes, 2006). However, active GLP-1R may also increase intracellular Ca^{2+} and phospholipase C levels, or stimulate other signal transduction pathways in a tissue-

dependent manner, including phosphoinositide 3-kinase (PI3K), insulin receptor substrate (IRS)-2, epidermal growth factor receptor transactivation, PKC, MAPK, cyclic AMP response element binding protein (CREB), pancreatic duodenal homeobox-1, and glucose transporter (GLUT)-2 (Baggio and Drucker, 2007; Holz *et al.*, 1995; Wheeler *et al.*, 1993).

Alongside its insulinotropic effects detailed above, GLP-1 may also suppress the postprandial glucagon secretion, delaying gastric emptying, promoting early satiety (and the subsequent decrement in food intake), slowing the rate of endogenous glucose production and, ultimately, promoting weight loss (particularly in T2D conditions) (Nauck *et al.*, 2011; Kim and Egan, 2008; Combettes, 2006). GLP-1 may also enhance pancreatic β-cell mass, most likely by stimulating cell proliferation and protecting against apoptosis (Farilla *et al.*, 2002; Perfetti *et al.*, 2000). Hence, the combination of these effects may allow the normalization of blood glucose levels in a highly efficient manner in T2D patients (Kim and Egan, 2008; Rachman *et al.*, 1996), thus rendering GLP-1 (rather than GIP) a very attractive target for the treatment of T2D. However, the use of native GLP-1 as a pharmacological approach was unfeasible, since immediately after its secretion and release by intestinal L-cells, GLP-1 degradation by dipeptidyl peptidase-4 (DPP-4) starts and may account for 50% of the hormone inactivation (Holst, 2007; Deacon *et al.*, 1996), which is further increased after the passage of the intact bioactive peptide through the liver, culminating in less than 10% of active GLP-1 reaching the circulation (Holst, 2007).

DPP-4 is an ubiquitous and multifunctional enzyme that can be found either solubilized in blood or anchored to the cell membrane in many tissues and cell types (Green and Flatt, 2007), including the kidney, lung, adrenal gland, pancreas, liver, thymus, lymph node, uterus, placenta, prostate, and on the surface of lymphocytes, macrophages and endothelial cells (Matheeussen *et al.*, 2011; Hansen *et al.*, 1999). This glycoprotein is also expressed in several brain areas (*e.g.*, hypothalamus, hippocampus, circumventricular organs, choroid plexus, and leptomeninges) (Alponti *et al.*, 2011; Vrang and Larsen, 2010). The most well-known function of DPP-4 is the degradation and inactivation of the native GLP-1. Indeed, the enzyme specifically cleaves different dipeptides (like GLP-1, GLP-2, GIP, fibronectin, substance P, chemokines, neuropeptide Y (NPY), peptide YY (PYY)) that possess an alanine, proline or hydroxyproline residue in the penultimate N-terminal position (Kim *et al.*, 2014;

Mulvihill and Drucker, 2014). In the case of GLP-1, the resulting GLP-1 (7-36)-amide is metabolized to GLP-1 (9-37) or GLP-1 (9-36)-amide, which constitute the major circulating forms of the hormone (with an estimated half-life of 8-10 min, as a result of renal clearance) (Tomas and Habener, 2010) and have a 1000-fold reduced affinity for GLP-1R, thus completely blunting its insulin-releasing activity (Green and Flatt, 2007; Deacon *et al.*, 1995). Adding to this, the neutral endopeptidase (NEP, a membrane-bound zinc metallopeptidase expressed in both the periphery and CNS) may further hydrolyze the GLP-1 (7-36)-amide into smaller peptides, further inactivating the native GLP-1 (Plamboeck *et al.*, 2005; Hupe-Sodmann *et al.*, 1995). Of note, DPP-4 has been also implicated in numerous pleiotropic cellular processes involving cell cycle regulation, proliferation, adhesion, immunomodulation and apoptosis (Kim *et al.*, 2014; Mulvihill and Drucker, 2014; Lambeir *et al.*, 2003).

To further intricate the above scenario, the other gut-derived incretin hormone, GIP, is known to inhibit the secretion of gastric acid and the gastrointestinal motility, and to stimulate the release of insulin under physiological conditions (Dupre *et al.*, 1973; Brown and Dryburgh, 1971), while in T2D patients its insulinotropic activity may be diminished (Holst and Gromada, 2004; Elahi *et al.*, 1994). Furthermore, studies revealed that T2D patients may exhibit a resistance to the action of GIP, alongside the deficiency in GLP-1 secretion (Nauck *et al.*, 2011; Hojberg *et al.*, 2009; Vilsboll *et al.*, 2003b; Vilsboll *et al.*, 2002). Since the GIP secretion was maintained or even increased under such pathological conditions, the apparent reduction in β-cell response to the hormone upon T2D may arise from a down-regulation of GIP receptor expression/activity (Lynn *et al.*, 2001; Vilsboll *et al.*, 2001). The evidence that GLP-1 appears to be more insulinotropic in hyperglycemic conditions than GIP (Mentis *et al.*, 2011; Nauck *et al.*, 1993) further reinforce the possible advantages of recovering GLP-1 levels and/or action to manage T2D. However, some authors also reported that the impaired insulin secretion upon T2D was not related with changes in GLP-1-related insulinotropic activity, thus reinforcing the hypothesis on the involvement of a decreased incretin effect herein (Pratley and Gilbert, 2008; Kjems *et al.*, 2003; Toft-Nielsen *et al.*, 2001).

Importantly, another player in this puzzle is the kidney, the tissue responsible for the adaptive maintenance of glucose homeostasis by filtering large amounts of glucose every day that are subsequently almost completely reabsorbed and used to meet the high

energy demands of the body, especially the brain (Alsahli and Gerich, 2017). Kidney's high capacity to reabsorb glucose is due to the sodium-glucose co-transporters (SGLTs), mainly its SGLT2 isoform and, to a lesser extent, to SGLT1 (Katz and Leiter, 2015). In T2D, glucose reabsorption may further increase until exceeding the kidney's maximal renal tubular reabsorptive capacity and the organ may be no longer able to efficiently excrete glucose through urine to normalize its levels in circulation (Gronda *et al.*, 2020; Nosadini and Tonolo, 2003).

Brain represents another main target of T2D pathophysiology, possibly due to the mixture between a dysfunction in neurotransmitter pools together with the decrease in incretin effect and in glucose uptake, plus the increase in hepatic glucose production, in lipolysis, in glucagon secretion and in glucose reabsorption. Indeed, T2D patients are characterized by insulin resistance in peripheral tissues, and compensatory hyperinsulinemia, which extends to the brain (Cardoso and Moreira, 2019; Arnold *et al.*, 2018; De Felice and Ferreira, 2014). Accordingly, the insulin resistant brain state and the related disturbances in brain insulin signaling have been increasingly suggested not only to underlie the disruption of glucose homeostasis, but also the onset of neurodegenerative diseases co-morbid to T2D (Maciejczyk *et al.*, 2019; Kullmann *et al.*, 2016; Candeias *et al.*, 2012). Recent studies also point the catecholamines (including dopamine), the vitamin D and testosterone deficiencies or the renin–angiotensin system antagonism as notable players in the T2D-mediated alterations in metabolic homeostasis and insulin sensitivity (Kalra *et al.*, 2013).

Aging is the main risk factor for the long-term complications of T2D, which often seriously affect the elderly patients with a poor management of glycemia (Freeman, 2019; Sesti *et al.*, 2018). These complications include cardiovascular disease (CVD), nephropathy, retinopathy, peripheral and autonomic neuropathy, and encephalopathy (Candeias *et al.*, 2012; Sims-Robinson *et al.*, 2010). It became clearer that the major impact of the metabolic imbalance associated to T2D in several brain areas may increase its susceptibility and lead to a broad spectrum of complications affecting the CNS, including deficits in memory, attention, intelligence, processing speed, and executive function (Zilliox *et al.*, 2016; Monette *et al.*, 2014; Roriz-Filho *et al.*, 2009). Irregularities in brain structure upon T2D, namely white matter abnormalities and brain atrophy were more pronounced in cortical, subcortical, and hippocampal areas (Correia *et al.*, 2012; Biessels *et al.*, 2002). Although the precise mechanisms

underlying the deleterious of chronic T2D in the brain remain incompletely understood, increasing evidence suggests a pivotal role for insulin resistance, chronic hyperglycemia, repeated episodes of severe hypoglycemia and/or vascular impairment in the cognitive dysfunction associated with T2D (Lyu *et al.*, 2020; Karvani *et al.*, 2019; Biessels and Despa, 2018). In addition, other co-morbidities often associated with diabetes (such as stroke, hypertension, dyslipidemia, and obesity) were also shown to potentiate its related cognitive decline (McNay and Recknagel, 2011). Furthermore, T2D has been widely shown to accelerate the brain aging process, exacerbating its harmful effects and increasing the risk for development of neurodegenerative diseases (Roriz-Filho *et al.*, 2009), namely Alzheimer disease (AD) and Parkinson disease (PD), as detailed below.

1.3 – Type 2 diabetes and neurodegenerative diseases: Alzheimer and Parkinson diseases

As referred in the previous section, T2D may constitute a risk factor for cognitive decline and dementia. Of the 20% of neurodegenerative diseases associated with *diabetes mellitus*, AD and PD constitute the first and second most common neurodegenerative disorders in the world, respectively (Morsi *et al.*, 2018; Ristow, 2004).

1.3.1 – Alzheimer disease

Dementia is an overall clinical syndrome characterized by a progressive decline of cognitive function, including memory, learning capacity, thinking, language, executive and visuospatial function, together with the deterioration in personality, motivation and behavior (Gale *et al.*, 2018; Weller and Budson, 2018). These alterations may occur in such an extent that may ultimately interfere with the daily life and activities of the patient and caregivers (World Health Organization (WHO), 2018b; Takizawa *et al.*, 2015). AD alone accounts for up to 80% of all dementia diagnoses, with estimates pointing to 36 million people affected worldwide in 2020 (Alzheimer's Association, 2020). With the ever-increasing aging of the population worldwide, estimates suggest that this numbers will more than triplicate by 2050 (Robinson *et al.*,

2017). Adding to this, the annual cost of dementia in the USA alone is expected to exceed the US$600 thousand million in 2020, thus rendering dementia a major societal concern (Alzheimer's Association, 2020; Takizawa *et al.*, 2015). At this respect, Portugal is no exception, since dementia was estimated to affect 1.88% of total population (approximately 193,516 people) in 2018, mainly in people aged over 70 (Alzheimer Europe, 2020; Goncalves-Pereira *et al.*, 2019; Ruano *et al.*, 2019). Moreover, estimates point towards an almost doubling in the number of individuals with dementia in Portugal by 2050, which exceeds the broader European estimates (Alzheimer Europe, 2020).

AD is a slowly, progressive, and fatal neurodegenerative disease that can remain asymptomatic for as long as 20 years (Bondi *et al.*, 2017). Its classical symptomatology includes forgetfulness of recent events, conversations and people's names, as well as signs of apathy and depression (Alzheimer's Association, 2020; Atri, 2019), whereas the typical neuropathological hallmarks comprise the extracellular accumulation of senile plaques enriched in the protein fragment amyloid-beta (Aβ), twisted intracellular strands of neurofibrillary tangles (NFT) enriched in the phosphorylated protein tau, alongside neuronal damage and death that affect mainly the hippocampus and cortex (DeTure and Dickson, 2019; Lane *et al.*, 2018). As Aβ accumulation increases and abnormal tau spreads throughout the brain, dysfunction of the interneuronal communication at synapses and blockage of nutrient transport into the neurons may lead to progressive cell death (Sengoku, 2020; Sery *et al.*, 2013). In line with this, the evolution of the diagnostic tools and the recognition that the pathology may start many years prior to the arousal of symptomatology led to the identification of three stages of AD: preclinical, mild cognitive impairment (MCI), and dementia (van Loenhoud *et al.*, 2019; Davis *et al.*, 2018; Lane *et al.*, 2018), thus allowing an earlier diagnosis, with increased molecular specificity that include the detection of putative biomarkers (such as Aβ and tau pathology), namely by positron emission tomography (PET) imaging or measurement in cerebrospinal fluid (CSF), together with structural magnetic resonance imaging (MRI) assessment of medial temporal lobe atrophy, of brain glucose metabolism visualization by fluorodeoxyglucose (^{18}F) (FDG)-PET imaging (Atri, 2019; Femminella *et al.*, 2018).

In terms of origin, AD can be classified as sporadic (which comprises the vast majority of AD cases) and familial (corresponding to a small percentage, <1%, of the

patients) (Villain and Dubois, 2019; Lane *et al.*, 2018). The familial AD form may result from mutations in any of three specific genes: amyloid precursor protein (APP), presenilin (PSEN)1 and PSEN2 (Cacace *et al.*, 2016) and often develop symptoms at earlier ages (typically between 30 and 50 years of age), in contrast with individuals with sporadic AD, whose symptoms appear later (typically at the age 65 or older) (Villain and Dubois, 2019). Although still debatable, the pathogenesis of sporadic AD may involve a complex interplay between genetic, environmental and physiological factors that are aggravated by aging (the only risk factor for sporadic AD established to date) and/or the apolipoprotein E (APOE) ε4 allele (the gene with the strongest risk association with the disease) (Munoz *et al.*, 2019). Furthermore, epidemiological studies point towards the influence of vascular factors, hypertension, obesity and diabetes *per se* on the increased risk for sporadic AD later in life (Armstrong, 2019; Silva *et al.*, 2019), a risk that can be further aggravated in the case of, *e.g.*, other pathologies co-morbid to T2D, such as obesity, hyperinsulinemia or metabolic syndrome (Bello-Chavolla *et al.*, 2019; Hayden, 2019). The first epidemiological evidence for the relation between T2D and AD date from the late 1990s, when several studies worldwide started to demonstrate that the relative risk for AD among T2D patients ranged from 1.8-4.4. These studies include the *Rotterdam Study* (in the Netherlands) (Ott *et al.*, 1999), the *Manitoba Study of Health and Aging* (in Canada) (Tyas *et al.*, 2001), the *Honolulu-Asia Aging Study* (in a population-based cohort of Japanese-American men) (Peila *et al.*, 2002), the *Framingham Study* (in the USA) (Akomolafe *et al.*, 2006) and, more recently, the *Hisayama Study* (in Japan) (Ohara *et al.*, 2011). In 2013, Chen and Zhong reported that individuals with metabolic syndrome or T2D were at a higher risk of developing MCI, and the AD affected two- to three-fold of the elderly T2D patients. These authors further observed that over 80% of AD patients were also T2D or pre-diabetic (Chen and Zhong, 2013). In addition, Dore *et al.* (2009) found that the presence of one or more APOE ε4 alleles raised the risk of cognitive dysfunction among T2D individuals (Dore *et al.*, 2009). Importantly, Yaffe *et al.* (2006) observed that each 1% elevation in glycosylated hemoglobin increased the risk for MCI and dementia in postmenopausal women, either T2D or not (Yaffe *et al.*, 2006).

Despite the intense research efforts over the last decades and the increasing evidence that implicate insulin signaling dysfunction, energy and cholesterol dysmetabolism, endosomal-vesicle recycling, inflammation, mitochondrial impairment

and apoptosis (*per se* or in combination) on the pathogenesis of sporadic AD, the later remains incompletely understood (Samant and Gupta, 2021; Berlanga-Acosta *et al.*, 2020; Holscher, 2019; Shoshan-Barmatz *et al.*, 2018; Xian *et al.*, 2018; Gamba *et al.*, 2019). This, together with the complexity inherent to T2D pathology (namely the disease duration, the level of glycemia management, the insulin treatment) and/or to the presence of other risk factors for dementia (such as atherosclerotic vascular disease and the APOE-ε4 allele) render the study of the crosslinking mechanisms between chronic T2D and its comorbid AD a matter of intense debate. However, the completion of this puzzle will be of the outmost medical, social and economic relevance, since the above-mentioned estimates for an increased prevalence of AD *per se* are further reinforced by its predicted increase to 420 million among T2D individuals (Robinson *et al.*, 2017). Therefore, it is not surprising that AD has been increasingly considered a type 3 diabetes or a "brain-specific T2D" (de la Monte and Wands, 2008). This hypothesis has been supported by studies demonstrating that the dysfunction in brain insulin signaling appears to constitute a pivotal crosslinking mechanism between T2D and AD (Berlanga-Acosta *et al.*, 2020; Arnold *et al.*, 2018; Tumminia *et al.*, 2018). These include the association between impaired brain/peripheral insulin and cerebral degenerative processes in T2D and AD (Chow *et al.*, 2019; Folch *et al.*, 2019; De La Monte, 2012) or the observation that insulin desensitization also occurs in AD brain (Holscher, 2020; Holscher, 2014c; Talbot *et al.*, 2012). Given the widely demonstrated neuroprotective role of this hormone, it is plausible that the loss of insulin actions (as occurs in T2D) may underlie the pathophysiology of several neurodegenerative disorders (Sebastiao *et al.*, 2014; Matsuzaki *et al.*, 2010)

1.3.2 – Brain insulin resistance

Insulin and insulin receptors (IRs) are ubiquitously expressed in many tissues, including the brain (Duarte *et al.*, 2012a; Schulingkamp *et al.*, 2000), where insulin can reach levels 10- to 100-fold greater than in plasma (especially in the hippocampus, cortex, hypothalamus, olfactory bulb, and pituitary) (Duarte *et al.*, 2012a; van der Heide *et al.*, 2006). Although still poorly understood, it has been hypothesized that insulin produced by pancreatic β-cells can be transported by the CSF into the brain and crosses the BBB by an active and saturable process (Plum *et al.*, 2005; Gasparini *et al.*, 2002).

However, insulin was also detected in immature nerve cell bodies and, in rodents, less than 1% of the peripherally administered hormone reached the CNS suggesting a probable local insulin biosynthesis (Duarte *et al.*, 2012a; Plum *et al.*, 2005). It has been shown that an increase in circulating insulin is associated with a concomitant increase in CSF insulin levels, which affects brain activity (van der Heide *et al.*, 2006). Once in the brain, insulin may activate the IRs that are largely localized in neurons and are less abundant in glia (Cole *et al.*, 2007; Schulingkamp *et al.*, 2000) (though the IRs at the CNS are slightly different from their peripheral counterparts (Schulingkamp *et al.*, 2000)), and/or the ubiquitously expressed insulin-like growth factor-1 (IGF-1) receptors (IGF-1Rs) throughout the brain (Bosco *et al.*, 2011; Gasparini *et al.*, 2002).

Both IRs and IGF-1Rs are homologous, membrane-bound receptors that belong to the superfamily of tyrosine kinase receptors, triggering similar intracellular signaling events (Schulingkamp *et al.*, 2000). Binding of insulin or IGF-1 promotes the receptor auto-phosphorylation, stimulating its tyrosine kinase activity and, subsequently, phosphorylating either the IRS proteins on tyrosine residues or the Src homology collagen (Shc) peptide, thus activating two parallel signaling cascades that are mediated by the PI3K and the MAPK (McNay and Recknagel, 2011; Cole *et al.*, 2007; Li and Holscher, 2007). After PI3K activation, downstream signaling proteins (such as serine (Ser)/threonine (Thr) kinase Akt), are recruited to the plasma membrane, being then translocated to the cytosol and nucleus, whereby they phosphorylate other target proteins (*e.g.*, glycogen synthase kinase-3β; GSK-3β) (Kim and Feldman, 2012; Lizcano and Alessi, 2002). The Ser/Thr protein kinase GSK-3β contains two distinct forms: an active form (dephosphorylated at Ser9) that is mostly found in nuclei, mitochondria, and membrane lipid rafts and the cytosolic inactive form (Cole *et al.*, 2007). Once activated by insulin/IGF-1, Akt, PKC or c-AMP-dependent protein kinase may inactivate GSK-3β, thus triggering *e.g.* the synthesis of proteins involved in neuronal glucose metabolism or in the protection against apoptosis and oxidative stress (van der Heide *et al.*, 2006; Fang *et al.*, 2000). Conversely, the overexpression of a constitutively active GSK-3β promoted cell death and its inhibition reduced apoptosis (Duarte *et al.*, 2012a). IR-/IGF-1R-induced activation of PI3K/Akt may also target forkhead box O (FOXO) 3, nuclear factor-kB (NF-kB), and CREB. Accordingly, Akt may phosphorylate and inhibit FoxO3, protecting against the disruption of mitochondrial membrane potential and cytochrome c release, and, promoting neuronal

survival (Cole *et al.*, 2007; van der Heide *et al.*, 2006), whereas NF-kB phosphorylation by Akt protected against oxidative stress and apoptosis by increasing the levels of copper/zinc and manganese superoxide dismutase (Cu/Zn SOD and MnSOD, respectively) (Duarte *et al.*, 2012a; Cole *et al.*, 2007). Moreover, CREB phosphorylation by Akt may increase neuronal glucose metabolism, mitochondrial membrane potential, ATP levels, nicotinamide adenine dinucleotide phosphate (NADPH) redox state, and hexokinase activity (Heras-Sandoval *et al.*, 2011). On the other hand, the activation of MAPK pathway may promote the expression of genes involved in cell and synapse growth, cellular repair and maintenance, while the compromised MAPK signaling pathways contribute to the pathology of diverse human diseases including cancer and neurodegenerative disorders such as AD, PD and amyotrophic lateral sclerosis (Kim and Choi, 2015). Adding to these effects associated to PI3K/Akt- and MAPK-mediated signaling *per se*, several studies suggest that their crosstalk may involve the phosphorylation of Bcl2-associated death promoter (Bad) to protect against apoptosis, by increasing the ability of mitochondria to withstand proapoptotic signals, thus reinforcing the neurotrophic and neuroprotective actions of insulin (Datta *et al.*, 2002; Datta *et al.*, 1997).

The classical effects of insulin at the periphery include glucose uptake, regulation of cell proliferation, gene expression, and the suppression of hepatic glucose production (Plum *et al.*, 2005; Lizcano and Alessi, 2002). Within the CNS, insulin-mediated activation of neuronal IRs and/or IGF-1Rs regulates not only peripheral physiological actions (like food intake, the inhibition of hepatic gluconeogenesis, the counter-regulation of hypoglycemia, the reproduction), but also exerts more "brain-like" effects, including the modulation of tau protein phosphorylation, AβPP metabolism, Aβ clearance, neuronal survival, and memory (McNay and Recknagel, 2011; Cole *et al.*, 2007; Plum *et al.*, 2005). Regarding the role of insulin on the regulation of food intake and peripheral energy homeostasis, evidence suggests that the hormone may activate the ATP-sensitive potassium (K^+ATP) channels, promoting the hyperpolarization of the hypothalamic glucosensing neurons and eliciting an anorexigenic signaling that may involve the inhibition of NPY and agouti-related peptide expression, together with the induction of proopiomelanocortin and cocaine- and amphetamine-regulated transcript production (Cole *et al.*, 2007; Plum *et al.*, 2005), ultimately reducing hepatic glucose production (Plum *et al.*, 2005). Although insulin may not constitute a major regulator of

brain glucose metabolism, several authors suggested that changes in its circulating levels may modulate the expression of GLUTs (Kim and Feldman, 2012; Cunnane *et al.*, 2011). For instance, increased brain insulin levels were shown to enhance brain GLUT4 expression and to stimulate glucose metabolism (Moreira *et al.*, 2009; Schulingkamp *et al.*, 2000).

As referred above, apart from these roles of cerebral insulin on peripheral and brain metabolism, the activation of IRs and IGF-1Rs at the CNS have been also involved in cortical and hippocampal synaptic plasticity, memory and learning (McNay and Recknagel, 2011; Cole *et al.*, 2007). Indeed, insulin-mediated PI3K activation may affect the long-term potentiation (LTP) and long-term depression (LTD), whereas the modulation of the glutamate α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors, and the GABA receptors (McNay and Recknagel, 2011; van der Heide *et al.*, 2006) through the activation of MAPK may induce LTP and memory consolidation (Correia *et al.*, 2011). Adding to this, mounting evidence also point towards a role for brain insulin in neurite outgrowth and axonal regeneration (Correia *et al.*, 2011; McNay and Recknagel, 2011; Cole *et al.*, 2007). Under this perspective, it is not surprising that the impairment of brain insulin signaling may affect the cellular function and survival, potentiating brain aging and age-related diseases such as AD. This prompted the intense research efforts developed in the last decades to unveil the neuroprotective potential of insulin and its downstream signaling at the CNS.

As the aging process occurs, the imbalance in the cellular oxidative defenses and/or in the generation of free radicals (mainly at the mitochondrial level) result in the accumulation of intracellular oxidative stress and damage that may potentiate the degeneration and eventually the cellular death (Anderton, 2002). This age-related oxidative redox shift may be also modulated by epigenetic factors that, together with a sedentary lifestyle, may attenuate mitochondrial metabolism and increase the cellular reliance on glycolysis (Brewer, 2010), thus exacerbating the damaging cycle through the involvement of oxidized membrane receptors, signaling molecules, transcription factors, and epigenetic transcriptional regulators (Duarte *et al.*, 2012a; Brewer, 2010). Accordingly, an impairment in insulin signaling was observed upon aging, mainly in the hippocampus, cortex, and choroid plexus. Similar deficits in insulin signaling have been increasingly associated with cognitive decline and the increased risk for dementia

(Correia *et al.*, 2012; de la Monte, 2009; Li and Holscher, 2007). Burns *et al.* (2012) reported that insulin resistance is associated with cognitive decline in non-demented elderly (Burns *et al.*, 2012). Although the precise mechanisms involved are still debatable, several studies showed an association between the decrease in CSF insulin levels and/or in CSF/plasma insulin ratio, while others suggested a role for the increase in fasting plasma insulin levels, for the decrease in IRs and IGF-1Rs levels (Duarte *et al.*, 2012a; Moloney *et al.*, 2010), tyrosine kinase activity or in the expression of the downstream IRS molecules upon the progression of AD (Bosco *et al.*, 2011; de la Monte, 2009; Li and Holscher, 2007). Accordingly, Muller *et al.* (Muller *et al.*, 2012) reported that IGF-1 signaling was deteriorated in the brain of aged mice. Other authors suggested that the oxidation (and the subsequent inhibition) of IRs and IGF-1Rs could underlie the age-related impairment in brain insulin signaling, a situation reinforced by the failure of oxidized protein tyrosine phosphatase-1B to promote their reactivation (Brewer, 2010; Fulop *et al.*, 2003). Importantly, the decrease in brain or CSF insulin (as well as IGF-1) levels upon peripheral hyperinsulinemia suggests that either its transport into the brain (de la Monte, 2009) or the BBB function might be compromised (Bosco *et al.*, 2011; Li and Holscher, 2007). Adding to this, we cannot exclude that the increase in membrane cholesterol levels and the subsequent decrease in membrane fluidity (Fulop *et al.*, 2003) upon brain aging and/or APOE4 genotype may affect the ligand–receptor binding or the internalization of IRs/IGF-1Rs, thus accounting for the chronic age-related insulin resistance (de la Monte, 2009; Li and Holscher, 2007).

In terms of downstream signaling, studies demonstrated that despite the increased glucose levels and the stimulation of insulin production upon such conditions, the brain glucose metabolism may become impaired, creating a vicious cycle that may further hamper insulin signaling and antioxidant mechanisms, aggravating mitochondrial dysfunction and the formation of advanced glycation end products (AGEs) formation, thus potentiating the age-related injury (Brewer, 2010; Plum *et al.*, 2005; Fulop *et al.*, 2003). Analogously, AD-related insulin resistance was demonstrated to affect predominantly the PI3K/Akt pathway, decreasing the expression and activation of brain GLUTs and thus reducing brain glucose and mitochondrial metabolism and the production of ATP (Bosco *et al.*, 2011). The increased levels of circulating glucose in CNS was also associated with increased AGEs formation and toxicity (Bosco *et al.*, 2011; Correia *et al.*, 2011). Conversely, the stimulation of MAPK in brains from AD

patients (Bosco *et al.*, 2011) was correlated with an increment in neuroinflammation, tau protein hyperphosphorylation, and AβPP trafficking (Bosco *et al.*, 2011; Sims-Robinson *et al.*, 2010). Indeed, mounting evidence suggests that the overactivation of MAPK, GSK-3β, and cyclin-dependent kinase 5 (Cdk5) (the major tau kinases involved in tau protein phosphorylation) may result in the hyperphosphorylation of tau protein (Correia *et al.*, 2011; de la Monte, 2009). Furthermore, the decreased phosphorylation of GSK-3β (Ser9) and its subsequent overstimulation may potentiate the activity of γ-secretase and the amyloidogenic AβPP processing, thus increasing the intracellular levels of Aβ (Kim and Feldman, 2012; Moreira *et al.*, 2009). The later may be further reinforced by the downregulated levels and activity of the hippocampal insulin-degrading enzyme (IDE, a zinc-metalloprotease that degrades several extracellular substrates and accounts for the modulation of extracellular Aβ degradation by insulin (Cole *et al.*, 2007; van der Heide *et al.*, 2006)) in severely affected AD patients, which was negatively correlated with their brain $A\beta_{1-42}$ content (Zhao *et al.*, 2007b). Adding to this, Bomfim *et al.* (Bomfim et al., 2012) reported that Aβ oligomers can activate the tumor necrosis factor α (TNFα)/c-Jun N-terminal kinase (JNK) pathway, induce IRS-1 phosphorylation at multiple serine residues, and inhibit physiologically phosphorylated IRS-1 (at Tyr896) in mature cultured hippocampal neurons. These observations were corroborated by the impairment of IRS-1 signaling in cynomolgus monkeys intracerebroventricularly injected with Aβ oligomers and in the APP/PS1 transgenic mouse model of AD (Bomfim *et al.*, 2012), as well as in human AD brains (Talbot *et al.*, 2012).

Although the increasing evidence reinforce the hypothesis that insulin/IGF-1 resistance and IRS-1 dysregulation may characterize the brains upon aging and/or AD (Muller *et al.*, 2012; Talbot *et al.*, 2012), and that increased insulin/IGF-1 signaling may exert neuroprotective effects (Bishop *et al.*, 2010; Parrella and Longo, 2010), other authors showed that a reduction in insulin/IGF-1 signaling may underlie the increased longevity in both model organisms (such as yeast, nematodes and flies) and in aged mammal brain (namely human, rhesus macaque, rat and mouse) (Bishop *et al.*, 2010). Accordingly, the reduction in insulin/IGF-1 signaling within the CNS was associated with an increased resistance to stress and an extended lifespan in worms and flies. A similar lifespan extension and an amelioration of AD pathology was also reported in the mammalian brain upon the reduction of insulin/IGF-1 signaling (*e.g.*, by neuron-

specific knockout of IRS2) (Bishop *et al.*, 2010). This was further supported by a study from Harries *et al.* (Harries *et al.*, 2012) demonstrating an inverse correlation between the expression of genes involved in insulin production, sensitivity (such as FOXO) and signaling [including phosphatase and tensin homolog (PTEN), PI3K, and 3-phosphoinositide-dependent protein kinase 1 (PDK1)] and the age of humans.

Although further research is needed to clarify such apparently opposite roles of insulin/IGF-1 and their downstream signaling pathways within the CNS across the aging process, accumulating evidence appears to point towards the hypothesis that AD could be a T2D (or insulin resistant state) of the brain, or even a type 3 diabetes (Arnold *et al.*, 2018; Kandimalla *et al.*, 2017; Leszek *et al.*, 2017).

1.3.3 - Brain mitochondrial dysfunction

Brain mitochondrial dysfunction is another common mechanism between T2D and AD, being responsible for defects in the coordination of energy metabolism and one of the major sources and targets of reactive oxygen species (ROS) (Cardoso *et al.*, 2017b).

Previous studies from our laboratory demonstrated that rat brain endothelial cells under chronic hyperglycemia showed an increase in mitochondrial ROS production and were more susceptible to $A\beta_{1-40}$ toxicity (Carvalho *et al.*, 2014b). This suggest that hyperglycemia may constitute a major risk factor for vascular injury associated with AD (Carvalho *et al.*, 2014b). This hypothesis was further supported by the observation that the triple transgenic mouse model of AD (3xTg-AD) had similar alterations in vasculature, brain mitochondrial bioenergetics and dynamics, in oxidative status, in autophagic mechanisms and in neurotoxic proteins burden compared to sucrose-induced T2D mice (Carvalho *et al.*, 2015; Sena *et al.*, 2015; Carvalho *et al.*, 2013; Carvalho *et al.*, 2012). The authors suggested that the impairment in intracellular quality control mechanisms may underlie the synaptic loss and cognitive impairment under such conditions (Carvalho *et al.*, 2015; Carvalho *et al.*, 2013). These data were in accordance with the increased autophagic degradation of *e.g.*, mitochondria in human AD postmortem brain, as given by the localization of the mitochondrial marker lipoic acid within the autophagic vacuoles (Moreira *et al.*, 2007b). Furthermore, other authors also demonstrated that the alterations in the brain mitochondrial morphology upon AD could

arise from an imbalance in fusion-fission, the reduction in mitochondrial content (either due to a compromised mitochondrial biogenesis and/or overactivation of mitophagy) and from a disruption of mitochondrial trafficking (Correia *et al.*, 2016; Zhu *et al.*, 2013). In addition, studies performed almost two decades ago demonstrated a higher susceptibility of isolated brain mitochondria from the T2D Goto-Kakizaki (GK) rats to oxidative damage (Santos *et al.*, 2001) and to the neurotoxic effects of $A\beta_{25-35}$ and $A\beta_{1-40}$ (Moreira *et al.*, 2003).

These and several other studies led to the "mitochondrial cascade hypothesis" for the origin of sporadic AD (Swerdlow, 2020; Swerdlow and Khan, 2004). According to this hypothesis, the baseline mitochondrial function of an individual's is genetically determined, but the rate at which the mitochondrial function declines across aging depends on both inherited and environmental factors, in such a way that, when mitochondrial decline exceeds a threshold, AD histopathology and symptoms are triggered (Swerdlow *et al.*, 2014; Swerdlow and Khan, 2004).

As mentioned above, besides brain insulin resistance and mitochondrial impairment, several other molecular mechanisms shared by AD and T2D and that may culminate in memory loss, such as inflammatory signaling and oxidative stress (Butterfield *et al.*, 2014; De Felice and Ferreira, 2014) (Figure 2).

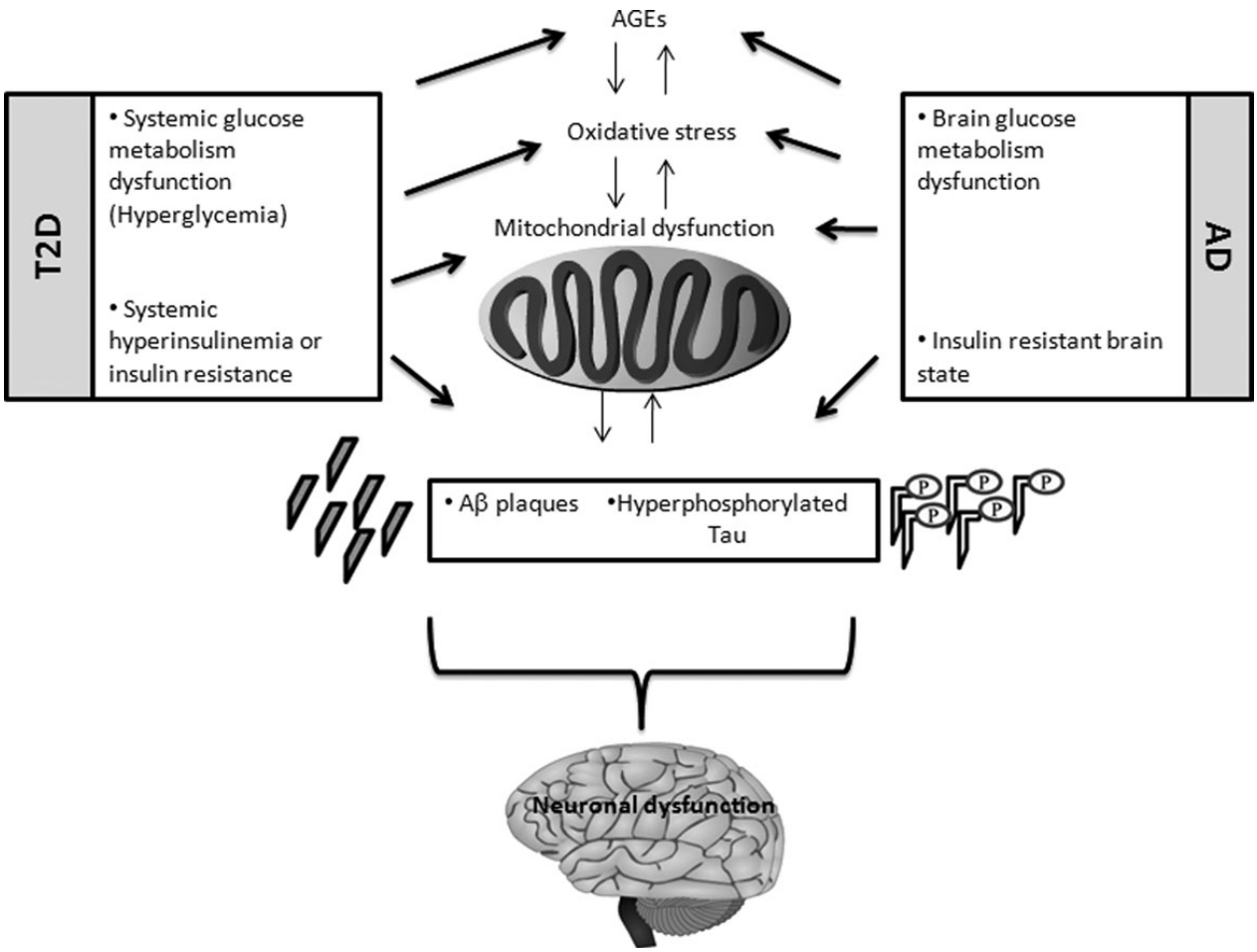


Figure 1.2 - Common pathological processes in AD and T2D. Type 2 diabets (T2D) and Alzheimer disease (AD) share many aspects. The features associated with both disorders are mainly influenced by abnormal systemic and/or central glucose and insulin metabolism. Hyperglycemia, chronic peripheral hyperinsulinemia, and insulin resistance compromise brain glucose metabolism and insulin signaling pathways, potentiating an energy crisis and creating a vicious cycle of oxidative stress and mitochondrial dysfunction. Moreover, impaired glucose metabolism-associated accumulation of advanced glycation end products (AGEs) potentiates amyloid beta (Aβ) aggregation and the formation of neurofibrillary tangles (NFT), which in turn exacerbate mitochondrial dysfunction and oxidative stress. Therefore, impaired glucose distribution and utilization as well as hyperinsulinemia and altered insulin signaling may result in neuronal damage and cognitive deficits that characterize both AD and T2D. *Adapted from* (Candeias *et al.*, 2012).

1.3.4 – Parkinson disease

PD is a chronic progressive age-related neurodegenerative disorder estimated to affecting more than 6.1 million people worldwide, a number that is expected to double by 2040 (Dorsey *et al.*, 2018). Similar to the previously mentioned for AD, Portugal is no exception, with a recent cross-sectional study pointing towards a prevalence of PD of 180/100 000 inhabitants aged over 50 years (Ferreira *et al.*, 2017).

Although the majority of PD patients develop the disease between 50 and 80 years of age (sporadic PD), most likely due to the interaction between a genetic predisposition with environmental factors (including age, male sex, unhealthy diet, infections, environmental toxins and trauma) (Hayes, 2019; Ascherio and Schwarzschild, 2016), a "young-onset" or "juvenile Parkinsonism" form of PD may also affect individuals between 21 and 40 years old, and appears to be largely of genetic origin (Mehanna and Jankovic, 2019). Among the putative genes underlying the young-onset PD, genetic studies suggest that 5%–10% of the cases have a monogenic cause related with the (at least) 11 forms of genetic parkinsonism described so far, being the prevalent genetic risk factor the gene for the glucocerebrosidase (GBA) (Blauwendraat *et al.*, 2019; Chang *et al.*, 2017). However, the genes that code for LRRK2 (leucine-rich repeat kinase 2), SNCA (α-synuclein), VPS35 (vacuolar protein sorting-associated protein 35), EIF4G1 (eukaryotic translation initiation factor 4 gamma 1) and CHCHD2 (coiled-coil-helix-coiled-coil-helix domain containing 2) have been also widely studied for autosomal dominants forms, the PRKN (Parkin ligase), PINK1 (PTEN-induced kinase 1) and DJ-1 (protein deglycase DJ-1, also known as Parkinson disease protein 7) for recessives, and RAB39B (Ras-related protein Rab-39B) has been associated to the X chromosome (Lunati *et al.*, 2018; Kim and Alcalay, 2017).

Clinically, motor symptomatology (bradykinesia, rest tremor, rigidity, and postural disturbances) is the most predominant in PD, which is often associated with several early non-motor symptoms (as hyposmia, rapid eye movements, sleep behaviour disorder, changes in personality, pain, paresthesias and depression), and troublesome late-onset symptoms (such as urinary disturbances, postural instability and falls, freezing of gait, speech, swallowing difficulties, dementia and hallucinations) (Cabreira and Massano, 2019; Hayes, 2019). Neurochemically, PD involves the progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc). As a result, the denervation of the nigrostriatal tract occurs, yielding a significant reduction of the striatal dopamine content (Zhai *et al.*, 2019; Surmeier, 2018; Kaasinen and Vahlberg, 2017). Another pathological hallmark of PD is the intracellular accumulation of SNCA proteins and the build-up of Lewy bodies (LBs) (comprising nitrated, phosphorylated, and ubiquitinated proteins surrounded by a halo of α-synuclein neurofilaments) in the surviving neurons (Walker *et al.*, 2019; Dehay *et al.*, 2015). Accordingly, SNCA protein misfolding, mitochondrial dysfunction, lysosome/proteasome impairment,

autophagy and neuroinflammation have been increasingly involved in the PD-associated neurodegenerative process (Candeias *et al.*, 2020; Lu *et al.*, 2020; Esteves *et al.*, 2018; Gelders *et al.*, 2018).

Similar to AD, besides the genetic testing in specific circumstances, the lack of accurate early biomarkers poses some limitations to the diagnosis of PD, which is currently based on worldwide stringent clinical criteria (Reich and Savitt, 2019; Tolosa *et al.*, 2006; World Health Organization (WHO), 2006). More recently, the advances in medical radioimaging allowed for the use of fluorodopa positron emission tomography (FDOPA-PET) and dopamine transporter imaging by single photon emission tomography (DAT-SPECT) to complement the clinical diagnosis of PD (Kuten *et al.*, 2020; Palermo and Ceravolo, 2019; Pagano *et al.*, 2016).

As discussed previously for AD, individuals with T2D have twice the risk to develop PD later in life and with a worse outcome/severity (Morsi *et al.*, 2018; Cereda *et al.*, 2012). This was further supported by a recent large cohort study based on the data from the English National Hospital Episode Statistics demonstrating a positive association between preexisting T2D and PD, whose magnitude was higher among those individuals with a poorer T2D management (De Pablo-Fernandez *et al.*, 2018). Conversely, more than 60% of the PD patients also present the main features of T2D, suggesting a role for impaired brain insulin signaling and glucose sensitivity herein (Bosco *et al.*, 2012). This was in line with evidence from *in vitro* PD models demonstrating that the insulin- or IGF-1-induced activation of the PI3K/Akt/GSK3 pathway rescued the toxicity in neurons (Ramalingam and Kim, 2016; Kao, 2009). Therefore, it is not surprising that the restoration of brain insulin signaling, *e.g.*, by repurposing anti-T2D drugs could be neuroprotective and ultimately constitute promising therapeutic strategies against PD (De Pablo-Fernandez *et al.*, 2018), as further detailed in section 1.5. Besides insulin resistance, other crosslinking mechanisms between PD and T2D that may condition its etiology and/or progression (Hong *et al.*, 2020; Sanchez-Gomez *et al.*, 2020) include metabolic and mitochondrial dysfunction, endoplasmic reticulum (ER) stress, impairment of the ubiquitin–proteasome and autophagy–lysosome systems, and inflammation (Santiago and Potashkin, 2013).

1.4 – Sex differences

Mounting evidence suggest the need to address sex and gender differences in biomedical research, namely on their impact in epidemiology, pathophysiology, therapy and outcome of many diseases (including depression, stroke, AD, PD and multiple sclerosis, among others) (Hanamsagar and Bilbo, 2016).

During the recent decades, a sexual dimorphism has been described, *e.g.* in terms of glucose homeostasis and energy balance (Kautzky-Willer *et al.*, 2016; Grant *et al.*, 2009). These include reports that Australian women had lower fasting plasma glucose, but higher plasma glucose following a 2h OGTT, suggesting a difference in the prevalence of glucose intolerance between men and women (Sicree *et al.*, 2008). Other authors observed a higher insulin sensitivity in women, most likely due to an enhanced glucose disposal by the skeletal muscle, and a decreased peripheral susceptibility to fatty acid-induced insulin resistance (Frias *et al.*, 2001; Nuutila *et al.*, 1995). Women also exhibited greater postprandial insulin and C-peptide concentrations, alongside a more effective insulin-dependent glucose uptake (Basu *et al.*, 2006). Therefore, it is not surprising that, despite the higher incidence of T2D in women, its global prevalence is greater among men (Mauvais-Jarvis, 2018). A similar profile was reported in 2015 among the Portuguese men, whose T2D prevalence is 15.9%, whereas in women is 10.9% (Sociedade Portuguesa de Diabetologia (SPD), 2016; Gardete-Correia *et al.*, 2010). However, this sexual dimorphism in the prevalence of T2D appears to be not so linear, but rather dependent on the stage of reproductive life (Wild *et al.*, 2004). Indeed, it has been long recognized that T2D is more prevalent among men at earlier ages (before the puberty), while women are diagnosed later in life (especially after the menopause) (Wild *et al.*, 2004). Though the precise mechanisms underlying such sexual dimorphism remain debatable, it seems plausible to highlight a putative role for the imbalance in sexual hormones (with a special emphasis on testosterone and estrogen) across the lifespan in the pathogenesis of T2D (Camporez *et al.*, 2019; Gyawali *et al.*, 2018; Mauvais-Jarvis, 2017; Ding *et al.*, 2006). In one hand, mounting evidence suggests the involvement of menopause on T2D etiogenesis, since estrogen deficiency was associated with altered insulin secretion and sensitivity, and with glucose effectiveness (Mauvais-Jarvis *et al.*, 2017). Furthermore, the epidemiologic EPIC-InterAct Study demonstrated that the age at menopause may determine future health outcome, since the hazard ratio of T2D was higher in women who became menopaused

before the age of 40 compared with women menopaused at the 50s (Brand *et al.*, 2013). Importantly, testosterone appears to constitute another variable in metabolic regulation, by altering β-cell function and insulin response in males and females (Gannon *et al.*, 2018; Mauvais-Jarvis, 2018; Muraleedharan and Jones, 2010). More specifically, testosterone deficiency (as occurs during andropause) predisposes men to hyperglycemia and T2D, while the excessive levels of testosterone present in postmenopausal women may increase their risk for T2D (Mauvais-Jarvis, 2018). Adding to this, a considerable sexual dimorphism appears to occur in chronic T2D-related complications and comorbidities (Clements *et al.*, 2020; Kautzky-Willer and Harreiter, 2017). For example, T2D women showed a stronger risk for cardiovascular complications compared with T2D men or non-diabetic women (Bancks *et al.*, 2020; Raparelli *et al.*, 2017; Kautzky-Willer *et al.*, 2016), while diabetic nephropathy has a faster progression (Gomez-Marcos *et al.*, 2015; de Hauteclocque *et al.*, 2014) and diabetic foot syndrome is more likely to develop in men (Morbach *et al.*, 2012; Peek, 2011). Although it remains controversial, some authors hypothesized that T2D attenuates the female biological advantage in the protection against long-term complications (Kautzky-Willer *et al.*, 2016).

To further intricate this scenario, sex-dimorphic features may also occur within the CNS, not only the levels of neuroactive steroids and neurosteroidogenic mechanisms, but also in their response both under physiological conditions and in neurodegenerative disorders (such as diabetic encephalopathy, AD, PD, stroke and multiple sclerosis). This raises the question on whether a sexually-biased function of the CNS or a sex-specific development of brain pathologies exist (Giatti *et al.*, 2020). In support of the later, despite the debate on the actual risk for AD in men and women of the same age, the latest report from Alzheimer's Association stated that AD has a 2/3 higher prevalence among women than men. This was primarily explained by the higher longevity of women and the age-related nature of AD (Alzheimer's Association, 2020). Regarding PD, twice of the clinically diagnosed cases are men, but women have a higher mortality rate and faster progression of the disease (Cerri *et al.*, 2019).

Although the other types of diabetes or pre-diabetic status were not the focus of our studies, several studies pointed towards a sexual dimorphism in the prevalence of pre-diabetic syndromes (Mauvais-Jarvis *et al.*, 2017), including impaired fasting glucose (which affected mainly the men), glucose intolerance (which more often affects

women) (Kautzky-Willer *et al.*, 2016) or metabolic syndrome (which often comprises high blood sugar, high blood pressure, high serum triglycerides, low serum high-density lipoprotein (HDL) and abdominal obesity, being more incident among US, chinese and indian women compared to men, and often predisposes for CVD, stroke and T2D) (Saklayen, 2018; Aguilar *et al.*, 2015; Gu *et al.*, 2005; Gupta *et al.*, 2004). For logical reasons, gestational *diabetes mellitus* (GDM, which corresponds to any degree of glucose intolerance with onset or first recognition during pregnancy) may also constitute a major independent and strong risk factor for a future T2D in 2-5% of pregnant women (Gilmartin *et al.*, 2008). Indeed, studies showed an incidence rate of diabetes 70% higher in women with a history of GDM than in pre-diabetic women (Chiefari *et al.*, 2017; Ratner *et al.*, 2008) and, also very important, fetal sex may impact either the risk for GDM or the future risk of developing T2D among women with GDM (Retnakaran and Shah, 2016; Retnakaran *et al.*, 2015). Conversely, T1D appears to predominate among men (1:7 in Caucasians) (Gale and Gillespie, 2001), possibly due to a stronger residual β-cell function in adolescent girls and to a pivotal role of gonadal hormones (Martinez *et al.*, 2016; Samuelsson *et al.*, 2013).

1.5 – Incretin-based anti-Type 2 diabetes drugs

Nearly 17 years have passed since relevant, new treatments have been established to deal with AD (Cummings *et al.*, 2019a). This, together with the current lack for treatments that alter the underlying AD or PD pathology or progression, and that can be tailored to each patient and/or to the disease progression demonstrate the urgent need for new efficient and safe anti-AD or -PD drugs (Cummings *et al.*, 2019b; Van Bulck *et al.*, 2019; Holscher, 2014b). The two classes of pharmacological therapies against AD currently available include the acetyl-cholinesterase inhibitors (AChEIs) (donepezil, galantamine and rivastigmine) and NMDA receptor antagonists (memantine) (Alzheimer's Association, 2020; Singh *et al.*, 2020). The AChEIs are recommended to treat patients with mild, moderate, or severe AD dementia, as well as those with PD dementia (Lane *et al.*, 2018; Weller and Budson, 2018). These drugs were described to improve the symptoms temporarily, by inhibiting the breakdown of Ach at the synapse, thus increasing its availability (Lane *et al.*, 2018). Conversely, memantine is approved for the treatment of patients with moderate to severe AD (mini-mental state examination (MMSE) score <15), and may act both as a non-competitive

N-methyl-D-aspartate receptor antagonist and as a dopamine receptor agonist (Weller and Budson, 2018).

Regarding the treatment and management of PD, the major focus has been the direct or indirect recovery of the dopaminergic deficits, namely throught the use of levodopa, dopamine agonists, dopa-decarboxylase inhibitors, monoamine oxidase inhibitors or catechol-Omethyl transferase inhibitors (Singh *et al.*, 2020; Van Bulck *et al.*, 2019; World Health Organization (WHO), 2006). These therapeutic approaches may be also complemented by the use of anti-cholinergics and amantadine as primary medications for the symptomatic treatment of PD, while functional surgery has recently become an important therapeutic or palliative approach (World Health Organization (WHO), 2006).

In face of the common molecular mechanisms between T2D, AD and PD, it has been increasingly suggested that novel, efficient and safer anti-T2D drugs may be also beneficial against both neurodegenerative diseases (Cardoso and Moreira, 2020; Cummings *et al.*, 2019b; Van Bulck *et al.*, 2019; Holscher, 2014b; Chen *et al.*, 2012).

1.5.1 – Type 2 diabetes control

The guidelines from WHO and IDF recommend that the first and main approach to control T2D progression and the development of long-term complications should be the maintenance of fed glycemia within the normal range, *i.e*, HbA1c <7% and to avoid blood glucose levels below 3 mmol/L (54 mg/dL)) (World Health Organization (WHO), 2019; American Diabetes, 2017; International Diabetes Federation (IDF), 2017). However, this is often difficult to implement at the earlier stages of T2D, when up to 50% of the individuals with T2D remain undiagnosed until the arousal of its chronic complications (Wu *et al.*, 2014). Thus, more active screening programs are recommended, mainly to individuals at a higher risk for T2D, namely those aged above 40 years, obese or with increased waist circumference, hypertensive and with a family history of diabetes (Wu *et al.*, 2014; Gray *et al.*, 2010). If well succeeded, these screenings may allow the earlier diagnosis of individuals with prediabetes or T2D and the immediate start of blood glucose control (International Diabetes Federation (IDF), 2017; Wu *et al.*, 2014).

Regarding the recommendations for an efficient management of glycemia upon T2D, the first approach usually includes the change in lifestyle (namely an increase in physical activity, a healthy diet with food rich in fiber and with a low-glycemic index, smoke cessation and avoiding the excessive alcohol intake) (Ashrafzadeh and Hamdy, 2019; Kirwan *et al.*, 2017). However, as the disease progresses the patients often lose the interest and the glycemic control becomes inefficient (American Diabetes, 2021b; Ashrafzadeh and Hamdy, 2019). In the long run, the persistent, untreated hyperglycemia may lead to the development of complications, as previously detailed. Thus, the pharmacological approach is often used to achieve an efficient T2D management (Gloyn and Drucker, 2018). According to the guidelines from WHO and IDF this pharmacological strategy against T2D commonly starts with the combination of monotherapy with metformin plus lifestyle changes (World Health Organization (WHO), 2018a; International Diabetes Federation (IDF), 2017). Then, if the baseline HbA1c level is ≥9% or if HbA1c target is not achieved within approximately 3 months the treatment should be intensified by adding a second agent (such as a sulfonylurea, a DPP-4 inhibitor (DPP-4i) or a SGLT2 inhibitor) (American Diabetes, 2021c; Thrasher, 2017). When this dual therapy is not sufficient to reach the HbA1c target or upon T2D progression, a third glucose-lowering drug is recommended (namely basal insulin or a GLP-1 receptor agonist) (Diabetes Canada Clinical Practice Guidelines Expert *et al.*, 2018; World Health Organization (WHO), 2018a). Importantly, the therapeutic involving combined drugs should depend on patient and disease-specific factors to efficiently address several targets within the T2D pathophysiology (Chawla *et al.*, 2020; International Diabetes Federation (IDF), 2017).

Currently, there are several classes of anti-T2D drugs used in the clinics:

- Biguanides: with metformin, or 1,1-dimethylbiguanide being for many years the first choice in the treatment of T2D due to its affordability and long-term safety and efficacy in lowering HbA1c, without promoting significant weight gain and with a negligible risk of hypoglycemia (Sanchez-Rangel and Inzucchi, 2017; Maruthur *et al.*, 2016). Metformin is a potent insulin sensitizer that also lowers the increased rate of hepatic gluconeogenesis (Sanchez-Rangel and Inzucchi, 2017). More specifically, metformin inhibits the mitochondrial respiratory chain complex I, binding in its "deactive" conformation and behaving as a non-competitive inhibitor of the

physiological electron pathway, and rises the AMP/ATP, stimulates the AMP-activated protein kinase (AMPK) and impairs cAMP and PKA signaling in response to glucagon, ultimately changing the overall peripheral cellular energy metabolism (Fontaine, 2018; Moreira, 2014). In terms of its neuroprotective potential, metformin was shown to cross the BBB (Ying *et al.*, 2014), and to enhance insulin action and prevent the development of neuropathological hallmarks of AD (namely, elevated tau phosphorylation was ameliorated and the activities of tau kinases, GSK-3β and extracellular signal-regulated kinases (ERK)-1/2, were restored to normal levels, $A\beta_{1-42}$ levels were decreased and acetylcholinesterase (AChE) activity was significant reduced) in cultured neuronal cells (mouse neuroblastoma cell line, Neuro-2a (N2a)) submitted to hyperinsulinemia-induced insulin resistance (Gupta *et al.*, 2011). Furthermore, in 2008 our group demonstrated that metformin protects GK rats against T2D-associated brain oxidative stress by decreasing thiobarbituric acid reactive substances (TBARS) and malondialdehyde (MDA) levels, and increasing reduced glutathione (GSH) levels and MnSOD activity in brain homogenates (Correia *et al.*, 2008). These evidences were complemented by the more recent population-based 4-year prospective study, the Singapore Longitudinal Aging Study, that found an association between the long-term treatment with metformin and a reduced risk of cognitive decline among individuals with T2D (Ng *et al.*, 2014). However, others reported that metformin administration to individuals with T2D patients or impaired glucose tolerance was associated with a worse cognitive performance (Moore *et al.*, 2013), and with a greater risk for AD among individuals aged 65 and older (Imfeld *et al.*, 2012).

- Sulfonylureas (SUs), whose second generation drugs include glyburide, glipizide, and glimepiride, often constitute the second line of anti-hyperglycemic agents used in T2D management (Webb *et al.*, 2019; Inzucchi *et al.*, 2012). SUs have comparable HbA1c-lowering effects with metformin, but the progressive decline in β-cell function worsens the durability of the glycemic control, and are also associated with a higher risk of hypoglycemia and weight gain (Thrasher, 2017). SUs act by closing K^+ATP channels at the plasma membrane of β-cells, thus increasing the

secretion of insulin (Lv *et al.*, 2020). Regarding the therapeutic potential of SUs against cognitive impairment, evidence are scarce. However, a prospective cohort study suggested that T2D patients treated with SUs may have a decreased risk of dementia, and the combination SUs plus metformin may reduce such risk by 35% over a period of 8 years (Hsu *et al.*, 2011).

- Thiazolidinediones (TZDs) include rosiglitazone and pioglitazone. This class of anti-T2D drugs show similar efficacy in decreasing HbA_{1C} levels as metformin and SUs, most likely by preserving β-cell function and extending the durability of glycemic control (Nanjan *et al.*, 2018; Inzucchi *et al.*, 2012). TZDs enhance insulin sensitivity in peripheral tissues (β-cell, muscle and adipocytes) and liver by activating the peroxisome proliferator-activated receptor gamma (PPAR-γ) (Nanjan *et al.*, 2018). However, their serious adverse effects (namely the increased weight gain, the risk for bone fractures and for chronic heart failure) render the use of TZDs in clinics less appealing (Consoli and Formoso, 2013). Several preclinical and clinical studies pointed towards some neuroprotective effects of TZDs (Li *et al.*, 2015c). For instance, De Felice *et al.*, observed that rosiglitazone protected mature cultured hippocampal neurons against Aβ oligomer-induced loss of synapses (De Felice *et al.*, 2009). In addition, the chronic administration of pioglitazone to a rat model of memory impairment induced by the intracerebroventricular (icv) injection of streptozotocin (STZ) enhanced cerebral glucose utilization, reduced oxidative stress and improved cognitive performance (Pathan *et al.*, 2006). These data were further complemented by the improvement in cognitive function of early to moderate AD patients from two small randomized double-blind trials on rosiglitazone therapy (Risner *et al.*, 2006; Watson *et al.*, 2005).

- SGLT2 inhibitors are the newest class of oral anti-T2D drugs and include canagliflozin, dapagliflozin, empagliflozin and ertugliflozin (van Baar *et al.*, 2018; Heerspink *et al.*, 2016). SGLT2 inhibitors decrease hyperglycemia by preventing glucose reabsorption by the kidney, in an insulin-independent mechanism (Rieg and Vallon, 2018; Abdul-Ghani and DeFronzo, 2008). The later property allows the combination of these drugs with other class(es) of glucose-lowering agents (Donnan and Segar, 2019; van Baar *et al.*, 2018).

SGLT2 inhibitors also reduce HbA1c, body weight and systolic blood pressure, with a lower risk of nephropathy progression and of heart failure in individuals affected by CVD (Brown *et al.*, 2019; Kelly *et al.*, 2019; Heerspink *et al.*, 2016). Although the information on the adverse effects of these drugs is still relative scarce, some cases of diabetic ketoacidosis without significant hyperglycemia, of a small increase in the risk of bone fractures and of acute kidney injury were reported (Diabetes Canada Clinical Practice Guidelines Expert *et al.*, 2018; Neal *et al.*, 2017; Watts *et al.*, 2016; Rosenstock and Ferrannini, 2015). In terms of the neuroprotective potential of SGLT2 inhibitors, recent studies demonstrated that they exerted neuroprotective actions in T2D mouse models (Sa-Nguanmoo *et al.*, 2017; Naznin *et al.*, 2017; Lin *et al.*, 2014). Interestingly, canagliflozin attenuated the obesity-induced neuroinflammation in the nodose ganglion and hypothalamus (Naznin *et al.*, 2017), whereas empagliflozin therapy reduced cerebral oxidative stress and the impairment of cognitive function in *db/db* mice (Lin *et al.*, 2014). Dapagliflozin ameliorated cognitive decline in high fat diet (HFD)-induced obese rats, most likely by improving their brain mitochondrial function, insulin signaling, apoptosis and hippocampal synaptic plasticity (Sa-Nguanmoo *et al.*, 2017).

- Insulin: The use of insulin alone or in a combination therapy should be preferred when T2D patients are unstable, with symptoms and signs of acute decompensation (acute hyperglycemia, dehydration, weight loss and presence of ketones) (Aschner, 2020; International Diabetes Federation (IDF), 2017; Thrasher, 2017). Although the hormone remains the most potent glucose-lowering agent with numerous studies showing its beneficial roles in the CNS by exogenously-added insulin and suggesting an enormous therapeutic potential for the restoration of brain insulin sensitivity against neurodegenerative disorders (Santiago and Hallschmid, 2019; Alagiakrishnan *et al.*, 2013), one must bear in mind that insulin administration is also associated with a significant risk for recurrent hypoglycemia episodes (American Diabetes, 2021c; Umpierrez and Korytkowski, 2016). In this perspective, insulin-induced hypoglycemia resulted in brain cortical and hippocampal oxidative imbalance, exacerbated

mitochondrial dysfunction and dysregulation of plasma amino acids and synaptosomal neurotransmitters in STZ-induced diabetic rats (Cardoso *et al.*, 2013; Cardoso *et al.*, 2011; Cardoso *et al.*, 2010). More recent evidence suggest that hypoglycemia may induce neurological deficits, seizures, coma and even neuronal death, particularly in brain areas involved in learning and memory (Hamed, 2017; Mohseni, 2014; Auer, 2004).

- Incretins: this class of novel and very efficient anti-T2D drugs comprise the subclasses of GLP-1R agonists (including exenatide (exendin-4, Ex-4), lixisenatide, liraglutide, albiglutide, dulaglutide and semaglutide) and DPP-4is (including alogliptin, linagliptin, saxagliptin and sitagliptin) (Duarte *et al.*, 2013). Incretins were shown to improve β-cell function and maintain the glycemic control for longer (Neumiller, 2015; Drucker and Nauck, 2006). From the section 1.2, one could hypothesize that the continuous administration of GLP-1 itself would effectively maintain glucose homeostasis. However, this is unfeasible due to the short half-life (less than 2 min in circulation) of the native GLP-1, since it is rapidly inactivated by the enzyme DPP-4 (Vilsboll *et al.*, 2003a; Deacon *et al.*, 1995). Hence, there has been a medical need to either develop synthetic, more DPP-4-resistant GLP-1R agonists/GLP-1 mimetics and/or efficient inhibitors of DPP-4, as we will detail in sections 1.5.2 and 1.5.3.

Besides the studies described above on the neuroprotective potential of each class of anti-T2D drugs, a recently updated systematic review in people with T2D concluded that any of the investigated pharmacological approaches (which included metformin, SUs and TZDs) could prevent or delay cognitive impairment (Areosa Sastre *et al.*, 2017). However, a pooled analysis from five cohorts failed to find an association between metformin or SUs and dementia risk and more interestingly, observed that insulin increased the risk for dementia by 50% (Weinstein *et al.*, 2019). Altogether, despite the promising potential of anti-T2D drugs to treat cognitive impairment and neurodegenerative events, this should be considered with caution in face of the multiple confounding effects.

1.5.2 – Glucagon-like peptide-1 receptor agonists: Exendin-4 and Liraglutide

GLP-1R agonists are synthetic analogues of the native human GLP-1 that were developed to overcome its rapid degradation by the DPP-4, with improved pharmacokinetic properties and more stable pharmacodynamic profiles than the native peptide (Aroda, 2018; Stolar *et al.*, 2013). The unique anti-T2D property of GLP-1R agonists rendered them highly efficient in the management of glycemia, with a self-limiting insulinotropic effect, thus reducing the risk of hypoglycemia alongside the promotion of insulin gene transcription, glucose-mediated glucagon suppression, enhanced β-cell function, proliferation and neogenesis, and inhibition of β-cell apoptosis (Brunton and Wysham, 2020; Aroda, 2018). GLP-1R agonists also delay gastric emptying and induce satiety, facilitating weight loss (Brown *et al.*, 2019; Ryan and Acosta, 2015).

Since the approval of the first GLP-1R agonist in 2006 (BYETTA® - Ex-4), the efficacy and safety of plus five drugs have been assessed in randomized controlled trials to assess their role in treating T2D. To date, two short-acting (the twice-daily Ex-4 and the once-daily lixisenatide) and five long-acting GLP-1R agonists (the once-daily liraglutide and the once-weekly Ex-4, albiglutide, dulaglutide and semaglutide) were approved for the treatment of T2D (Gentilella *et al.*, 2019). The key results from head-to-head comparative trials of these GLP-1R agonists were summarized in reviews from Madsbad and Aroda (Aroda, 2018; Madsbad, 2016).

Among all GLP-1R agonists, Ex-4 and liraglutide are the two most widely clinically used and best studied to treat T2D (Aroda, 2018). Ex-4 is a mimetic isolated from the saliva of the Gila monster (*Heloderma suspectum*) that shares 53% structural similarity and is more effective in lowering glucose levels than the native GLP-1 (Yap and Misuan, 2019). Taking into account the short-action of Ex-4 and its main effect in regulating postprandial glucose, the drug should be administered before meals (Abd El Aziz *et al.*, 2017). In a triple-blind, placebo-controlled, 30-week study, Ex-4-treated (10 μg *b.i.d.*) subjects with inadequate glycemic control by metformin and/or sulfonylurea, showed HbA1c reductions of ∼1.0–1.2%, of ∼20 mg/dL in fasting plasma glucose levels and of ~1 to 2 kg in body weight (DeFronzo *et al.*, 2005). Additionally, in a randomized, controlled trial involving metformin-treated T2D patients, treatment with

Ex-4 for one year improved β-cell function compared with insulin glargine (Bunck *et al.*, 2009).

Liraglutide is a true, long-acting, GLP-1R agonist that shares 97% sequence homology with human GLP-1 (Rossi and Nicolucci, 2009). Importantly, liraglutide resulted from the modification of native GLP-1 by replacing a lysine residue at position 34 by arginine plus and addition of a 16 carbon fatty-acid side-chain to lysine at position 26 (Madsen *et al.*, 2007; Knudsen *et al.*, 2000). These alterations promoted the non-covalent binding of this modified GLP-1 analogue to serum albumin to resist to DPP-4 degradation, to protect against renal clearance and self-association to form heptamers, thus slowing its absorption rate and prolonging half-life in plasma to 13h (in contrast with the 2h half-life of Ex-4) (Iepsen *et al.*, 2015). These changes resulted in the first daily-administered GLP-1R agonist, liraglutide (Knudsen and Lau, 2019). To date, several studies demonstrated the beneficial effect of liraglutide treatment in T2D individuals (Pratley *et al.*, 2019; Tamborlane *et al.*, 2019; Mann *et al.*, 2017; Marso *et al.*, 2016). For example, in 2009 the Liraglutide Effect and Action in Diabetes (LEAD) studies reported that liraglutide reduced HbA1C levels by 0.9-1.4%, fasting plasma glucose by ~30-50 mg/dL and body weight by ~1-3 kg in T2D patients (Russell-Jones *et al.*, 2009; Zinman *et al.*, 2009; Garber *et al.*, 2009; Nauck *et al.*, 2009; Marre *et al.*, 2009). These observations were also accompanied by their decrease in blood pressure and the increase in heart rate (Russell-Jones *et al.*, 2009; Zinman *et al.*, 2009; Garber *et al.*, 2009; Nauck *et al.*, 2009; Marre *et al.*, 2009). Interestingly, the LEAD-6 trial showed that liraglutide was more efficient than Ex-4 *b.i.d.* in reducing HbA1c levels (by 1.12% vs 0.79% respectively) and in improving the HOMA-β (homeostasis model assessment of β-cell function) index by 41.93% vs 16.81% in T2D patients treated for 26 weeks (Buse *et al.*, 2009). In a 14-week extension of the LEAD-6 study, the patients who started and responded well to Ex-4 *b.i.d.* treatment could even further ameliorate some parameters (HbA1C levels by 0.32%, body weight by 0.9 kg and systolic blood pressure by 3.8 mmHg) when switching to liraglutide (Buse *et al.*, 2009). Adding to these effects, in the double-blind SCALE Obesity and Prediabetes trial, the administration of liraglutide (3.0 mg dose, daily) for 56 weeks to individuals with a $BMI \geq 30\ kg/m^2$ reduced their body weight by $8.0 \pm 6.7\%$ (8.4 ± 7.3 kg) and markedly reduced their appetite (Pi-Sunyer *et al.*, 2015). Importantly, a weight loss of 5% or greater occurred in 54.3% of the overweight and obese participants with T2D upon a

similar liraglutide administration in the SCALE Diabetes Randomized Clinical Trial (Davies *et al.*, 2015). More recent, data from the 3-year follow-up of the SCALE Obesity and Prediabetes trial, the once-daily subcutaneous (s.c.) liraglutide (3.0 mg) administration to individuals with obesity and pre-diabetes reduced their risk by 4% for T2D (le Roux *et al.*, 2017).

Despite these increasingly demonstrated benefits of the most commonly used GLP-1R agonists Ex-4 and liraglutide, the choice of a GLP-1R agonist for the treatment of T2D is still limited by their high cost and the need for an injectable administration (American Diabetes, 2021c) (noteworthy that a recent oral version of semaglutide was approved for medical use in the United States in September 2019 and in the European Union in April 2020) (Anderson *et al.*, 2020). Furthermore, the adverse events most frequently associated with GLP-1R agonists include gastrointestinal complications (nausea, vomiting and diarrhea) that tend to diminish as treatment progresses (Madsbad, 2016; Handelsman *et al.*, 2015), as well as some risk for pancreatitis (Thrasher, 2017). The later adverse effect renders these drugs contraindicated in patients with personal or family history of medullary thyroid carcinoma and in patients with multiple endocrine neoplasia syndrome type 2.

As previously detailed, since secretion and action of incretin hormones account for 70% of the insulin response to postprandial circulating glucose. This, together with the ubiquitous expression and actions of GLP-1/GLP-1R within the brain, and the described crosslinking mechanisms between T2D, neurodegenerative diseases and/or normal aging, suggest that the incretin hormones and their pharmacological mimetics may constitute appealing alternatives to restore insulin action within the CNS under such conditions (Erbil *et al.*, 2019; Vilsboll and Holst, 2004). Indeed, GLP-1R agonists have been demonstrating consistent results in the prevention or attenuation of T2D-associated neuronal and cognitive deficits, being also potentially neuroprotective against AD and PD (Candeias *et al.*, 2015; McClean *et al.*, 2010). More specifically, Gault and Holscher (2018) showed that GLP-1R agonists (namely Ex-4, liraglutide and (Val8)GLP-1(Glu-PAL)) improved memory formation, synaptic plasticity, neuronal growth and repair, and reduced inflammation, apoptosis and oxidative stress in animal models of T2D (namely HFD and *ob/ob* mice, and HFD/STZ-induced T2D rats) (Gault and Holscher, 2018). Analogous observations were described in the brains from mouse models of AD (namely APP/PS1 and 3xTg-AD mice), treated with GLP-1R agonists

(namely Ex-4, liraglutide and (Val8)GLP-1(Glu-PAL)), which mitigated apoptosis, neuronal oxidative stress, inflammatory responses and the detrimental effects of β-amyloid and plaque formation, while inducing neurite outgrowth, synaptic plasticity and memory formation (Holscher, 2010).

The beneficial impact of the GLP-1 analogue Ex-4, within the CNS appears to range from a hippocampal neurogenic effect in adult rodents controls to the improvement in hippocampal-associated cognitive performance (as given by the radial maze test and decreased immobility in the forced swim test) (Isacson *et al.*, 2011), in cognition (as given by the Y-maze test) and in the locomotor activity (as given by the open field test) of T2D rodents, most likely by exerting a brain anti-oxidant action, and by restoring their brain-derived neurotrophic factor (BDNF) gene and synaptophysin expression in the frontal cortex (Abdelwahed *et al.*, 2018). Adding to this, Ex-4 may have a potential therapeutic against AD, since it attenuated mitochondrial toxicity (in a PI3K/Akt-dependent pathway), Aβ accumulation and the activity of AChE within the hippocampus and pre-frontal cortex from $A\beta_{1-42}$-induced cognitive deficit rats (Garabadu and Verma, 2019). These effects of Ex-4 were accompanied by the attenuation of Aβ-induced memory-deficits, as given by the Morris water maze (MWM) and Y-maze test protocols in AD-like animals (Garabadu and Verma, 2019). These preclinical data were only partially corroborated by an 18-month, double-blind, randomized placebo-controlled phase II clinical trial (Clinical Trial.gov Identifier: NCT01255163) involving a small number of AD individuals, aiming to examine the efficacy and safety of Ex-4 (Mullins *et al.*, 2019). Although peripheral Ex-4 treatment did not improve the clinical and cognitive markers in AD compared to placebo individuals, it reduced their Aβ-42 in plasma neuronal extracellular vesicles (Mullins *et al.*, 2019). Regarding PD, Ex-4 reduced the amphetamine-induced rotations in the 6-hydroxydopamine (6-OHDA) rat model of the disease and increased the number of their tyrosine hydroxylase (TH)- and vesicular monoamine transporter 2 (VMAT2)-positive neurons in the substantia nigra (Bertilsson *et al.*, 2008). In line with these observations, a single-blinded, pilot clinical trial demonstrated that the s.c. Ex-4 injection for 12 months clinically improved the motor and cognitive measures in 45 PD patients compared with the control group (Clinical Trial.gov Identifiers: NCT01174810) (Aviles-Olmos *et al.*, 2013).

Liraglutide-mediated neuropotection against T2D appears to involve the recovery of brain metabolic homeostasis (as given by the increased mitochondrial transcription factor A (TFAM), sirtuin (SIRT)1, and AMPK phosphorylation), the improved brain mitochondrial regulation (via peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α)), and the decrease in hippocampal lipid oxidation (upon determination of 4-hydroxynonenal levels), ultimately promoting the synaptic plasticity (as given by the BDNF-tropomyosin receptor kinase B (TrkB) signaling) in the UCD-T2D rats over the disease progression (Agrawal *et al.*, 2014; Cummings *et al.*, 2010). Analogously, McClean *et al*. (2011) first demonstrated that liraglutide exerts neuroprotective effects against AD, by preventing the memory impairments in object recognition and water maze tasks in the APP/PS1 mouse model of AD (McClean *et al.*, 2011). These and other authors further described that the intraperitoneal (i.p.) administration of liraglutide once daily, for 8 weeks reduced brain amyloid plaque formation by 30%-50%, the levels of soluble amyloid oligomers (by 25%) and APP, the activation of microglia by up 50% and the Aβ-associated astrocytic activation, while increasing IDE and synaptophysin, LTP formation and the number of hippocampal neuronal progenitor cells in APP/PS1 mice (McClean and Holscher, 2014; Long-Smith *et al.*, 2013; McClean *et al.*, 2011). These and other preclinical studies prompted the evaluation of liraglutide's therapeutic potential against degenerative disorders and AD in two ongoing clinical trials (Clinical Trial.gov Identifiers: NCT01469351 and NCT01843075). The first observations from the trial proposed by the University of Aarhus suggest that the treatment of AD with liraglutide for 6-month may prevent the decline in glucose metabolism and, therefore, the cognitive impairment, synaptic dysfunction and disease evolution (Gejl *et al.*, 2016). More recently, preventing the AD-associated decline of cerebral metabolic rate for glucose in a 26-week, randomized, placebo-controlled, double-blinded intervention in patients with AD (Gejl *et al.*, 2017). Of note, Yang *et al.* (2013) reported that liraglutide normalized brain Akt and GSK-3β activity and reduced tau phosphorylation in HFD/STZ-induced T2D rats, suggestion that the drug could prevent the arousal of AD in individuals with T2D (Yang *et al.*, 2013).

Regarding the therapeutic potential of liraglutide against PD, *in vivo* studies showed that administration of liraglutide (25 nmol/kg i.p. once-daily for 7 days) reversed the motor impairment (assessed by the rotarod and grip strength tests), partially

reversed dopamine synthesis (indicated by the levels of TH) in the substantia nigra and striatum, reduced the activated microglia and astrocytes, and enhanced the expression of the neuroprotective growth factor glial derived neurotrophic factor (GDNF) in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropypridine (MPTP) mouse model of PD (Yuan *et al.*, 2017). Interestingly, recent studies also reported that s.c.-administered liraglutide may neuroprotect against ischaemia-reperfusion injury, by reducing the infarct size by 48%, improving the neuroscore by 6.0 72h following a 90min middle cerebral artery occlusion (MCAO), and protecting against apoptosis (Basalay *et al.*, 2019). These effects may involve the reduction of ischemia-associated ROS, and the activation of the PI3K/Akt and MAPK pathways (Zhu *et al.*, 2016).

1.5.3 – Dipeptidyl peptidase-4 inhibitors: Linagliptin

DPP-4i, as GLP-1R agonists, are second-line glucose-lowering medications that can be used as monotherapy or in combination for the treatment of T2D (Diabetes Canada Clinical Practice Guidelines Expert *et al.*, 2018; International Diabetes Federation (IDF), 2017). Their anti-T2D effects involve primarily an inhibition of the serum DPP-4 by ≥80%, consequently inhibiting the degradation of its endogenous substrates (namely the incretin hormones, whose GLP-1 levels in circulation were increased by approximately four-fold) (Gilbert and Pratley, 2020; Pathak and Bridgeman, 2010). Thus, DPP-4i may prolong the postprandial insulin secretion and insulin-sensitizing effects (Deacon and Holst, 2013). Meta-analyses studies indicated that DPP-4i have a moderate glycemic efficacy, reducing HbA1C by 0.6–0.8%, and modest effects in lowering the systolic and diastolic blood pressure (Zhang and Zhao, 2016; Aroda *et al.*, 2012). However, as described for GLP-1R agonists, DPP-4i often lose effectiveness with the progression of insulin resistance and deterioration of pancreatic β-cells function (Hamilton *et al.*, 2011). Furthermore, the adverse effects often reported with DPP-4i include angioedema/urticarial, other immune-mediated dermatological effects, and rare cases of acute pancreatitis (Diabetes Canada Clinical Practice Guidelines Expert *et al.*, 2018; Thrasher, 2017).

The orally-given DPP-4i currently approved for T2D treatment include linagliptin, alogliptin, saxagliptin, vildagliptin and sitagliptin (Ahren, 2019). Linagliptin (8-[(3R)-3-aminopiperidin-1-yl]-7-but-2-ynyl-3-methyl-1-[(4-methylquinazolin-2-yl)

ethyl]purine-2,6-dione) constitutes the most attractive DPP-4i for the management of T2D, mostly because it is primarily cleared by non-renal mechanisms (85% of the drug is eliminated unchanged in the feces) and, therefore, there is no restriction for its use in patients with renal dysfunction (Keshavarz *et al.*, 2017). Linagliptin has demonstrated a similar efficacy as the other DPP-4i drugs (Ahren, 2019), being rapidly absorbed after oral administration and reaching a maximal plasma concentration after approximately 90min, with a half-life of 10h with 5 mg dosing (Retlich *et al.*, 2015).

Although under physiological conditions DPP-4i do not cross the BBB, numerous studies demonstrated a neuroprotective role in stroke, AD or PD experimental models (Darsalia *et al.*, 2019; Isik *et al.*, 2017; Svenningsson *et al.*, 2016). Hence, it is possible that the neuroprotective effects of DPP-4i may rely on indirect peripheral mechanisms (rather than the DPP-4i-mediated inhibition of the degradation of GLP-1), *e.g.*, the involvement of other substrates (like GIP and the pituitary adenylate cyclase-activating polypeptide (PACAP)) on the regulation of glycaemia (Al-Badri *et al.*, 2018). Strikingly, the neuroprotective effects of linagliptin against stroke in the normal and T2D mouse brain were correlated with the increased plasma GLP-1, but independent from GLP-1R (Chiazza *et al.*, 2018; Darsalia *et al.*, 2016) and/or from glycemic control (Darsalia *et al.*, 2013). Accordingly, Mi *et al.*, (2019) showed that linagliptin-mediated neuroprotection was correlated with the SIRT1/hypoxia-inducible factor (HIF)-1α/vascular endothelial growth factor (VEGF) pathway (Mi *et al.*, 2019), while Ma *et al.*, (2015) demonstrated an association with the attenuation of BBB disruption and of oxidative stress (Ma *et al.*, 2015). Regarding linagliptin, Darsalia *et al.* (2014, 2013) observed that it enhanced neural stem cell (NSC) proliferation and increased neuronal survival by ∼30% in the brains from T2D mice submitted to stroke (Darsalia *et al.*, 2014b; Darsalia *et al.*, 2013). Moreover, linagliptin administration to T2D GK rats for 4 weeks decreased endothelial toll-like receptor (TLR)2 expression, and increased their nitric oxide (NO) bioavailability, resulting in lowered plasma endothelin-1 (ET-1) levels and reduced ET-1-induced cerebrovascular contraction, ultimately recovering their ET-1-mediated cerebrovascular dysfunction (Hardigan *et al.*, 2016). Interestingly, oral administration of linagliptin to 9-month-old 3xTg-AD mice for 8 weeks mitigated their cognitive deficits (as given by an improved performance on the MWM and Y-maze tests), reduced their brain $A\beta_{1-42}$ levels, tau phosphorylation and neuroinflammation markers (Kosaraju *et al.*, 2017). Similar beneficial effects of acute administration of

linagliptin were reported in a randomized, crossover, placebo-controlled trial involving 46 T2D patients (Fadini *et al.*, 2016). Indeed, the drug increased the number of vasculo-regenerative and anti-inflammatory cells, suggesting a decrease of vascular risk in T2D (Fadini *et al.*, 2016). In another randomized study involving 29 T2D individuals treated for 16 weeks, linagliptin (5 mg/day) improved endothelial function (Shigiyama *et al.*, 2017).

Chapter 2

Objectives

2.1 - Hypothesis and Objectives

As previously described, some of the most prevalent age-related diseases nowadays, like T2D, AD and PD, share numerous mechanisms. Among them, we emphasize the brain abnormalities, cognitive decline and increased risk for dementia, which may arise from the complex interaction between normal brain aging and central insulin signaling dysfunction. Moreover, the differential male and female sex-related hormone profiles throughout the lifespan may also differentially affect the brain, and may, thus, explain the increased susceptibility of women (especially after attaining the menopause) to AD.

Regarding the association of T2D with PD, it has been hypothesized that the compromise of the nigrostriatal pathway and the loss of protection from parvalbumin (PV) interneurons in aging and in obesity/diabetes may enlighten the causes for the increased risk of diabetics to develop PD.

Drugs developed to treat a disease could be effective against similar conditions. Considering the impressive effects of incretin-based therapies in T2D patients and the improvement in the CNS function achieved with these compounds, both GLP-1R agonists (Ex-4 and liraglutide) and DPP-4is (linagliptin) demonstrated potential as therapeutic tools against neurodegenerative diseases, like AD and PD.

From the above, we hypothesized that, on one hand, the sexual dimorphism in brain hormone-mediated intracellular signaling pathways in T2D brain contributes to the different vulnerability for neurodegenerative conditions and, on the other hand, the peripheral administration of anti-T2D incretin drugs protect against the neurodegenerative events associated with T2D at midlife, and AD and PD upon aging.

In this perspective, we aimed to unveil: 1) the differential effect of a sex-specific hormonal pattern in the interaction between normal brain aging and the dysfunctional CNS insulin/IGF-1-mediated signaling pathways, and on the risk for the development of AD-like pathological hallmarks in middle-aged T2D rat brains; 2) the neuroprotective effects of the anti-T2D incretin drugs Ex-4, linagliptin or liraglutide against T2D-, AD or PD-associated neurodegeneration.

More specifically, we aimed to evaluate the effect of sex on the peripheral and brain estrogen/IGF-1/insulin-related signaling, and on the brain cortical accumulation of

oxidative stress and AD-like hallmarks in middle-aged (8 months old) Wistar control and non-obese, T2D GK rats (Chapter 3) (Candeias *et al.*, 2017). Given the increased susceptibility of middle-aged male GK rat brains to the deposition of those markers, then we aimed to analyze the effect of a chronic peripheral Ex-4 administration on their brain cortical: 1) IR-/IGF-1R-/GLP-1R-mediated signaling, autophagic and cell death mechanisms (Chapter 4) (Candeias *et al.*, 2018), alongside its effect on 2) glucose homeostasis and metabolism, mitochondrial function and dynamics (Chapter 5). In parallel, we aimed to determine the impact of aging and/or obesity-induced T2D on PV-positive (PV+) interneurons in the striatum of young and middle-aged (2 and 14-month-old) male C57BL/6 mice, and the neuroprotective potential of a chronic treatment with linagliptin against their striatal pathology (Chapter 6) (Lietzau *et al.*, 2020).

Finally, we aimed to uncover the protective effect of a chronic peripheral administration of liraglutide against the peripheral T2D-like and inflammatory markers, together with its neuroprotective role against AD-like changes in brain cortical sex and metabolic hormones levels/signaling, glucose transport and metabolism, mitochondria dynamics, intracellular stress mechanisms, and AD-like neuropathological hallmarks and cognitive function in the mature (10-month-old), female, 3xTg-AD mouse model (Chapter 7) (Duarte *et al.*, 2020).

Chapter 3

Sex effects in adult rat brains: Type 2 diabetes and Alzheimer disease hallmarks

Adapted from: Candeias E, Duarte AI, Sebastiao I, Fernandes MA, Placido AI, Carvalho C, Correia S, Santos RX, Seica R, Santos MS, Oliveira CR, Moreira PI (2017) Middle-Aged Diabetic Females and Males Present Distinct Susceptibility to Alzheimer Disease-like Pathology. *Mol Neurobiol,* 54**,** 6471-6489. doi: 10.1007/s12035-016-0155-1.

Middle-Aged Diabetic Females and Males Present Distinct Susceptibility to Alzheimer Disease-like Pathology

3.1 - ABSTRACT

Type 2 diabetes (T2D) is a highly concerning public health problem of the twenty-first century. Currently, it is estimated that T2D affects 422 million people worldwide with a rapidly increasing prevalence. During the past two decades, T2D has been widely shown to have a major impact in the brain. This, together with the cognitive decline and increased risk for dementia upon T2D, may arise from the complex interaction between normal brain aging and central insulin signaling dysfunction. Among the several features shared between T2D and some neurodegenerative disorders (*e.g.*, Alzheimer disease (AD)), the impairment of insulin signaling may be a key link. However, these may also involve changes in sex hormones' function and metabolism, ultimately contributing to the different susceptibilities between females and males to some pathologies. For example, female sex has been pointed as a risk factor for AD, particularly after menopause. However, less is known on the underlying molecular mechanisms or even if these changes start during middle-age (perimenopause). From the above, we hypothesized that sex differentially affects hormone-mediated intracellular signaling pathways in T2D brain, ultimately modulating the risk for neurodegenerative conditions. We aimed to evaluate sex-associated alterations in estrogen/insulin-like growth factor-1 (IGF-1)/insulin-related signaling, oxidative stress markers, and AD-like hallmarks in middle-aged control and T2D rat brain cortices. We used brain cortices homogenates obtained from middle-aged (8-month-old) control Wistar and nonobese, spontaneously T2D Goto-Kakizaki (GK) male and female rats. Peripheral characterization of the animal models was done by standard biochemical analyses of blood, plasma, or serum. Steroid sex hormones, oxidative stress markers, and AD-like hallmarks were given by specific ELISA kits and colorimetric techniques, whereas the levels of intracellular signaling proteins were determined by Western blotting. Albeit the high levels of plasma estradiol and progesterone observed in middle-aged control females suggested that they were still under their reproductive phase, some gonadal dysfunction might be already occurring in T2D ones, hence, anticipating their menopause. Moreover, the higher blood and lower

brain cholesterol levels in female rats suggested that its dysfunctional uptake into the brain cortex may also hamper peripheral estrogen uptake and/or its local brain steroidogenic metabolism. Despite the massive drop in IGF-1 levels in females' brains, particularly upon T2D, they might have developed some compensatory mechanisms towards the maintenance of estrogen, IGF-1, and insulin receptors function and of the subsequent Akt- and ERK1/2-mediated signaling. These may ultimately delay the deleterious AD-like brain changes (including oxidative damage to lipids and DNA, amyloidogenic processing of amyloid precursor protein and increased tau protein phosphorylation) associated with T2D and/or age (reproductive senescence) in female rats. By demonstrating that differential sex steroid hormone profiles/action may play a pivotal role in brain over T2D progression, the present study reinforces the need to establish sex-specific preventive and/or therapeutic approaches and an appropriate time window for the efficient treatment against T2D and AD.

Keywords Sex, type 2 diabetes, Alzheimer disease-like hallmarks, insulin, sex steroids

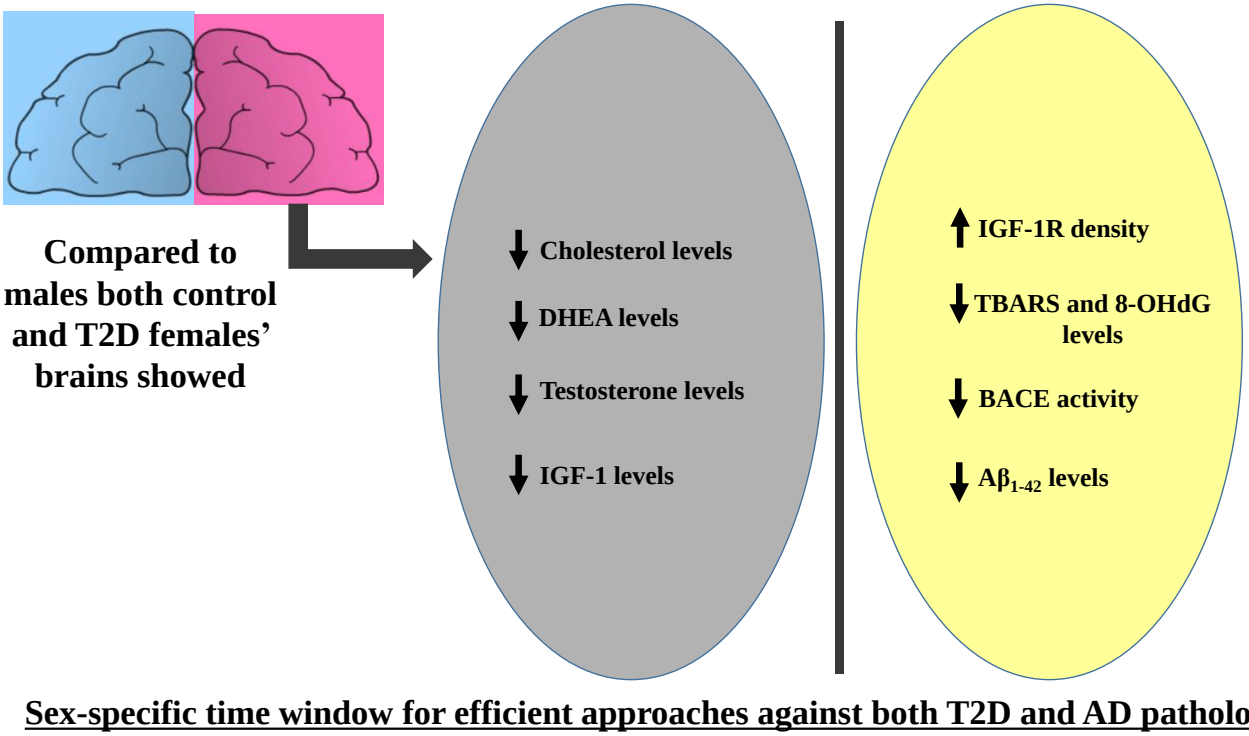


Figure 3.1 – Graphical abstract.

3.2 - INTRODUCTION

The prevalence of type 2 diabetes (T2D) has risen tremendously in the last decades, with estimates pointing towards 1.3 million deaths per year worldwide (NCD Risk Factor Collaboration (NCD-RisC), 2016; Maruthur, 2013; World Health Organization (WHO), 2015). This has recently rendered T2D an pandemic and a highly socioeconomic concern, mainly due to the increasingly aged population, the risk factors associated with modern lifestyle, and the morbidity and mortality associated with its severe long-term complications (particularly those affecting the central nervous system (CNS)) (Rouquet *et al.*, 2013; Duarte *et al.*, 2012a; World Health Organization (WHO), 2015). Such secondary effects of T2D may lead to brain degeneration, dysfunction, and, ultimately, cognitive impairment and dementia (*e.g.*, Alzheimer disease (AD)) (Carvalho *et al.*, 2014a; De Felice and Ferreira, 2014; Wang *et al.*, 2014a; Sima, 2010). Albeit the precise molecular mechanisms underlying T2D-related cognitive dysfunction and AD remain incompletely understood, increasing evidence points towards a crucial role for brain insulin resistance, not only in T2D-related cognitive impairment, but also in AD pathophysiology (Correia *et al.*, 2011; Frisardi *et al.*, 2010). In line with this, it has been increasingly suggested that, besides constituting a potential missing link between both pathologies (reinforcing the novel idea that AD could be a brain-specific insulin resistance), insulin could provide a successful therapeutic approach herein, most likely via the restoration of its brain signaling (Holscher, 2014a; Sebastiao *et al.*, 2014; Rettberg *et al.*, 2014; Duarte *et al.*, 2013; Hunter and Holscher, 2012). Although growing evidence suggests that males and females have significant differences in terms of incidence, progression, and severity of diabetes pathology (Kautzky-Willer *et al.*, 2016; Wandell and Carlsson, 2014; Grant *et al.*, 2009), only a few studies compared the consequences of the disease in males and females and their results are highly contradictory. This renders the knowledge on the subcellular mechanisms underlying the effects of sex in diabetic brain very scarce.

Although the first studies reported a slightly higher diabetes prevalence in men and a higher number of women diagnosed with the disease (most likely explained by the increased women longevity) (Wild *et al.*, 2004), increasing evidence suggests that the ever changing hormonal profiles throughout women's life (particularly their massive drop in estrogen levels upon menopause) render them more prone to metabolic disorders (namely T2D and obesity) and AD, especially upon aging (Rettberg *et al.*,

2014; Alzheimer's, 2016; Pereira *et al.*, 2015; Arnetz *et al.*, 2014; Davey, 2013; Hirata-Fukae *et al.*, 2008; Green and Simpkins, 2000; Bachman *et al.*, 1992). Interestingly, besides sex and aging, T2D may also potentiate AD incidence in women (Wang *et al.*, 2012a). Although higher hippocampal volume reductions and more severe cognitive impairments were found in diabetic women than in men (Hempel *et al.*, 2012; Sakata *et al.*, 2010), Ding *et al.* (Ding *et al.*, 2010) observed that the cognitive decline associated with diabetic retinopathy was significantly higher in men.

It seems unquestionable that the sharp decrement in estrogen levels (and its benefits) in aged menopausal and in estrogen-depleted women may at least partially play a pivotal role in their higher incidence of neurodegeneration, cognitive dysfunction, and memory deficits, similarly to the observations in AD women (Sakata *et al.*, 2010; Ding *et al.*, 2010; Long *et al.*, 2012; Lopez-Grueso *et al.*, 2010; Yue *et al.*, 2005; Sherwin, 2003). Moreover, decreased sex steroid hormones constitute a risk factor for AD both in men and women, whereas therapy with androgens or estrogens could be neuroprotective (Vest and Pike, 2013; Winkler and Fox, 2013), particularly against AD- and diabetes-related neurodegeneration (Caruso *et al.*, 2008; Lapchak and Araujo, 2001). Accordingly, several authors showed a correlation between the impairment in brain mitochondrial estrogen receptors (ERs) and mitochondrial dysfunction in menopausal and AD female rodent brains (Long *et al.*, 2012; Zhao *et al.*, 2012; Yao *et al.*, 2009). Additionally, ovariectomized (OVX, surgically induced menopause) or reproductively senescent females also had lower brain insulin-like growth factor-1 (IGF-1) expression and higher amyloid-β (Aβ) formation (Zhao *et al.*, 2012), whereas estrogen exposure rescued brain insulin/IGF-1 signaling, glucose metabolism, and Aβ accumulation (Rettberg *et al.*, 2014; Zhao *et al.*, 2012; Moran *et al.*, 2013; Alonso *et al.*, 2010). This suggested a synergistic interaction between brain ER, insulin receptor (IR), and IGF-1-receptor (IGF-1R) (probably via PI3K/Akt signaling) that, upon the peripheral estrogen fluctuations in females, could induce brain mitochondrial, metabolic, and synaptic dysfunction, neuronal death, cognitive dysfunction, and ultimately, AD (Rettberg *et al.*, 2014; Zhao *et al.*, 2012; Moran *et al.*, 2013; Alonso *et al.*, 2010). To further aggravate this, diabetes was shown to decrease the levels and benefits of neuroactive steroids in plasma and nervous system (Vikan *et al.*, 2010; Leonelli *et al.*, 2007; Oh *et al.*, 2002), whereas exposure to some of these hormones (such as dehydroepiandrosterone (DHEA), testosterone, or estradiol),

protected against diabetic damage (Mitkov *et al.*, 2013; Munoz *et al.*, 2012; Saravia *et al.*, 2006; Aragno *et al.*, 2002).

The success of (sex-specific) preventive/therapeutic approaches against T2D-associated AD risk may rely mostly on the clarification of the precise molecular links (using the appropriate models for each phase of life) between changes in sex hormones (particularly in the mostly unknown pre- and perimenopausal phases) and T2D-related neurodegeneration. As (1) most studies relied on OVX females to show the potential benefits/damage of estrogen administration/depletion and its pivotal role on sex differences (Lopez-Grueso *et al.*, 2010) and (2) controversy exists on the long-lasting influence of early female fertility experience (or contraception pills) on learning and memory upon aging (Cui *et al.*, 2014; Pawluski and Galea, 2006; Love *et al.*, 2005; Kinsley *et al.*, 1999), we hypothesized that sex-specific hormonal patterns differentially affect insulin/IGF-1/estrogen-mediated signaling in middle-aged T2D brains, thus modulating their vulnerability to AD-like pathology. Accordingly, in this study, we took advantage on our wide experience with the T2D Goto-Kakizaki (GK) rats (Carvalho *et al.*, 2014a; Santos *et al.*, 2014b; Duarte *et al.*, 2004; Moreira *et al.*, 2003; Santos *et al.*, 2000) to analyze the role of sex on insulin/IGF-1/estrogen-related signaling, oxidative stress, and AD-like hallmarks in brain cortical homogenates from previous breeder, middle-aged (8-month-old) male and female control Wistar and T2D GK rats. The GK rat is a non-obese, spontaneously T2D animal model, characterized by moderate (but stable) fasting hyperglycemia, hyperinsulinemia, and hyperleptinemia (when young) that progress towards a deficient glucose-induced insulin secretion and peripheral insulin/leptin resistance in older animals (Moreira *et al.*, 2007c; Moreira *et al.*, 2007d). Although GK rats do not present severe complications at the beginning of disease and, to our knowledge, no direct evidence of AD-like pathology was described in these animals, some studies reported the occurrence of brain-specific alterations in the expression of genes related to neurotransmission, lipid metabolism, neuronal development, insulin secretion, oxidative damage, and DNA repair in hippocampus, pre-frontal cortex, and striatum of 10-week-old male rats (Abdul-Rahman *et al.*, 2012). Additionally, an age-related oxidative imbalance was observed in brain vessels and synaptosomes that may increase their susceptibility to neurodegenerative events upon disease progression (Carvalho *et al.*, 2014a). These findings were in line with our previous reports of increased dysfunction of GK rat brain mitochondria in the presence

of Aβ (Moreira *et al.*, 2003; Moreira *et al.*, 2005b). Recently, Hussain *et al.* (Hussain *et al.*, 2014) observed a significant neuronal loss and increased microglia activation in 13-month-old GK rat brain cortex. Additionally, some impairment in their exploratory activity and learning was found already at 4 months of age (Moreira *et al.*, 2007d), being such memory deficits proportional to the grade of insulin resistance (Li *et al.*, 2013). This, together with the evidence that GK rats develop human-like features of T2D complications upon aging (Moreira *et al.*, 2007c; Moreira *et al.*, 2007d), render them a valuable model to study the peripheral and CNS changes occurring during the progression of the disease *per se* (Moreira *et al.*, 2007c; Moreira *et al.*, 2007d).

3.3 - MATERIALS AND METHODS

3.3.1 - Materials

Ketamine chloride was from Parke-Davis (Ann Arbor, MI, USA) and chlorpromazine chloride was purchased from Laboratórios Vitória (Portugal). Commercial cocktails of protease and phosphatase inhibitors were from Roche Applied Science. Cholesterol RTU test was purchased from Biomérieux SA, (Marcy-l'Etoile, France). DHEA ELISA kit was purchased from Abnova Co. (Taipei, Taiwan). Testosterone ELISA kit was purchased from IBL International (Hamburg, Germany). Estradiol EIA kit and 8-hydroxy-2-deoxy guanosine EIA kit were purchased from Cayman Chemical (Ann Arbor, USA). Rat Estrogen E ELISA kit and Rat Amyloid Beta Peptide 1-42 ELISA kit were purchased from EIAab Science Co. (Wuhan, China). Progesterone ELISA kit was purchased from Uscn Life Science Inc. (Wuhan, China). Rat insulin enzyme immunoassay kit was purchased from SPI-BIO, Bertin Pharma (Montigny le Bretonneux, France). Rat IGF-1 ELISA kit was purchased from Biosensis Pty Ltd. (Thebarton, South Australia). β-secretase activity assay kit fluorogenic and trichloroacetic acid (TCA) were purchased from Calbiochem (Merck KGaA, Darmstadt, Germany). Tau [pS396] human ELISA kit was purchased from Invitrogen (Camarillo, CA, USA). Protein G Plus-Agarose beads and rabbit polyclonal P-Tau Thr181, rabbit polyclonal estrogen receptor (ER)-α, and rabbit polyclonal IGF-1-β antibodies were obtained from Santa Cruz Biotechnology (Heidelberg, Germany). Mouse monoclonal total Tau (BT2) antibody was obtained from Thermo Scientific (Waltham, MA, USA). Rabbit polyclonal P-ERβ (S105) and rabbit polyclonal total ERβ were obtained from

Abcam (Cambridge, England, UK). Rabbit monoclonal insulin receptor (IR)-β, mouse monoclonal P-Akt (Ser473), rabbit monoclonal P-p44/42 ERK (Thr202/Tyr204), rabbit polyclonal total p44/42 ERK, mouse monoclonal p-tyrosine, and rabbit monoclonal α-tubulin antibodies were obtained from Cell Signaling (Leiden, The Netherlands). Mouse monoclonal total Akt was obtained from BD Biosciences. Thiobarbituric acid (TBA), 1,4-dithiotreitol (DTT), phenylmethanesulfonyl fluoride (PMSF), Tween 20, and mouse monoclonal actin antibody were purchased from Sigma Chemical Co. (St. Louis, MO). Polyvinylidene difluoride (PVDF) Hybond-P membranes, anti-mouse, and anti-rabbit secondary antibodies, and ECF substrate for Western Blotting were purchased from GE Healthcare (Little Chalfont, UK). Polyacrylamide was obtained from BioRad (Hercules, CA) and Spin-X centrifuge tube filters used in immunoprecipitation were obtained from Costar (NY, USA). All the other chemicals were of the highest grade of purity commercially available.

3.3.2 - Animals

Following EU and Portuguese legislation (Directive 2010/63/EU; DL113/2013, August 7th), 8-month-old (middle-aged, retired breeders) male and female Wistar control rats and T2D GK rats (a non-obese model that spontaneously develop T2D early in life, resulting from the selective breeding of Wistar rats with high glucose levels) (Santos *et al.*, 2000) 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. Thus, following the "3Rs" Reduction principle established by FELASA, we used the brain cortical levels of estrogen E depicted in Fig. 3.2D to estimate the number of animals required for this study. 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. In line with this and in order to increase the power of our hypothesis, we used at least n = 4 rats/group, obtained from our local animal facilities (conventional animal facilities of Faculty of Medicine, University of Coimbra). Rats were kept in pairs of two animals from the same sex in a static microisolator cage with a filter top and bedding and nesting materials, under controlled light (12-h day/night cycle) and humidity (45–65 %), and *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.

3.3.3 - Peripheral Blood Collection and Routine Biochemical Analysis

After an overnight fasting, blood samples were collected by terminal cardiac puncture from anesthetized [ketamine chloride (75 mg/kg, i.p.) and chlorpromazine chloride (2.65 mg/kg, i.m., Lab. Vitória, Portugal)] 8-month-old rats, as previously described by Matafome *et al.* (Matafome *et al.*, 2011). Briefly, blood was centrifuged at 572×*g* in a Sigma 2-16 PK centrifuge, for 10 min at 4°C and the resultant plasma and serum were snap frozen for subsequent analyses. Blood glucose levels were determined, immediately after the euthanasia of the animals, through the glucose oxidase reaction, using a glucose analyzer (Glucometer Elite, Bayer SA, Portugal) and compatible reactive tests. Serum triglycerides; cholesterol (total, LDL, and HDL); and alkaline phosphatase levels were determined using commercial kits (Olympus-Diagnóstica Portugal, Produtos de Diagnóstico SA, Portugal).

3.3.4 - Isolation and Homogenization of Brain Cortex

Rats were weighed, anesthetized, and euthanized by decapitation, and brains were immediately removed. Brain cortices were immediately dissected and snap frozen for further studies. Immediately before the experiments, brain cortices were homogenized at 0–4 °C, in lysis buffer containing (in mM): 25 HEPES, 2 $MgCl_2$, 1 EDTA, 1 EGTA, pH 7.4, supplemented with 2 mM DTT, 100 μM PMSF and cocktails of protease and phosphatase inhibitors. Then, the mixture was centrifuged at 17,968×*g* for 10 min, at 4 °C in a Sigma 2-16K centrifuge, to remove the nuclei. The resulting supernatant was collected, and the pellet was resuspended in supplemented buffered solution and centrifuged again at 17,968×*g* for 10 min. The supernatant was added to the previously obtained one and the protein was measured using the Sedmak method (Sedmak and Grossberg, 1977).

3.3.5 - Determination of Cytosolic Cholesterol and Steroid Hormone Levels

Brain cortical cytosolic cholesterol levels were measured by the cholesterol RTU test, according to manufacturer's instructions. Briefly, 10 µL of each brain cortical homogenate were added to 1 mL of Calimat reagent and the absorbance read at 550 nm, 37 °C, in a SpectraMax Plus 384 multiplate reader (Molecular Devices, Wokingham, UK). Results were expressed as milligrams per milliliter per milligram protein. Brain cortical cytosolic DHEA content was determined in 10 µL of each rat brain cortical homogenate by the DHEA ELISA kit, according to manufacturer's instructions (with the remaining volumes decreased to half). Absorbance was read at 405 nm in a SpectraMax Plus 384 multiplate reader. Results were expressed as pictogram per milliliter per milligram protein.

Plasma and brain cortical cytosolic testosterone levels were determined in 5 µL of each plasma or brain cortical homogenate by the testosterone ELISA kit, according to manufacturer's instructions. Absorbance was measured at 450 nm, in a SpectraMax Plus 384 multiplate reader. Results were expressed as nanograms per milliliter and nanograms per milliliter per milligram protein, for plasma and brain cortical testosterone levels, respectively.

Plasma estradiol levels were measured by the Estradiol EIA kit, according to manufacturer's instructions. Absorbance was read at 450 nm, in a SpectraMax Plus 384 multiplate reader. Results were expressed as picograms per milliliter.

Brain cortical estrogen levels were determined in 50 µL of each sample by using Rat Estrogen E ELISA kit, according to manufacturer's instructions (with the remaining volumes decreased to half). Absorbance was determined by a SpectraMax Plus 384 multiplate reader, at 450 nm. Results were expressed as picograms per milliliter per milligram protein.

Plasma and brain cortical cytosolic progesterone levels were measured by the Progesterone ELISA kit, according to manufacturer's instructions. Absorbance was measured at 450 nm in a SpectraMax Plus 384 multiplate reader. Results were expressed as nanograms per milliliter for plasma samples and as nanograms per milliliter per milligram protein for brain homogenates.

3.3.6 - Co-Immunoprecipitation and Western Blotting Analysis

Co-immunoprecipitation studies were done accordingly to a previously described procedure (Duarte *et al.*, 2008), with slight modifications. Briefly, 100 μg of brain cortical homogenates were incubated with Protein G Plus-Agarose for 30 min at 4°C, with gentle shaking, and then centrifuged for 5 min at 572×*g* in a Sigma 2-16 PK centrifuge. At this step, 20 μL of the supernatant (the total control) were collected and kept on ice for further analysis by western blotting. The remaining supernatant was incubated with 5 μL of primary antibody (rabbit ERα, rabbit IRβ, and rabbit IGF-1Rβ antibodies) for 1 h at 4 °C with gentle shaking. Then, the immunoprecipitates were collected by discarding the supernatant, following another incubation with Protein G Plus-Agarose and centrifugation. Pellets were then washed four times with phosphate buffer saline (PBS). After the first washing step, 20 μL of the supernatant (non-immunoprecipitated control, non-IP) were collected and kept on ice for further western blotting analysis. After the final wash, the pellets containing the immune complexes were denatured with 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 100 °C, for 5 min. The samples were centrifuged at 17,968×*g* for 10 min, at 4 °C, using a Spin-X centrifuge tube filter (0.45 μm cellulose acetate in 2.0 mL polypropylene tube) to separate the Protein G Plus-Agarose. Samples containing immunoprecipitated denatured proteins or brain cortical homogenates (50 μg per lane) were subjected to SDS/PAGE (10 %) analysis and transferred onto PVDF membranes. Then, membranes were blocked for 1 h at room temperature in Tris-buffered saline (TBS, pH 7.4) plus 5 % nonfat dry milk or bovine serum albumin (BSA), plus 0.1 % Tween 20. Membranes were then incubated overnight at 4 °C, with mouse Phospho-tyrosine (1:2000) (in the case of membranes containing immunoprecipitated rabbit ERα, rabbit IRβ, or rabbit IGF-1Rβ antibodies) or rabbit IRβ antibodies (in the case of membranes containing immunoprecipitated rabbit ERα) and, in the case of membranes containing brain cortical homogenates, with rabbit Phospho-Tau pThr181 (1:250), rabbit ERα (1:1000), rabbit Phospho-ERβ (1:1000), rabbit IRβ (1:1000), rabbit IGF-1Rβ (1:1000), mouse Phospho-Akt (1:1000), and rabbit Phospho-ERK p44/42 (1:1000) antibodies. Membranes were then incubated with the respective anti-rabbit or anti-mouse secondary IgG antibodies (1:20,000), for 2 h at room temperature, and developed using ECF. Immunoreactive bands were visualized by the VersaDoc Imaging System

(BioRad, Hercules, CA, USA). Fluorescence signal was analyzed using the QuantityOne software and the results given as INT/mm2. Of note, membranes were then reprobed with the corresponding mouse total Tau (1:1000), rabbit total ERβ (1:1000), rabbit total Akt (1:1000), rabbit total p44/42 ERK, mouse β-actin (1:5000), or rabbit α-tubulin (1:1000) antibodies. Results were presented as phosphorylated protein/total protein or protein levels (corresponding to the ratio of each protein vs. β-actin or α-tubulin).

3.3.7 - Determination of Plasma and Brain Cortical Insulin and IGF-1 Levels

Plasma and brain cortical cytosolic insulin levels were measured in 25 μL of each plasma or brain cortical homogenate by the rat insulin enzyme immunoassay kit, according to manufacturer's instructions. Absorbance was read at 405 nm in a SpectraMax Plus 384 multiplate reader. Results were expressed as nanograms per milliliter and nanograms per milliliter per milligram protein, for plasma and brain cortical insulin levels, respectively.

Brain cortical cytosolic IGF-1 levels were measured by the Rat IGF-1 ELISA kit, according to manufacturer's instructions. Absorbance was read at 450 nm in a SpectraMax Plus 384 microplate reader. Results were expressed as pictograms per milliliter per milligram protein.

3.3.8 - Measurement of Lipid and DNA Oxidation

The extent of lipid oxidation was determined by measuring thiobarbituric acid reactive substances (TBARS), using the TBA assay (Ernster and Nordenbrand, 1967), with slight modifications. Briefly, 10 μL of each brain cortical homogenate were boiled at 100 °C, for 10 min, in 100 μL of reaction medium containing: 0.375 % TBA, 15 % TCA, 0.25 M HCl, and 6.8 mM β-hydroxytoluene (BHT). Then, samples were chilled on ice and centrifuged at 825×*g* for 10 min at 4 °C in a Sigma 2-16 PK centrifuge. A volume of 125 μL from the resulting supernatant was collected and absorbance measured at 530 nm in a SpectraMax Plus 384 multiplate reader, against a blank prepared under similar conditions, but in the absence of protein. The amount of TBARS

formed was calculated using a molar extinction coefficient of 1.56 × 105 mol^{-1} cm^{-1} and expressed as picomoles per milligram protein.

Levels of the DNA oxidation marker 8-hydroxy-2-deoxyguanosine (8-OHdG) were determined in 10 μL brain cortical homogenate by using 8-OHdG EIA kit from Cayman Chemical Co., according to manufacturer's instructions. Absorbance was read at 405 nm, in a SpectraMax Plus 384 multiplate reader. Results were expressed as picograms per milliliter per milligram protein.

3.3.9 - Determination of β-Secretase-1 Activity and $A\beta_{1–42}$ Levels

β-secretase (BACE) activity was evaluated fluorimetrically in 10 μL of brain cortical homogenates by using the β-secretase Activity Assay Kit, Fluorogenic, according to manufacturer's instructions. Fluorescence was measured in a SpectraMax Gemini EM (Molecular Devices, Wokingham, UK) fluorescence plate reader, with an excitation wavelength of 355 nm and emission wavelength of 510 nm. BACE activity was expressed as relative fluorescent unit (RFU) per milligram protein.

Brain cortical $A\beta_{1–42}$ levels were determined in 50 μL brain cortical homogenates by the rat amyloid beta peptide 1-42 ELISA kit, according to manufacturer's instructions (with the remaining volumes decreased to half). Absorbance was determined at 450 nm, in a SpectraMax Plus 384 multiplate reader. Results were expressed as picograms per milliliter per milligram protein.

3.3.10 - Determination of Phosphorylated Tau Protein (Ser396) Levels

Levels of phosphorylated tau protein at the serine 396 residue were quantified in 10 μL brain cortical homogenates by the tau [pS396] Human ELISA Kit, according to manufacturer's instructions. Absorbance was read at 450 nm in a SpectraMax Plus 384 multiplate reader. Results were expressed as pictograms per milliliter per milligram protein.

3.3.11 - Statistical Analysis

Results are expressed as mean ± SEM of the indicated number of rats. Statistical analysis and graphic artwork were obtained using the GraphPad Prism software. The identification of outliers was done with the ROUT test. Statistical significance was determined using the two-way ANOVA with Fisher's least significant difference (LSD) post-test. A *P* value <0.05 was considered statistically significant.

3.4 - RESULTS

3.4.1 - Effect of Sex on Blood Biochemical Features of Middle-Aged Control and T2D Rats

GK rats were previously described to present a polygenic, non-obese, and spontaneous T2D profile, accompanied by insulin resistance and abnormal glucose metabolism (Janssen *et al.*, 2003; Galli *et al.*, 1999). As in our previous studies (Duarte *et al.*, 2004; Santos *et al.*, 2000), GK rats used herein were hyperglycemic compared to their respective age-matched Wistar cohorts, with the major source of variation being due to diabetes itself ($P < 0.001$) (Table 3.I). A significant effect of sex on plasma insulin levels ($P < 0.0001$) was also observed, with significantly higher plasma insulin levels in both non-diabetic and T2D females than the respective male cohorts (204 and 101 %, respectively) (Table 3.I). Moreover, T2D rat cohorts had higher serum alkaline phosphatase (ALP) and cholesterol (total, HDL and LDL) levels than the non-diabetic animals. Interestingly, the changes in both ALP and cholesterol parameters were dependent on both diabetes (ALP: $P < 0.0001$; HDL: $P < 0.0001$; total: $P < 0.001$) and sex (ALP: $P < 0.01$; LDL: $P < 0.01$; HDL: $P < 0.01$; total: $P < 0.05$), with a significant interaction between both variables being also observed in ALP levels ($P < 0.01$). Type 2 diabetic females had decreased levels of ALP, whereas control ones had increased levels of HDL and LDL (Table 3.I). Notably, the triglycerides levels showed a significant increase in Wistar females compared to male ones, and a ∼17% higher level in these Wistar females than in GK ones ($P < 0.05$ for sex) (Table 3.I).

Table 3.I- Blood biochemical characterization of middle-aged Wistar and GK male and female rats.

	Wistar rats		*GK rats*	
	Males	**Females**	**Males**	**Females**
Blood glucose levels (mg/dL blood)	83.13 ± 5.76 (n=8)	79.25 ± 8.0 (n=4)	163.0 ± 20.59** (n=7)	170.20 ± 27.81$^{\$\$}$ (n=5)
Plasma insulin levels (ng/mL)	0.29 ± 0.13 (n=3)	0.88 ± 0.04*** (n=6)	0.40 ± 0.11 (n=6)	0.81 ± 0.03$^{\text{££}}$ (n=5)
Alkaline phosphatase (ALP) (IU/L)	93.60 ± 7.71 (n=5)	104.80 ± 16.59 (n=4)	235.20 ± 22.45**** (n=5)	134.80 ± 8.39$^{\text{£££}}$ (n=5)
Triglycerides (mg/dL blood)	64.20 ± 10.78 (n=5)	148.80 ± 35.72** (n=4)	104.0 ± 8.23 (n=5)	123.40 ± 17.30 (n=5)
Total cholesterol (mg/dL blood)	45.40 ± 4.0 (n=5)	56.75 ± 4.52 (n=4)	68.0 ± 2.26** (n=5)	75.20 ± 5.27$^{\$\$}$ (n=5)
HDL cholesterol (mg/dL blood)	31.86 ± 4.58 (n=5)	51.60 ± 5.52** (n=4)	62.94 ± 2.19**** (n=5)	72.94 ± 3.63$^{\$\$}$ (n=5)

LDL cholesterol (mg/dL blood)	4.02 ± 1.56 (n=5)	20.70 ± 9.13** (n=3)	15.74 ± 1.78* (n=5)	20.78 ± 2.40 (n=5)

Data are mean ± SEM of the indicated number of animals. Statistical significance: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****P <0.0001 vs. Wistar males; $$ $P < 0.01$ vs. Wistar females; ££ $P < 0.01$, £££P < 0.001 vs. GK males, by two-way ANOVA for multiple comparisons, with Fisher's LSD post-test.

3.4.2 - Sex Steroid Hormones' Dysmetabolism in Female Brains Seems to Precede Detectable Changes in their Peripheral Estrogenic Profile, particularly upon T2D

Given the above-mentioned increment in blood cholesterol levels in both normal and T2D females (Table 3.I) and that cholesterol is the main precursor of sex steroid hormones (Tsutsui, 2012; Mellon and Griffin, 2002) and a pivotal constituent of brain plasma membrane (particularly in lipid rafts), we next analyzed the brain cortical intracellular cholesterol levels in our experimental conditions (Fig. 3.2A). A significant effect of diabetes ($P < 0.001$) and sex ($P < 0.0001$) in brain cholesterol levels was observed. More specifically, the higher brain cholesterol levels in both T2D males and females than their respective Wistar controls (Fig. 3.2A) mirrored their peripheral total cholesterol pattern (Table 3.I). However, in contrast with peripheral cholesterol levels, both Wistar and GK female rats had significantly lower brain cortical cholesterol levels than their respective male cohorts (by 57 and 34 %, respectively) (Fig. 3.2A). Following the sex steroid hormones' metabolic cascade, we then analyzed brain cortical DHEA levels. In line with the previous results, a significant effect for sex ($P < 0.0001$) and T2D ($P < 0.05$) was detected in brain DHEA levels (Fig. 3.2B). These were higher in both T2D cohorts, while 76% and 57 % lower brain cortical DHEA levels occurred in both non-T2D and T2D female rats than in the respective age-matched males (Fig. 3.2B). This was further accompanied by significant effects in both plasma and brain cortical levels of the main sexual hormones—testosterone, estrogen, and progesterone (Table 3.II and Fig. 3.2C–E). More specifically, both sex and T2D altered the brain cortical testosterone levels ($P < 0.001$ and $P < 0.01$, respectively) (Fig. 3.2C). Similar to the previous profiles, brain cortical testosterone levels were significantly increased in

both GK rat cohorts than in Wistar rats (Fig. 3.2C). Moreover, a significant decrement in brain cortical testosterone content occurred in both female cohorts compared to the respective age-matched males (a 56 % difference comparing Wistar and 46 % between GK rats) (Fig. 3.2C). These results were in accordance with sex-induced significant alterations in plasma testosterone levels ($P < 0.01$ for sex) (Table 3.II), with 78 % and 73 % lower plasma testosterone levels occurring in both non-T2D and T2D females than in the respective male groups (Table 3.II). Conversely, plasma estradiol levels were 49 % higher in Wistar females than in males (revealing a significant effect of sex ($P < 0.05$)), whereas those from GK rat females were similar to their males (Table 3.II). Plasma estradiol levels were also 37 % lower in T2D females than in the non-diabetic ones (yielding a significant impact for T2D and for its interaction with sex ($P < 0.05$)) (Table 3.II). Regarding brain cortical estrogen profiles, a significant interaction between T2D and sex ($P < 0.01$) appeared to account for the different pattern observed (Fig. 3.2D). Notably, despite no significant differences between Wistar males and females brain cortical estrogen levels, in GK females, the levels of estrogen were massively decreased compared to their age-matched male cohort (Fig. 3.2D). These results suggested that, despite no evident signs of gonadal failure in middle-aged, retired breeder Wistar female rats, either their estrogen uptake from the periphery into CNS and/or their local steroidogenic metabolism (from cholesterol towards estrogen) might be already compromised, being possibly further accelerated by T2D (as given by the 49 % lower brain estrogen content in GK females than in Wistar ones) (Fig. 3.2D). To further support this hypothesis, we also found a significant effect of sex on plasma progesterone levels ($P < 0.05$) that accounted for the 300 % and 243 % higher plasma progesterone levels in both Wistar and GK females than in males (Table 3.II). This was accompanied by 143 % higher brain cortical progesterone levels in control females than in the respective male cohort (Fig. 3.2E).

Table 3.II- Plasma sex steroid hormones levels in middle-aged Wistar and GK male and female rats.

	Wistar rats		*GK rats*	
	Males	**Females**	**Males**	**Females**
Testosterone levels (ng/mL plasma)	2.05 ± 0.22 (n=3)	0.45 ± 0.03* (n=4)	1.70 ± 0.54 (n=6)	0.46 ± 0.19$^{£}$ (n=5)
Estradiol levels (pg/mL plasma)	77.09 ± 5.07 (n=3)	115.10 ± 9.38** (n=6)	72.94 ± 4.78 (n=6)	72.01 ± 8.26$^{\$\$\$}$ (n=6)
Progesterone levels (ng/mL plasma)	12.09 ± 7.50 (n=3)	48.34 ± 15.28 (n=6)	11.71 ± 6.09 (n=6)	40.12 ± 10.55 (n=6)

Data are mean ± SEM of the indicated number of animals. Statistical significance: *$P < 0.05$, **$P < 0.01$ vs. Wistar males; \$\$\$ $P < 0.001$ vs. Wistar females; £ $P < 0.05$ vs. GK males, by two-way ANOVA for multiple comparisons, with Fisher's LSD post-test.

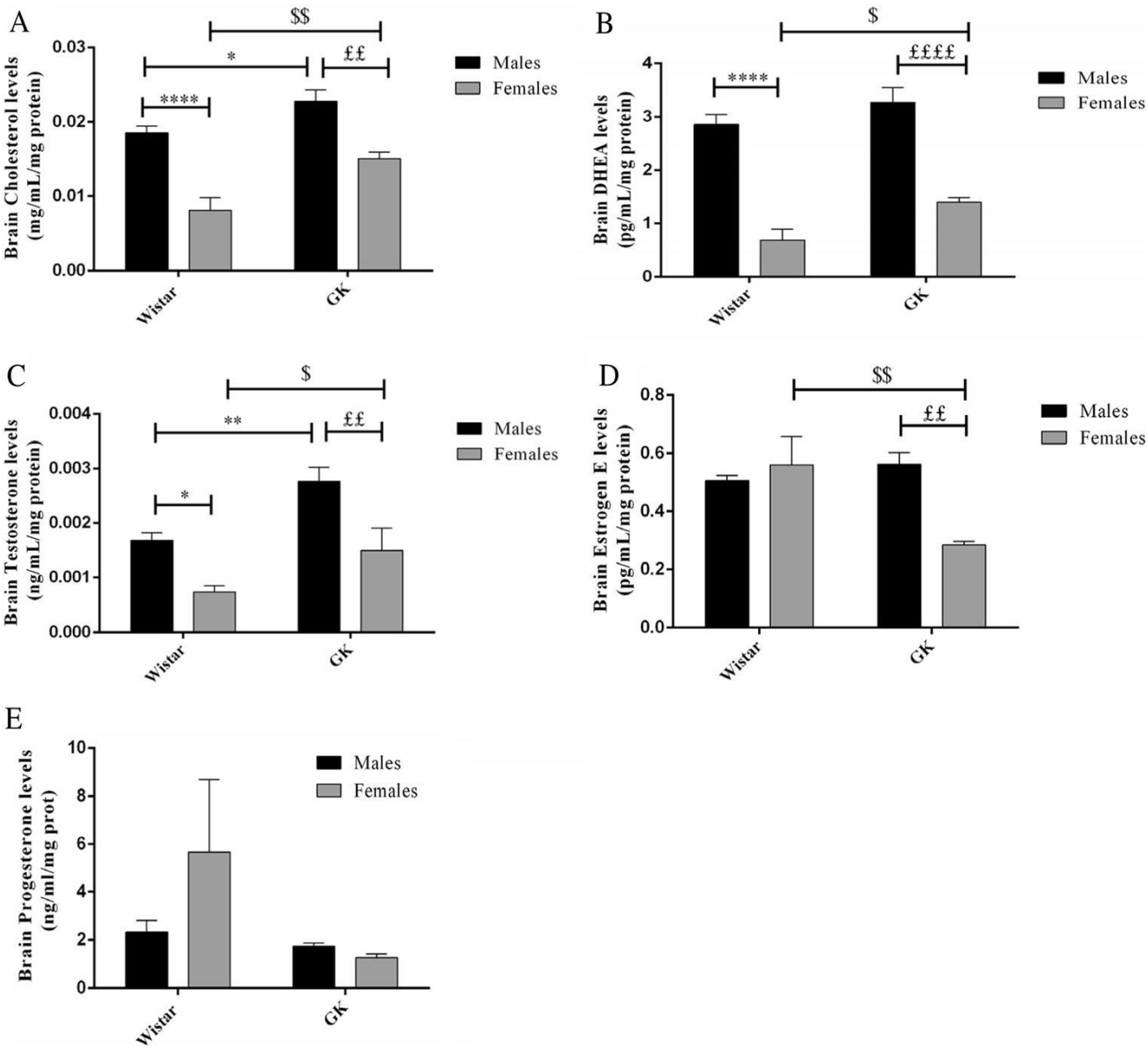


Figure 3.2 - Effect of sex and T2D on middle-aged rat brain cortical sex steroid hormones' metabolism. Brain cortical cholesterol (n = 4) (A), DHEA (n = 4) (B), testosterone (n = 4–6) (C), estrogen E (n = 4) (D), and progesterone levels (n = 4–5) (E). Data are the mean ± SEM of the indicated number of animals. Statistical significance: **P* < 0.05, **P < 0.01, *****P* < 0.0001 vs. Wistar males; $*P* < 0.05, $$*P* < 0.01 vs. Wistar females; ££*P* < 0.01, £££*P* < 0.001 vs. GK males, by two-way ANOVA for multiple comparisons, with Fisher's LSD post-test.

3.4.3 - Effect of Sex and T2D on Brain Estrogen Receptors Density and Phosphorylation

Brain cortex function is highly dependent on estradiol (Alonso *et al.*, 2008), with both male and female cortices being highly enriched in both ERα and β isoforms (Montague *et al.*, 2008; McEwen and Alves, 1999), particularly in neurons and glia (Rettberg *et al.*, 2014; Zhao *et al.*, 2011; Rosario *et al.*, 2010). In line with this and the above results, we next evaluated the role of sex on both brain cortical ERα and β densities and phosphorylation upon T2D (Fig. 3.3A–C). The significant reduction in brain cortical ERα density induced by T2D was reflected by the 45 % lower brain cortical ERα density in GK rat males than in Wistar ones (Fig. 3.3A), suggesting that T2D may partially impair ERα protein expression in males' brains. However, this was not accompanied by a lower ERα tyrosine phosphorylation in brain cortex (Fig. 3.3B). Conversely, the tendentiously higher (by 33 %) ERα density in brain cortices from T2D rat females than males (Fig. 3.3A), pointed towards an eventual compensatory ERα protein expression to overcome their above-mentioned lower brain cortical estrogen levels (Fig. 3.2D), thus accounting for the maintenance of brain cortical ERα tyrosine phosphorylation under such cohorts (Fig. 3.3B). No significant differences were found in brain cortical phosphorylation of the ERβ isoform (Fig. 3.3C).

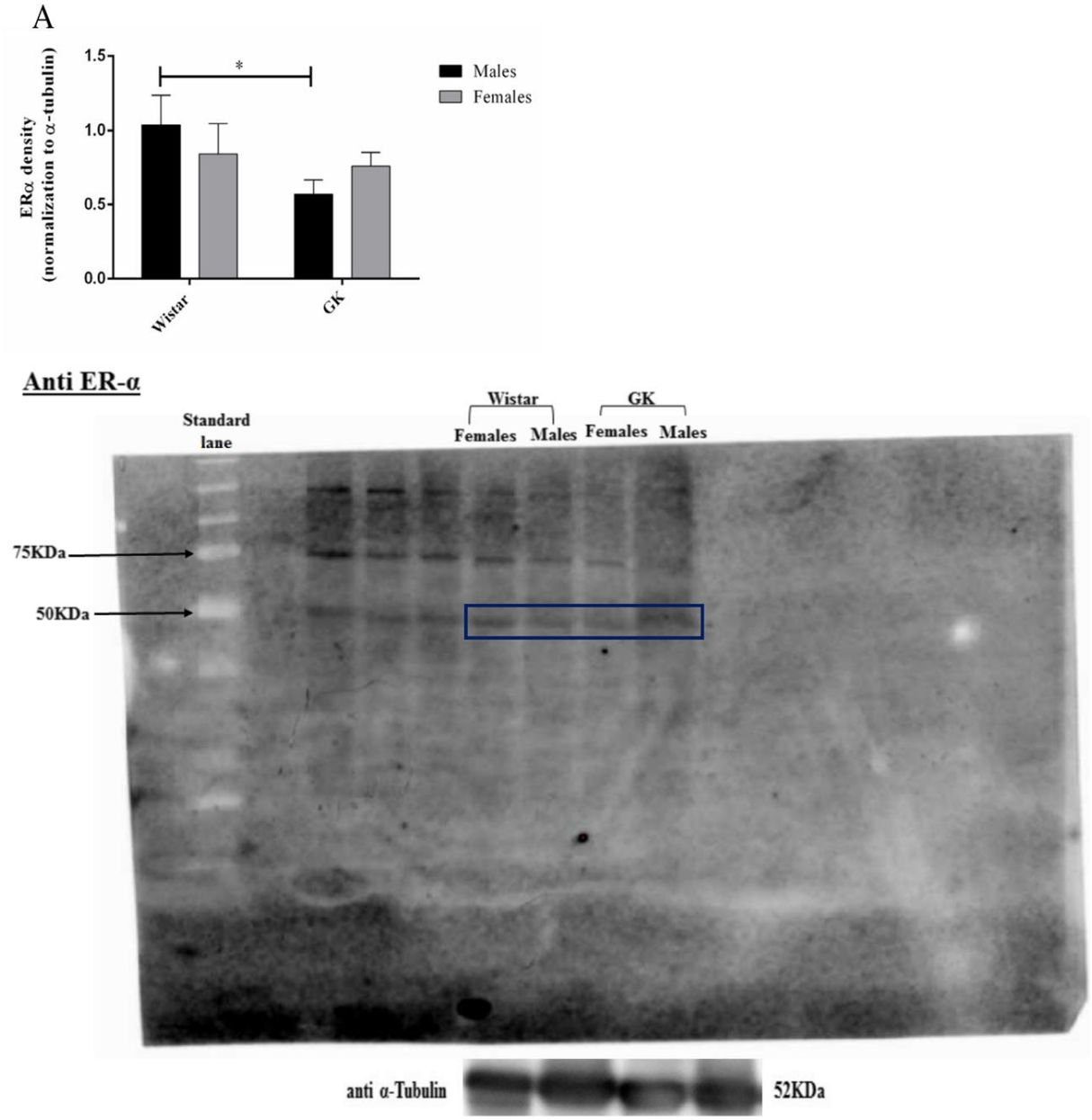

A
ERα density (normalization to α-tubulin)
1.5
1.0
0.5
0.0
*
Males
Females
Wistar
GK
Anti ER-α
Standard lane
Wistar
GK
Females
Males
Females
Males
75KDa
50KDa
anti α-Tubulin
52KDa
B
P-Tyrosine/P-ERα
0.4
0.3
0.2
0.1
0.0
Males
Females
Wistar
GK
Wistar
GK
Females
Males
Females
Males
1 2 3 1 2 3 1 2 3 1 2 3
IP: anti P-Tyrosine
WB: anti ER-α
50KDa
IP: anti ER-α
WB: anti ER-α
50KDa
C
P-ERβ/Total ERβ
8
6
4
2
0
Males
Females
Wistar
GK
Wistar
GK
Females
Males
Females
Males
anti P-ERβ
55KDa
anti Total ERβ
55KDa

Figure 3.3 - Effect of sex and T2D on middle-aged rat brain cortical ERs. ERα density (n = 6) (A) and activation, as given by tyrosine phosphorylation (n = 3) (B), and ERβ activation by phosphorylation (n = 4) (C). Data are the mean ± SEM of the indicated number of animals. Statistical significance: **P* < 0.05, vs. Wistar males, by two-way ANOVA for multiple comparisons, with Fisher's LSD post-test. 1 - total, 2 - non-IP, 3 - IP.

3.4.4 - The Lower Brain IGF-1 Levels in Female Rats was Followed by Increased IGF-1R Densities and the Maintenance of its Receptor Function and Downstream Signaling

Once activated by estrogen, ER (particularly ERα) may interact with IGF-1R/IR or their downstream signaling effectors to protect against AD-like neuropathology (Alonso *et al.*, 2008; Cardona-Gomez *et al.*, 2002a; Cardona-Gomez *et al.*, 2002b; Patrone *et al.*, 1996; Kato *et al.*, 1995). On the other hand, brain IGF-1/insulin resistance in areas highly enriched in their receptors (as cortex and hippocampus) and/or reduced insulin transport across the blood-brain barrier (BBB) occurred in diabetes and AD (Duarte *et al.*, 2013; Moran *et al.*, 2013; Cholerton *et al.*, 2011). In this perspective, we next analyzed the impact of sex and T2D in brain cortical insulin/IGF-1 levels and pivotal downstream signaling markers. Despite no statistically significant differences in brain cortical IGF-1 levels of non-T2D and T2D males (Fig. 3.4A), the massive drop in brain cortical IGF-1 levels in both Wistar and GK females (by 90 % and 78 %, respectively, and with a $P = 0.058$ between T2D cohorts) compared to the respective male cohorts may highly account for a significant effect of sex ($P < 0.05$) herein (Fig. 3.4A). Interestingly, this was also accompanied by 174 % and 68 % higher IGF-1R densities in both non-diabetic and T2D females, respectively (Fig. 3.4B), that may further render sex as the putative responsible for such changes ($P < 0.05$). Additionally, this may contribute for the overall maintenance in IGF-1R phosphorylation on tyrosine residues and subsequent activation (Fig. 3.4C). Surprisingly, no significant changes were observed on brain cortical insulin levels, IR density or phosphorylation (Fig. 3.4D–F). Given the role for the crosstalk between active ERα and IRs/IGF-1Rs in the regulation of common downstream signaling cascades that may, ultimately, control, *e.g.*, cognitive function (Garcia-Segura *et al.*, 2010; Garcia-Segura *et al.*, 2006), we next evaluated the interaction between anti-ERα and anti-IR by co-immunoprecipitation (Fig. 3.4G). Although it was observed that both receptors (ERα and IR) interact with each other in all cohorts, there were no differences in the strength of the interaction between

any of the groups (Fig. 3.4G). In line with the above-mentioned maintenance of hormone-related receptor activation in brain cortices from all cohorts, the expression of the active forms of Akt (by phosphorylation at Ser743) and ERK1/2 were not significantly influenced by sex or T2D (Fig. 3.5A, B).

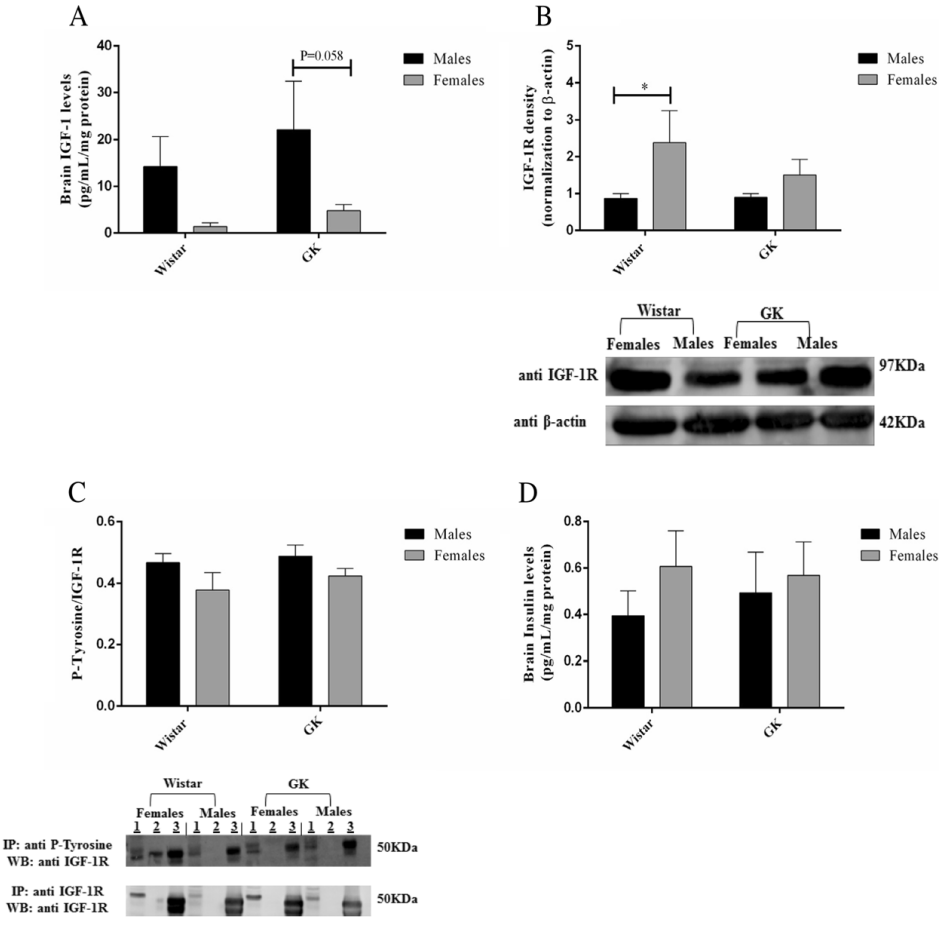

A
Brain IGF-1 levels (pg/mL/mg protein)
P=0.058
Males
Females
Wistar
GK
B
IGF-1R density (normalization to β-actin)
*
Males
Females
Wistar
GK
Wistar
GK
Females
Males
Females
Males
anti IGF-1R
97KDa
anti β-actin
42KDa
C
P-Tyrosine/IGF-1R
Males
Females
Wistar
GK
D
Brain Insulin levels (pg/mL/mg protein)
Males
Females
Wistar
GK
Wistar
GK
Females
Males
Females
Males
1 2 3 1 2 3 1 2 3 1 2 3
IP: anti P-Tyrosine
WB: anti IGF-1R
50KDa
IP: anti IGF-1R
WB: anti IGF-1R
50KDa
E
IR density (normalization to β-actin)
Males
Females
Wistar
GK
F
P-Tyrosine/IR
Males
Females
Wistar
GK
Wistar
GK
Females
Males
Females
Males
anti IR
95KDa
anti β-actin
42KDa
Wistar
GK
Females
Males
Females
Males
1 2 3 1 2 3 1 2 3 1 2 3
IP: anti P-Tyrosine
WB: anti IR
50KDa
IP: anti IR
WB: anti IR
50KDa
G
IR/ER-α
Males
Females
Wistar
GK
Wistar
GK
Females
Males
Females
Males
1 2 3 1 2 3 1 2 3 1 2 3
IP: anti IR
WB: anti ER-α
50KDa
IP: anti ER-α
WB: anti ER-α
50KDa

Figure 3.4 - Effect of sex and T2D on middle-aged rat brain cortical insulin and IGF-1 levels and receptor densities/activation. Brain cortical IGF-1 levels (n = 4–5) (A), IGFR-1 density (n = 4–5) (B) and activation, as given by its tyrosine phosphorylation (n = 3) (C), as well as brain cortical insulin levels (n = 5–8) (D), IR density (n = 5–6) (E), and activation, as given by its tyrosine phosphorylation (n = 3) (F), and ERα interaction with IR (n = 3) (G). Data are the mean ± SEM of the indicated number of animals. Statistical significance: **P* < 0.05 vs. Wistar males, by two-way ANOVA for multiple comparisons, with Fisher's LSD post-test. 1 - total, 2 - non-IP, 3 - IP.

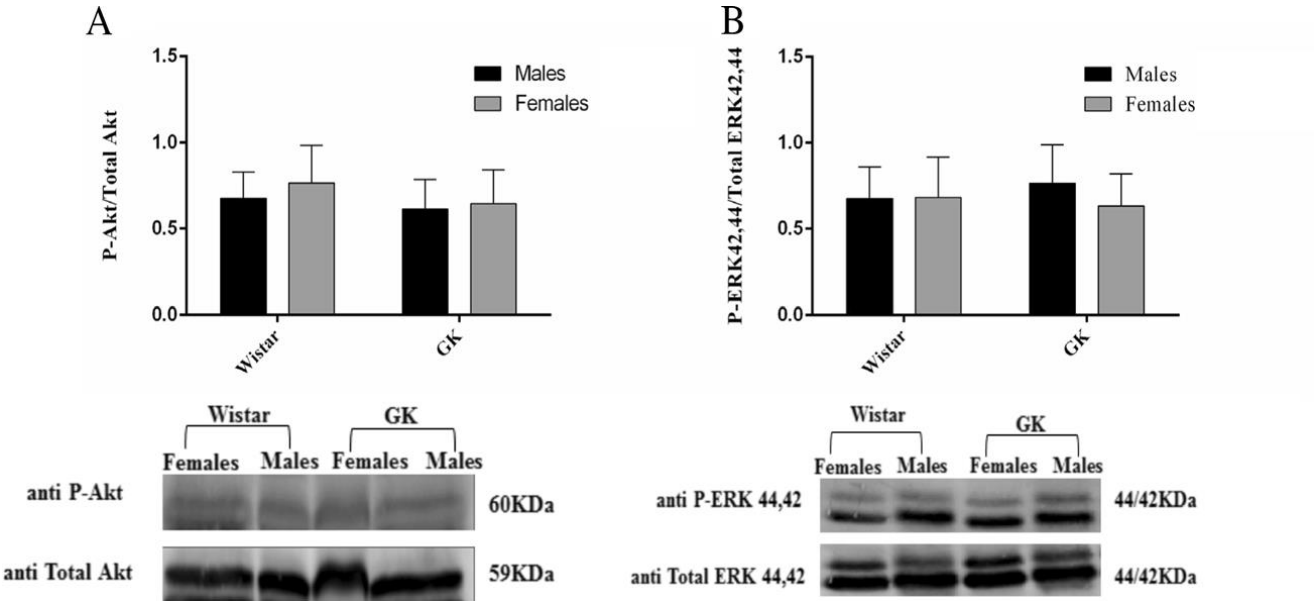


Figure 3.5 - Effect of sex and T2D on middle-aged rat brain cortical downstream signaling cascades. Phosphorylated (active) Akt (n = 5–6) (A) and ERK1/2 activation (n = 5) (B). Data are the mean ± SEM of the indicated number of animals.

3.4.5 - Middle-Aged Female Rats Were less Susceptible to Brain Cortical Lipid and DNA Oxidation

Diabetes-associated hyperglycemia has been highly correlated with several damaging reactions (including oxidative stress, crosslinking of amyloid fibrils, modification of cytoskeletal tau proteins and inflammation) (Srikanth *et al.*, 2011) that may accelerate the cognitive decline in mildly cognitively impaired patients (Qiu *et al.*, 2008) or even increase the risk for AD (den Heijer *et al.*, 2003). As such, we then determined the impact of sex and T2D in brain cortical markers for lipid and DNA

oxidation. A massive difference in both TBARS (Fig. 3.6A) and 8-OHdG contents (Fig. 3.6B) ($P < 0.01$ and $P < 0.0001$ in TBARS and 8-OHdG levels, respectively) was observed, being these levels much higher in the brain cortices from both Wistar male (by 91 % and 51 %, respectively) and GK male rats (by 58 % and 31 %, respectively) (Fig. 3.6A, B). Moreover, both T2D male and female rats had 24 % and 74 % higher brain cortical 8-OHdG levels ($P < 0.001$ for T2D) (Fig. 3.6B), whereas TBARS content was massively increased (by 402 %) only in the case of T2D females (Fig. 3.6A). These results suggested that brain cortices from both Wistar and GK female rats were less vulnerable to oxidative damage than the age-matched male cohorts.

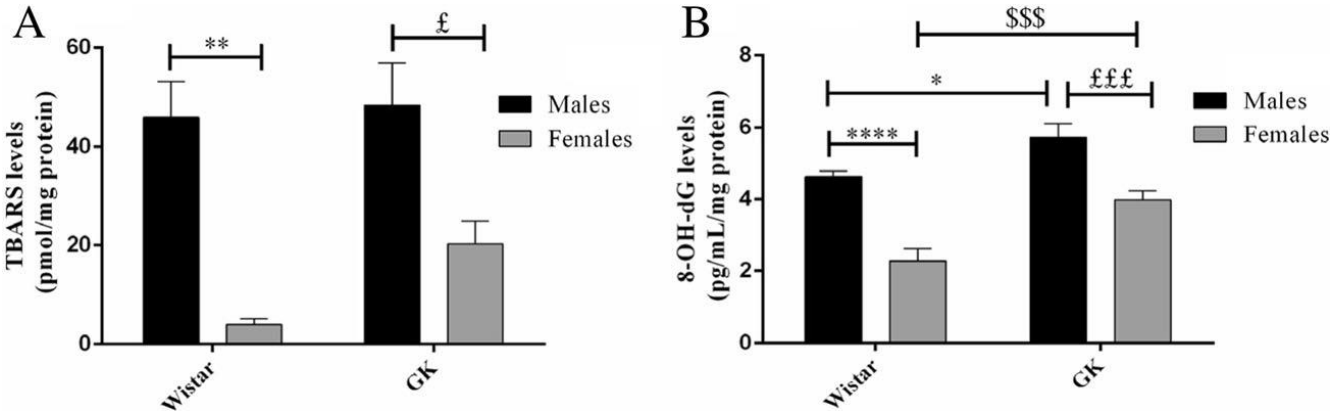


Figure 3.6 - Effect of sex and T2D on middle-aged rat brain cortical oxidative stress markers. Lipid oxidation levels, as given by the colorimetric determination of TBARS levels (n = 3–7) (A) and DNA oxidation, as given by the 8-OHdG levels (n = 5) (B). Data are the mean ± SEM of the indicated number of animals. Statistical significance: *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$ vs. Wistar males; $$$$P < 0.001$ vs. Wistar females; £$P < 0.05$, £££$P < 0.001$ vs. GK males, by two-way ANOVA for multiple comparisons, with Fisher's LSD post-test.

3.4.6 - Female Rat Brain Cortices were Less Prone to the Accumulation of AD-Related Neuropathological Hallmarks

Insulin resistance and its subsequent hyperinsulinemia may render either non-diabetic or pre-diabetic individuals more prone to cognitive dysfunction and dementia, including AD (Rettberg *et al.*, 2014). Additionally, Matsuzaki *et al*. (Matsuzaki *et al.*, 2010) found that hyperinsulinemia arising a decade or more before death was correlated with the presence and severity of amyloid plaques. Hence, we next evaluated the effect

of sex and T2D in brain cortical AD-like neuropathological features. Although there were no significant differences on brain cortical amyloid precursor protein (APP) levels among experimental groups (Fig. 3.7A), a significant effect of T2D ($P < 0.001$), sex ($P < 0.0001$), and their interaction ($P < 0.05$) was found in brain cortical BACE activity (Fig. 3.7B). Particularly, a massive drop in BACE activity occurred in both Wistar and GK female rat brains (by 63 % and 35 %, respectively) than in age-matched males (Fig. 3.7B), thereby suggesting a delayed amyloidogenic processing of APP. In line with this, sex (but not T2D) also affected brain cortical $A\beta_{1-42}$ content (but not $A\beta_{1-40}$ levels) (Fig. 3.7C, D), with both female groups showing a 23 % (with a $P = 0.08$ between Wistar cohorts) and 37 % lower $A\beta_{1-42}$ content than the respective age-matched male cohorts (Fig. 3.7C). Additionally, a significant increment in brain cortical tau protein phosphorylation at Thr181 (a known residue phosphorylated in early AD pathology (Augustinack *et al.*, 2002; Goedert *et al.*, 1995)) was seen in T2D female rats (by 98 %) (Fig. 3.7E). The opposite profile was found for tau protein phosphorylation at Ser396 (a known intermediary phosphorylated residue in AD pathology (Augustinack *et al.*, 2002; Hoffmann *et al.*, 1997)), with sex significantly affecting this profile ($P < 0.05$) (Fig. 3.7F). More specifically, the non-T2D female cohort displayed a 55 % lower brain cortical tau protein phosphorylation at Ser396 than their respective male cohort (Fig. 3.7F). As most of the 85 putative phosphorylation sites on Tau protein are Ser (∼50 %) and Thr (∼41 %) residues, being each of them controlled by one or more protein kinases (*e.g.*, GSK3β, Cdk5) (Martin *et al.*, 2011; Johnson and Stoothoff, 2004), we also analyzed the potential involvement of GSK3β and Cdk5 herein. As no significant changes were found in phospho-GSK3βSer9 (inactive form) nor in phospho-GSK3βTyr216 (active form) between groups (data not shown), it seems unlikely that this kinase may be directly involved herein. Moreover, since Cdk5 has been also involved in APP regulation (Martin *et al.*, 2011), our observation that neither total Cdk5 nor its catalytic and truncated subunits (p35 and p25, respectively) changed significantly between groups (data not shown) appear to be in line with our results from both APP and Tau protein phosphorylation. Hence, the decrement in brain cortical Tau phosphorylation at Ser396 in control females may not involve any of these kinases or even ERK1/2. These results suggested that both Wistar and GK female rats may be less susceptible to the accumulation of AD-like neuropathological markers than the age-matched control and T2D males.

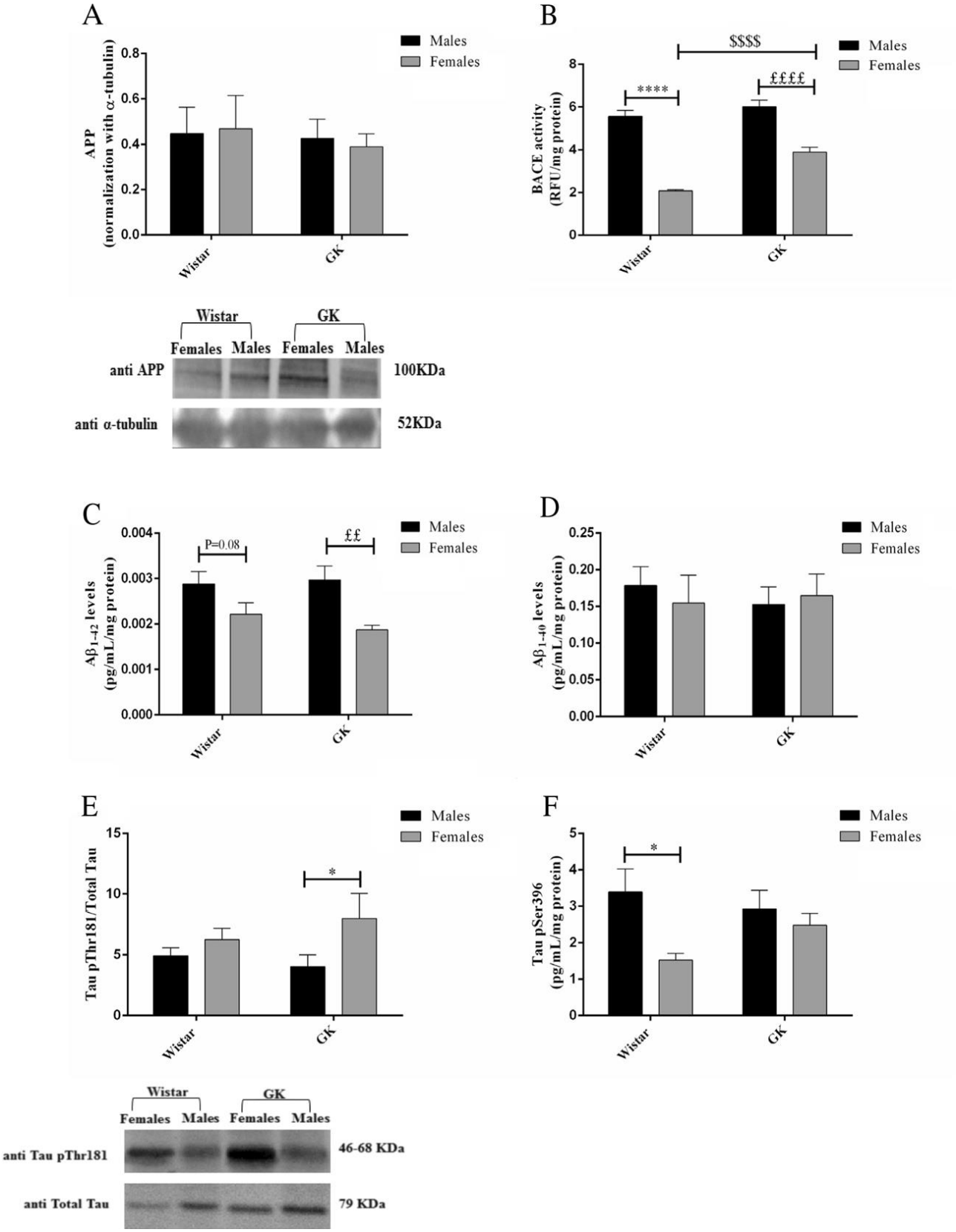


Figure 3.7 - Effect of sex and T2D on middle-aged rat brain cortical amyloidogenic APP processing and AD-like neuropathological hallmarks. APP protein levels (n = 4–5) (A), BACE activity (n = 5–6) (B), $A\beta_{1\text{-}42}$ (n = 4–5) (C), and $A\beta_{1\text{-}40}$ levels (n = 4–7) (D), and Tau

protein phosphorylation in Thr181 (n = 6) (E) and Ser396 residues (n = 4–6) (F). Data are the mean ± SEM of the indicated number of animals. Statistical significance: *P < 0.05, ****P < 0.0001 vs. Wistar males; $$$$$P$ < 0.0001 vs. Wistar females; ££P < 0.01, ££££P < 0.00001 vs. GK males, by two-way ANOVA for multiple comparisons, with Fisher's LSD post-test.

3.5 - DISCUSSION

To our knowledge, this is the first study showing that, whereas middle-aged, retired breeder control females were still under their reproductive phase, the age-matched T2D ones were suffering already a partial peripheral impairment in gonadal estrogen production. Moreover, both female cohorts were already undergoing an imbalance in brain steroid hormone metabolism (from cholesterol to estrogen) that was more pronounced in T2D. This suggests that females' brain steroid hormonal changes may precede those at periphery. Strikingly, our results also point towards the development of compensatory mechanisms, most likely involving highly complex interactions between hormone/hormone receptors in brain cortices from both female cohorts, which may rely mainly in the maintenance of ER, IR, and IGF-1R activation and crosstalk. As a result, their common downstream signaling cascades appear to be maintained, ultimately protecting perimenopausal females against brain cortical oxidative stress and the accumulation of the neuropathological AD hallmarks.

Although hyperglycemia and insulin resistance may underlie cognitive dysfunction and AD upon chronic T2D (Duarte *et al.*, 2012a; Holscher, 2014a; Rettberg *et al.*, 2014; Duarte *et al.*, 2013; Hunter and Holscher, 2012; Cardona-Gomez *et al.*, 2002a), this may not be enough to explain the increased risk of menopausal females for cognitive dysfunction and AD, nor the role for endogenous sex hormones on sex-dependent T2D etiology (Wang *et al.*, 2012a; Long *et al.*, 2012; Yue *et al.*, 2005; Xing *et al.*, 2013; Guarner-Lans *et al.*, 2011; Carroll *et al.*, 2010; Schafer *et al.*, 2007). In line with this, we found that sex and/or T2D may differentially affect some common peripheral blood biochemical features in 8 months old, middle-aged retired breeder rats. Despite some controversial findings among the several colonies worldwide, the impairment of β-cell mass/function appears to constitute an early event in GK rats, detectable already in neonatal animals, and leading to a basal hyperglycemia, as well as decreasing insulin sensitivity and affecting glucose-induced insulin release (Tourrel *et*

al., 2002; Movassat *et al.*, 1997). In accordance, plasma insulin levels are also affected, with a decrease occurring at first weeks of age (1–3 weeks old) (Movassat *et al.*, 2007), followed by a hyperinsulinemia from the first month to adulthood (observed at 6 months old) (Noll *et al.*, 2011; Sena *et al.*, 2009), and then a state of euinsulinemia (8–12 months old) (Zhong *et al.*, 2012; Schrijvers *et al.*, 2004), with reports of a decrease in insulin levels at more advanced ages (13–18 months old) (Hussain *et al.*, 2014; Murakawa *et al.*, 2002). Thus, despite the significant effect of female sex on plasma insulin levels reported in our study, the euinsulinemic levels observed in middle-aged GK rats compared to Wistar ones appear to be in accordance with Zhong *et al.* (Zhong *et al.*, 2012). However, we must bear in mind that, despite the similar blood insulin content between Wistar and GK rats, their β-cells may be unable to cope with chronic hyperglycemia and, thus, they may suffer from insulin resistance, a secretory/exocytosis defect and/or impaired β-cell function (Guest *et al.*, 2002). Additionally, we cannot exclude the possible involvement of a deregulation of the glucose-induced insulin release from β-cells that may compel β-cells to overproduce insulin in order to compensate for hyperglycemia (Amiri *et al.*, 2015; Koyama *et al.*, 1998). Strikingly, Östenson *et al.* (Ostenson *et al.*, 2007) found that the marked impairment in insulin exocytosis in Wistar rat islet was not further decreased in GK rats.

Moreover, while Wistar females were still under their fertile phase (showing higher plasma estradiol and lower testosterone levels than males), the T2D ones showed some gonadal dysfunction, being most likely under perimenopause that, in normal rats, may occur from 9 to 12 months (Maffucci and Gore, 2006; Rubin, 2000). This was accompanied by a similar brain estrogen pattern between Wistar males and females that contrasted with its massive drop in T2D females. As most CNS estrogen comes from gonads and reaches the brain via the BBB (Brinton, 2008; Balthazart and Ball, 2006; Garcia-Ovejero *et al.*, 2005; Rune and Frotscher, 2005; Prange-Kiel *et al.*, 2003), our results suggest that estrogen uptake into the brain might be already compromised. This could result (at least partially) from an impaired cholesterol uptake in females' brain (despite their higher total blood cholesterol levels) and its subsequent decrement in brain cortex (Guarner-Lans *et al.*, 2011; Kolovou and Bilianou, 2008) that may ultimately inhibit its local synthesis of estrogen (particularly upon T2D) (Brinton, 2008; Balthazart and Ball, 2006; Garcia-Ovejero *et al.*, 2005; Rune and Frotscher, 2005; Prange-Kiel *et al.*, 2003), even before the advent of a clear gonadal dysfunction

(Brinton, 2013). Our results further suggested that such dysfunctional brain cholesterol uptake in both female cohorts could also contribute to an overall brain metabolic inhibition involving decreased levels of DHEA and testosterone (Alonso *et al.*, 2008) and, ultimately, impaired CNS estrogen synthesis (as in GK females) or, alternatively, to a partial metabolic "deviation" of cholesterol towards the formation of progesterone. However, this could also provide a potential neuroprotective mechanism as, despite some controversy on the role of serum cholesterol in Aβ toxicity (Wang *et al.*, 2012a; Acharya *et al.*, 2013; Jung *et al.*, 2013; Sano *et al.*, 2011; Feldman *et al.*, 2010; McGuinness *et al.*, 2016; Stefani and Liguri, 2009; Whitmer *et al.*, 2005; Mielke *et al.*, 2010; Mielke *et al.*, 2005) AD has been increasingly considered a cholesterol dysmetabolism-related pathology (Zhao *et al.*, 2012; Hannaoui *et al.*, 2014; Vaya and Schipper, 2007).

Despite no significant changes in brain cortical ERα density in control females, its tendentiously higher density in T2D ones was mirrored by an overall maintenance in ERα and ERβ phosphorylation at tyrosine residues. This suggested that T2D females may have developed mechanisms to overcome their brain steroid hormonal dysmetabolism. Conversely, the massive decrement in ERα density in GK males compared to Wistar ones, besides pointing towards some inability to compensate for the lack of such receptor, was partially in accordance with an age-related decrease in ERα expression and sensitivity to estradiol that could underlie cognitive dysfunction and dementia (Waters *et al.*, 2011). Additionally, we cannot exclude that this could be further potentiated by increased ERα splice variants, with the subsequent inhibition of ERα and increased risk for AD (Rettberg *et al.*, 2014; Ryan *et al.*, 2014; Foster, 2012; Ishunina and Swaab, 2012; Ishunina *et al.*, 2007). Nevertheless, others reported a higher expression of ERα splicing variants in elderly women than men (Foster, 2012) that, together with their higher occurrence of ERα polymorphisms (particularly in the presence of the APOEε4 allele) (Carroll *et al.*, 2010), and/or the occurrence of ERβ splicing variants that turn it into a dominant negative receptor (with lower affinity for estradiol) that may preferentially dimerize with ERα, may blunt ERα-mediated neuroprotective and neurogenic actions, ultimately rendering females more susceptible to AD (Heldring *et al.*, 2007; Weiser *et al.*, 2008; Zhao *et al.*, 2007a; Lu *et al.*, 1998; Chu and Fuller, 1997). This may not be the case herein, as we will further discuss.

Knowledge on the direct effect of estrogen in brain IGF-1 levels is still limited. The lower brain cortical IGF-1 and/or estrogen levels observed in both control and T2D females were partially in accordance with other studies involving OVX or reproductive senescence models, that related the lower IGF-1 gene expression with higher soluble Aβ oligomers or $A\beta_{1–42}$, and the subsequent lowering of circulating IGF-1 and/or its signaling (Zhao *et al.*, 2012; Zhao *et al.*, 2008; Carro *et al.*, 2002; Mao *et al.*, 2012). Despite the brain IGF-1 depletion in our female cohorts, IGF-1R density was tendentiously higher and may account for the maintenance of IGF-1R activation, thus suggesting that, similarly to ERs, a compensated IGF-1R-mediated signaling may a role herein. Indeed, numerous studies showed that the loss of synergism between peripheral estrogen/ER (particularly ERα) and insulin/IR (namely during female's hormonal fluctuations) may underlie peripheral insulin resistance, glucose imbalance (Rettberg *et al.*, 2014; Gao *et al.*, 2012; Leiter and Chapman, 1994), as well as brain mitochondrial, metabolic, neuronal, and cognitive dysfunction, ultimately resulting in AD (Rettberg *et al.*, 2014; Zhao *et al.*, 2012; Moran *et al.*, 2013; Alonso *et al.*, 2010). Thus, our observation of an increment in plasma insulin content in control and T2D female rats, together with the above-mentioned effects in brain cortical IGF-1R density/activation and the clear physical interaction between ERα and IR in both female cohorts, further pointed towards a compensatory mechanism to maintain the brain ER/IR/IGF-1R-mediated signaling in female rats. This was reinforced by their maintained activation of brain cortical Akt and ERK1/2. These signaling pathways appear to be of high relevance in post-menopausal women, whose MAPK may regulate Aβ production and tau phosphorylation (Rettberg *et al.*, 2014). Others showed that estrogen-induced activation of monomeric ERs (α and β) upon $A\beta_{1–42}$ exposure promoted Src/PI3-K and Ras/Raf/MEKK signaling, with the concomitant phosphorylation (via Akt and ERK1/2) and upregulation of genes for neuronal survival (*e.g.*, Bcl2) (Rettberg *et al.*, 2014; Rosario *et al.*, 2010).

Although an increased GSK3β phosphorylation at Ser9 was described in streptozotocin-induced type 1 diabetic rats (Clodfelder-Miller *et al.*, 2005), we did not find significant changes between cohorts on GSK3β phosphorylation (Tyr216 or Ser9), Cdk5 catalytic p35 subunit nor its truncated p25 subunit, similarly to (Clodfelder-Miller *et al.*, 2006). Thus, neither GSK3β nor Cdk5 activation may underlie the inhibition of brain cortical BACE, and decreased $A\beta_{1–42}$ and phosphorylated tau protein in both

female groups. Alternatively, it is plausible that a nearly normal lipid raft formation within their brain cortical membranes may at least partially be involved, since an excessive cholesterol synthesis (and its abnormal increase in membranes and in cholesterol-laden lipid rafts) has been correlated with γ-secretase activation and APP cleavage into $A\beta_{1-40}$ and $A\beta_{1-42}$ (Zhao *et al.*, 2012; Vaya and Schipper, 2007).

Despite the controversy on estrogen/ER regulation of oxidative stress upon AD, our results clearly suggest that, during middle age (perimenopause), other hormone receptor-related signaling (as IR/IGF-1R) and/or their crosstalk with ERs may still induce PI3-K/Akt-mediated antioxidant defenses, thus overcoming the loss of estrogen's benefits and rendering both control and T2D females at a lower risk for lipid and DNA oxidation and ultimately increasing their longevity (Borras *et al.*, 2007; Vina *et al.*, 2006; Vina *et al.*, 2005).

Although most evidence on this field relies on menopausal-related estrogen deficits and its multitude of metabolic changes (Rettberg *et al.*, 2014), by using perimenopause females, here, we show that such changes may start in brain and then spread peripherally. Importantly, we also cannot exclude the involvement of their previous breeding experience (and their highly fluctuating hormonal levels), as others correlated early changes in serum estrogen levels with cognitive dysfunction years later in aged women (Laughlin *et al.*, 2010) and with cortical and hippocampal plaque formation and memory dysfunction in female AD mice (Aragno *et al.*, 2002; Li *et al.*, 2013; Heys *et al.*, 2011; Colucci *et al.*, 2006; Sobow and Kloszewska, 2004; Ptok *et al.*, 2002). However, this is still controversial, as Christensen *et al*. (Christensen *et al.*, 2010) failed to report an effect of pregnancy and motherhood in cognitive deterioration, whereas others showed long-lasting effects of multiparity on hippocampal neuroplasticity and function (including the stimulation of neurogenesis and spatial working memory, despite an impaired spatial reference memory) (Barha *et al.*, 2015). We also must not forget that changes in one component of such highly integrated hormonal system (as the loss in estrogen regulation) may force the others (as insulin) to adapt and compensate (Rettberg *et al.*, 2014). These compensations might be highly personalized, as some females may be very well adapted throughout their lives, whereas others may not cope with those changes and/or only compensate for a relatively short time. Accordingly, women at risk for late-onset AD may possibly belong to the later two groups (Rettberg *et al.*, 2014).

3.6 - CONCLUSION

Altogether, these findings reinforce our hypothesis of a crucial role for differential sex steroid hormones profiles/action in CNS over T2D progression with aging and this should deserve (1) a clarification on the precise mechanisms underlying AD risk and progression over each phase of life using appropriate models and (2) a deliberate stratification by sex of future clinical trials aiming at new therapies for AD (Mielke *et al.*, 2014), hence establishing a sex-specific time window for successful preventive measures, hormonal or simply other new therapies that efficiently reduce both T2D and AD incidence (Rettberg *et al.*, 2014; Vagelatos and Eslick, 2013; Sanz *et al.*, 2012; Williams *et al.*, 2010), reinforcing the sex medicine.

Chapter 4

Exendin-4 therapy in type 2 diabetic Goto-Kakizaki rats: brain glucagon-like peptide-1 receptor-mediated signaling pathways, autophagy and apoptosis

Adapted from: Candeias E, Sebastiao I, Cardoso S, Carvalho C, Santos MS, Oliveira CR, Moreira PI, Duarte AI (2018) Brain GLP-1/IGF-1 Signaling and Autophagy Mediate Exendin-4 Protection Against Apoptosis in Type 2 Diabetic Rats. *Mol Neurobiol,* 55**,** 4030-4050. doi: 10.1007/s12035-017-0622-3.

Brain GLP-1/IGF-1 Signaling and Autophagy Mediate Exendin-4 Protection Against Apoptosis in Type 2 Diabetic Rats

4.1 - ABSTRACT

Type 2 diabetes (T2D) is a modern socioeconomic burden, mostly due to its long-term complications affecting nearly all tissues. One of them is the brain, whose dysfunctional intracellular quality control mechanisms (namely autophagy) may upregulate apoptosis, leading to cognitive dysfunction and Alzheimer disease (AD). Since impaired brain insulin signaling may constitute the crosslink between T2D and AD, its restoration may be potentially therapeutic herein. Accordingly, the insulinotropic anti-T2D drugs from glucagon-like peptide-1 (GLP-1) mimetics, namely, exendin-4 (Ex-4), could be a promising therapy. In line with this, we hypothesized that peripherally administered Ex-4 rescues brain intracellular signaling pathways, promoting autophagy and ultimately protecting against chronic T2D-induced apoptosis. Thus, we aimed to explore the effects of chronic, continuous, subcutaneous (s.c.) exposure to Ex-4 in brain cortical GLP-1/insulin/insulin-like growth factor-1 (IGF-1) signaling, and in autophagic and cell death mechanisms in middle-aged (8 months old), male T2D Goto-Kakizaki (GK) rats. We used brain cortical homogenates obtained from middle-aged (8 months old) male Wistar (control) and T2D GK rats. Ex-4 was continuously administered for 28 days, via s.c. implanted micro-osmotic pumps (5 μg/kg/day; infusion rate 2.5 μL/h). Peripheral characterization of the animal models was given by the standard biochemical analyses of blood or plasma, the intraperitoneal glucose tolerance test, and the heart rate. GLP-1, insulin, and IGF-1, their downstream signaling and autophagic markers were evaluated by specific ELISA kits and Western blotting. Caspase-like activities and other apoptotic markers were given by colorimetric methods and Western blotting. Chronic Ex-4 treatment attenuated peripheral features of T2D in GK rats, including hyperglycemia and insulin resistance. Furthermore, s.c. Ex-4 enhanced their brain cortical GLP-1 and IGF-1 levels, and subsequent signaling pathways. Specifically, Ex-4 stimulated protein kinase A (PKA) and phosphoinositide 3-kinase (PI3K)/Akt signaling, increasing cyclic guanosine monophosphate (cGMP) and AMPK levels, and decreasing GSK3β and JNK activation in T2D rat brains. Moreover, Ex-4 regulated several markers for autophagy in GK rat brains (as mTOR,

PI3K class III, LC3 II, Atg7, p62, LAMP-1, and Parkin), ultimately protecting against apoptosis (by decreasing several caspase-like activities and mitochondrial cytochrome c, and increasing Bcl2 levels upon T2D). Altogether, this study demonstrates that peripheral Ex-4 administration may constitute a promising therapy against the chronic complications of T2D affecting the brain.

Keywords Exendin-4, type 2 diabetes, brain cortex, GLP-1 signaling, autophagy, apoptosis

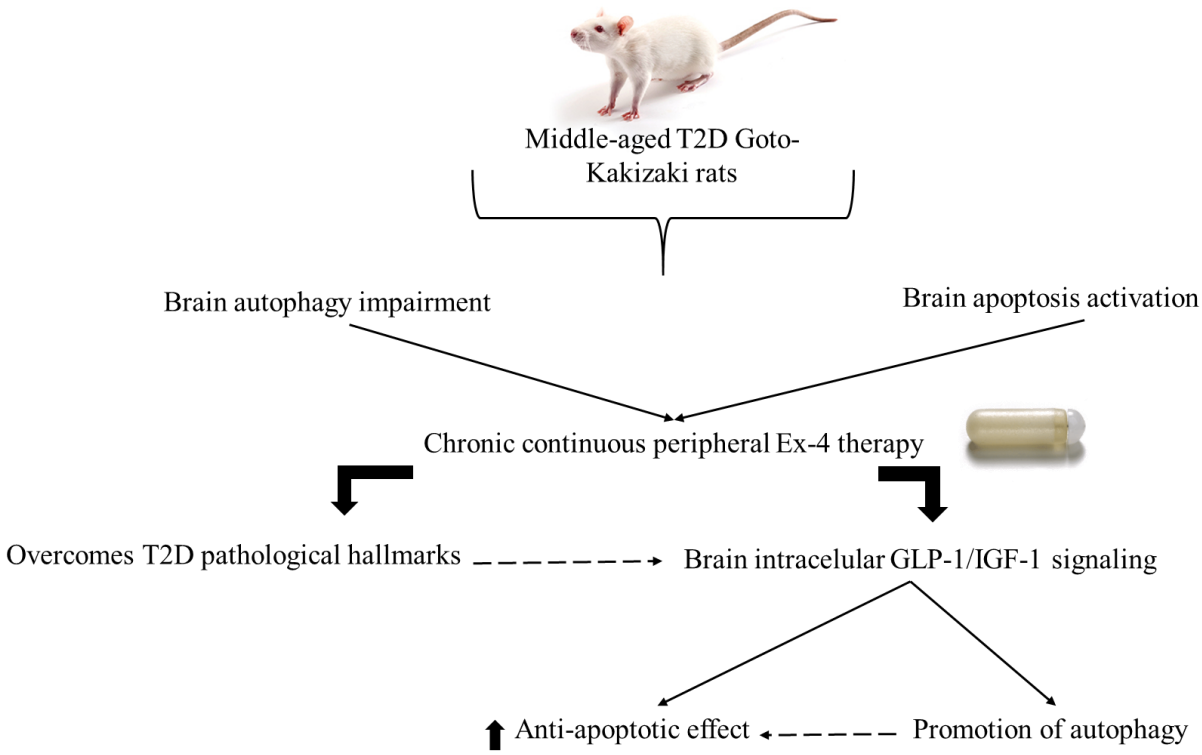


Figure 4.1 – Graphical abstract.

4.2 - INTRODUCTION

Type 2 diabetes (T2D), responsible for 1.5 million of deaths in 2012, is characterized by persistent hyperglycemia due to a deficient insulin action (World Health Organization (WHO), 2016). One of the most concerning aspects of T2D is its long-term complications that may affect, *e.g.*, the brain (Sebastiao *et al.*, 2014). Central effects of T2D include brain insulin resistance, glucose dysmetabolism, alterations in autophagic pathway, neuronal death, and cognitive impairments, ultimately increasing the risk for neurodegenerative diseases (Pugazhenthi *et al.*, 2017; Duarte *et al.*, 2013). Indeed, not only T2D has been associated with 20% of the neurodegenerative disorders, particularly with Alzheimer disease (AD), as each pathology appears to be a risk factor for the other (Nguyen and Le, 2016; Candeias *et al.*, 2012).

Albeit the precise links between T2D and neurodegeneration remain incompletely understood, dysfunctional insulin signaling and autophagic pathways may play an important role (Hsu and Shi, 2017; Ribe and Lovestone, 2016). Accordingly, both T2D and AD were associated with severe deficiency in insulin signaling pathway that might accelerate neurodegeneration (Liu *et al.*, 2011). Moloney *et al.* (Moloney *et al.*, 2010) found a relation between defects in insulin receptor (IR)/insulin-like growth factor-1 receptor (IGF-1R) levels and their downstream signaling kinases (namely, insulin receptor substrate (IRS)-1 and -2) in human AD neurons, especially in those with neurofibrillary tangles. Inhibition of insulin/phosphoinositide 3-kinase (PI3K)/Akt signaling pathway in brains from T2D or AD patients may also affect glycogen synthase kinase-3β (GSK3β) activity and, ultimately, the levels of Tau protein phosphorylation (Liu *et al.*, 2011). This led to the hypothesis that AD could be an "insulin-resistant brain state", and thus, the stimulation of IR/IGF-1R-mediated signaling could constitute a potential therapeutic target in dementia (Duarte *et al.*, 2013; Kullmann *et al.*, 2016). In line with this, we previously showed that insulin administration maintained brain mitochondrial efficiency and sheltered organelles from oxidative stress in type 1 diabetic streptozotocin (STZ) rats exposed to amyloid-β peptide (Aβ) (Moreira *et al.*, 2005b). Intranasal administration of insulin to older subjects with T2D was also neuroprotective, modulating their connectivity between hippocampal regions and thus regulating memory and cognitive performance (Zhang *et al.*, 2015a).

As T2D progresses, patients may need exogenous insulin to control glycemia (Zimmet *et al.*, 2001). Moreover, given the frequent side effects of the most common

anti-T2D drugs and their loss of efficacy over time (Sebastiao *et al.*, 2014; Cardoso *et al.*, 2010; Gavin *et al.*, 2010; Suh *et al.*, 2005; MacLeod *et al.*, 1993), several glucagon-like peptide-1 (GLP-1) receptor (GLP-1R) agonists were developed for the treatment of T2D.

GLP-1R agonists belong to the class of incretins, a group of hormones (Campbell and White, 2008; Drucker and Nauck, 2006; Elrick *et al.*, 1964) that potently lower glycated hemoglobin (HbA1C), fasting glucose levels and body weight, with a low risk of hypoglycemia (Candeias *et al.*, 2015). Among them, one of the most clinically used and best studied is exenatide (exendin-4, Ex-4) (Gallwitz, 2005). Ex-4 is a highly insulinotropic and anti-hyperglycemic agent that shares a 53% amino acid sequence homology with human GLP-1, being resistant to degradation by dipeptidyl peptidase-4 (DPP-4) (Bhavsar *et al.*, 2013; Eng *et al.*, 1992). Ex-4 may also exert wide beneficial effects, namely, by protecting pancreatic β-cells against apoptosis (Natalicchio *et al.*, 2013) and/or by increasing their proliferation, most likely via the PI3K/Akt pathway (Wang *et al.*, 2015a). Ex-4 also attenuated markers for cardiovascular risk in T2D patients (Wysham *et al.*, 2015) and decreased the number of apoptotic cardiomyocytes, probably via GLP-1R activation (Mangmool *et al.*, 2015). Notably, a promising neuroprotective role has been increasingly suggested for Ex-4. Darsalia *et al.* (Darsalia *et al.*, 2014a) found that Ex-4 protected against ischemic brain damage in normal and aged obese T2D mice by modulating neuroinflammatory processes. Others also reported that even the exposure to Ex-4 for 1 week improved cognition in rats submitted to traumatic brain injury (Eakin *et al.*, 2013).

Degradation of damaged intracellular components is regulated mainly by the ubiquitin–proteasome system (UPS) and the lysosomal/autophagic pathway (Svenning and Johansen, 2013). Though traditionally autophagy was considered a mere quality control mechanism, more recently, it has been also considered a non-selective intracellular pathway that respond to nutrient starvation (Svenning and Johansen, 2013; Zaffagnini and Martens, 2016). Importantly, autophagy deregulation (as in T2D or AD) may underlie the accumulation of, *e.g.*, neuropathological markers for AD (neurofibrillary tangles (NFTs) and amyloid plaques) rendering it one of the main features in AD (Menzies *et al.*, 2015; White *et al.*, 2010; Wong and Cuervo, 2010), and possibly affecting the clearance of dysfunctional mitochondria (mitophagy), parts of the endoplasmic reticulum (ER) (ERphagy), protein aggregates (aggrephagy), and

lysosomal defects (also affecting the chaperone-mediated autophagy) (Menzies *et al.*, 2015). Importantly, the regulation of the autophagic induction depends on several conserved metabolic cell sensors (such as mTOR and 5′ adenosine monophosphate-activated protein kinase (AMPK)), which are also common links to insulin/IGF-1 intracellular signaling (Petrovski and Das, 2010). Accordingly, the decrease in AMPK activity in normal aging, T2D and AD (Du *et al.*, 2015; Salminen and Kaarniranta, 2012) was shown to repress autophagy and block protein clearance pathways, culminating in the accumulation of misfolded or aggregated proteins (as in pancreatic β-cells and neurons) that may further exacerbate T2D and AD (Cai *et al.*, 2012b; Gonzalez *et al.*, 2011; Kim *et al.*, 2011).

To our knowledge, there are no studies on the role of Ex-4 on brain insulin signaling, autophagy, and cell death upon chronic T2D *per se*. Hence, we hypothesized that peripherally administered Ex-4 rescues brain intracellular signaling pathways, promoting autophagy and ultimately protecting against chronic T2D-induced apoptosis. Thus, we took advantage on our wide experience with the T2D Goto-Kakizaki (GK) rats (Candeias *et al.*, 2017; Carvalho *et al.*, 2014a; Santos *et al.*, 2014b; Duarte *et al.*, 2004; Moreira *et al.*, 2003; Santos *et al.*, 2000) to investigate the impact of a chronic, continuous, peripheral administration of Ex-4 on brain IR- and GLP-1R-mediated signaling, autophagic mechanisms, and cell death in non-obese, middle-aged (8 months old), male, T2D GK rats.

4.3 - MATERIALS AND METHODS

4.3.1 - Materials

Ex-4 and GLP-1R, Phospho-IRS-2 (Ser731), Beclin, Parkin, receptor-interacting serine/threonine-protein kinase (RIP)1, and RIP3 antibodies were obtained from Abcam (Cambridge, UK). Micro-osmotic pumps (model 2ML4) were obtained from Alzet® (Cupertino, CA, USA). Bovine serum albumin (BSA), phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), Tween 20, Ac-YVADpNa, Ac-VDAV-pNa, Ac-IETD-pNA, Ac-LEDH-pNA, and Ac-DEVD-pNa substrates and p62, microtubule-associated protein 1A/1B-light chain 3 (LC3) and β-actin antibodies were obtained from Sigma-Aldrich (St. Louis, MO, USA). Butorfanol and isoflurane were obtained from Lab. Vitória (Portugal). Cyclic AMP (cAMP) Direct Immunoassay Kit was purchased to

BioVision, Bio Portugal (Porto, Portugal). D-Glucose, polyvinylidene difluoride (PVDF), Immobilon-P membranes, and Phospho-cAMP response element-binding protein (CREB) (Ser133) antibody were obtained from Millipore (Billerica, MA, USA). Commercial protease and phosphatase inhibitor cocktails were obtained from Roche Applied Science (Amadora, Portugal). Rat Insulin Enzyme Immunoassay kit was purchased from SPI-BIO, Bertin Pharma (Montigny le Bretonneux, France). Rat GLP-1 ELISA Kit and Rat AMPK ELISA Kit were purchased from Elabscience (Wuhan, Hubei, China). PKA kinase activity kit was purchased from Enzo Life Sciences, Grupo Taper SA (Sintra, Portugal). Rat IGF-1 ELISA kit was purchased from Biosensis Pty Ltd. (Thebarton, South Australia). Cyclic GMP XP Assay Kit, Phospho-Akt (Ser473), PI3K p110, Phospho-ERK1/2 (Thr202/Tyr204), Phospho-c-Jun N-terminal kinase (JNK) (Thr183/Tyr185), Phospho-mTOR (Ser2448), PI3K class III, autophagy-related protein (Atg7), lysosomal-associated membrane protein (LAMP)-1, caspase-12, IRβ (48B), Bcl2, Bcl-2-associated X protein (Bax), Total CREB, Total ERK, Total JNK, and Total mTOR antibodies were purchased from Cell Signaling Technology (Leiden, The Netherlands). Phospho-GSK-3β (Tyr 216), IGF-1Rβ, Total GSK-3β, Total IRS-2, and TOM-20 antibodies were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). Cytochrome c and Total Akt antibodies were purchased from BD Biosciences (Allschwil, Switzerland). Anti-mouse, anti-rabbit, and anti-goat secondary antibodies, and enhanced chemifluorescence (ECF) reagent were purchased from Amersham Biosciences (Little Chalfont, UK). All other chemicals used were of the highest grade of purity commercially available.

4.3.2 - Animal Housing and Treatment

Following EU and Portuguese legislation (Directive 2010/63/EU; DL113/2013, August 7), 8-month-old (middle-aged) male Wistar control and T2D GK rat (a non-obese model that spontaneously develop T2D early in life) (Santos *et al.*, 2000) 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. Thus, following the "3Rs" reduction principle established by FELASA, in a first approach, we used the brain cortical GLP-1 levels in GK rats treated or not with Ex-4 (Fig. 4.2A) to estimate the number of animals required for this study. Briefly, by using the t test applied to the difference between those two independent means on the G-Power

software (Faul *et al.*, 2007), an alpha error of 0.05 and a power of 80%, we estimated that a total of six rats should be used for the overall study. In line with this and aiming to increase the power of our hypothesis, we used at least n = 4 rats per parameter. Wistar and GK rats were obtained from Charles River (Barcelona, Spain) and Taconic (Ejby, Denmark), respectively, maintained at our animal colony (Animal Research Center, University of Coimbra), under controlled light (12 h day/night cycle) and humidity (45–65%), *ad libitum* standard hard pellets chow. Signs of distress were carefully monitored and glucose tolerance tests (GTT) were used as selection index. Analogously to our previous study in mice (Duarte *et al.*, 2011), 6 male Wistar and 12 GK rats (8 months old) were implanted subcutaneously (s.c.) with a micro-osmotic pump (2ML4, Alzet®), according to the manufacturer's instructions. Rats were divided into three experimental groups. In one group, six GK rats were continuously infused with Ex-4 (5 μg/kg/day; infusion rate 2.5 μL/h), for 28 days (from the 8 to 9 months old), whereas the remaining two groups (n = 6 Wistar and n = 6 GK rats) received sterile saline infusion. Accuracy of micro-osmotic pumps was verified according to manufacturer's instructions and also by weighing each pump before implantation and after removal from the animal. All surgical procedures were performed under anesthesia with inhalable isofluorane (4–5% during the induction of sedation and then 1.5–2% for maintenance) and local s.c. butorphanol (2 mg/kg) injection. Although not expected, a rapid decrease in body weight >15–20% was defined as a potential humane endpoint for the study.

4.3.3 - Body Weight

Body weight was monitored once per week, from 7.5 (pretreatment) to 9 months old (post-treatment). Results were expressed as body weight (g).

4.3.4 - Evaluation of Heart Rate

Heart rate was evaluated after treatment, using a LE 5001 Non-Invasive Blood Pressure Meter (Panlab Harvard Apparatus, Reagente 5 Quimica Electronica, Porto, Portugal). For rats, the detection range was from 270 to 960 beats/min (BPM).

4.3.5 - Intraperitoneal GTT

The clearance of an intraperitoneally (i.p.) injected glucose load (2 mg D-glucose/g body weight) from the rats' organism was determined by intraperitoneal glucose tolerance tests (ipGTT), performed after treatment, early in the afternoon, as described by Bowe *et al*. (Bowe *et al.*, 2014) and Assis *et al*. (de Assis *et al.*, 2009) with slight modifications. Briefly, rats were fasted for ~6 h (starting early in the morning) and glucose levels were determined before i.p. injection of 2 mg D-glucose/g body weight (basal glycemia) and after 15, 30, 60, and 120 min. At the end of the test, cages were supplied with wet food. Results were expressed as milligrams glucose per deciliter blood and as area under the curve (AUC).

4.3.6 - Peripheral Blood Collection and Routine Biochemical Analyses

Before the above mentioned i.p. D-glucose load (0 min), blood from the caudal vein of fasted rats was collected and centrifuged at 572×*g* in a Sigma 2-16 PK centrifuge, for 10 min at 4 °C. The resulting plasma was used to determine fasting insulin levels through the Rat Insulin Enzyme Immunoassay kit, according to the manufacturer's instructions. Absorbance was read at 405 nm in a SpectraMax Plus 384 multiplate reader, when maximum binding (B0) wells ranged from 0.2 to 0.8 a.u. Results were expressed as nanograms per milliliter for plasma insulin levels. Homeostasis assessment model-insulin resistance (HOMA-IR) index was calculated using the formula: HOMA-IR = (fasting insulin [μU/mL] × fasting glucose [mmol/L])/22.5, while the homeostasis assessment model-β cell function (HOMA-β) index was given by: HOMA-β = (20 × fasting insulin [μU/mL]) / (fasting glucose [mmol/L] – 3.5) (Matthews *et al.*, 1985; Wallace *et al.*, 2004). Immediately after animal's euthanasia, total blood was collected to determine occasional blood glucose, (HbA1c, cholesterol, and triglycerides levels. Briefly, glycemia was given by the glucose oxidase reaction, using a glucometer (Glucometer-Elite, Bayer SA, Portugal) and compatible stripes. Results were expressed as milligrams glucose per deciliter blood. HbA1c levels were measured using a Multi-Test HbA1c (A1C Now+, Bayer SA, Portugal), whereas cholesterol and triglycerides were determined by an Accutrend Plus meter and compatible reactive strips (Accutrend® Plus system, Roche, Amadora,

Portugal). Results were expressed as percent, milligrams cholesterol per milliliter blood and milligrams triglycerides per deciliter blood, respectively.

4.3.7 - Isolation and Preparation of Brain Cortical Homogenates

Rats were weighed and euthanized by decapitation, and brains were immediately removed. Brain cortices were immediately dissected and snap-frozen for further studies. Immediately before the experiments, brain cortices were homogenized at 0–4 °C in lysis buffer, containing (in mM) the following: 25 HEPES, 2 $MgCl_2$, 1 EDTA, 1 EGTA, (pH 7.4), supplemented with 2 mM DTT, 100 μM PMSF, and commercial protease and phosphatase inhibitors cocktails. The crude homogenate was centrifuged at 17,968×*g* for 10 min, at 4 °C in a Sigma 2-16K centrifuge to remove the nuclei, and the resulting supernatant was collected. The pellet was further resuspended in supplemented buffered solution and centrifuged again at 17,968×*g* for 10 min, at 4 °C. The supernatant was added to the previously obtained one and protein content was measured by the Sedmak method (Sedmak and Grossberg, 1977).

4.3.8 - Mitochondrial Fraction Isolation

Mitochondrial fraction was isolated as previously described (Rosenthal *et al.*, 1987), with slight modifications (Moreira *et al.*, 2001). Briefly, brain tissue (except cerebellum) was isolated, washed, and homogenized at 4 °C in 10 mL isolation medium, containing (in mM) the following: 225 mannitol, 75 sucrose, 5 HEPES, 1 EGTA, and 1 mg/mL essentially fatty acid-free BSA (pH 7.4), and supplemented with 1.5 mg of bacterial protease type VIII. Then, brain homogenates were centrifuged at 750×*g*, 4 °C, for 5 min in a Sorvall RC-5B Refrigerated Superspeed Centrifuge, and the resulting pellet was resuspended in 10 mL of the isolation medium supplemented with 0.02% digitonin (to release mitochondria from the synaptosomal fraction), and centrifuged again at 11,950×*g* for 10 min, at 4 °C. The resulting pellet (mitochondrial fraction) was resuspended in 10 mL of isolation medium and centrifuged again at 11,950×*g* for 5 min, at 4 °C. After resuspension of the resulting pellet in 10 mL of washing medium, containing (in mM) the following: 225 mannitol, 75 sucrose, and 5 HEPES (pH 7.4), a final centrifugation was made at 11,950×*g* for 5 min, at 4 °C. The

obtained pellet was resuspended in 100 μL of washing medium and protein determined by the biuret method, using known concentrations of BSA as standard (Gornall *et al.*, 1949).

4.3.9 - Quantification of Pivotal GLP-1R/IR/IGF-1R Signaling Markers' Levels

Brain cortical GLP-1R/IR/IGF-1R-mediated downstream signaling cascades were given by the determination of the pivotal molecules: GLP-1, insulin, IGF-1, cAMP, PKA, cyclic guanosine monophosphate (cGMP), and AMPK, using commercially available colorimetric ELISA kits, according to manufacturers' instructions with slight modifications.

GLP-1 levels were measured in 20 μL of each sample (working dilution of 1:5) by the Rat GLP-1 ELISA Kit. Absorbance was determined at 450 nm, in a SpectraMax Plus 384 multiplate reader. Results were expressed as pictograms per milligram protein.

Brain cortical insulin levels were measured in 25 μL of each sample by using the above mentioned Rat Insulin Enzyme Immunoassay kit (with the remaining volumes decreased to half) and the results were expressed as nanograms per milligram protein.

Brain cortical cytosolic IGF-1 levels were measured in 5 μL of each sample (working dilution of 1:20) by the Rat IGF-1 ELISA kit. Absorbance was read at 450 nm, in a SpectraMax Plus 384 microplate reader. Results were expressed as picograms per milliliter per milligram protein.

cAMP levels were determined in 5 μL of each sample (working dilution of 1:10) with the cAMP Direct Immunoassay Kit. Absorbance was read at 450 nm, in a SpectraMax Plus 384 multiplate reader. Results were expressed as picomoles per milligram protein.

PKA activity was determined in 5 μL of each sample (working dilution of 1:6) by the PKA kinase activity kit. The absorbance was determined at 450 nm, in a SpectraMax Plus 384 multiplate reader. Results were expressed as nanograms per assay.

cGMP content was measured in 5 μL of each sample (working dilution of 1:10) by the Cyclic GMP XP Assay Kit. Absorbance was read at 450 nm, in a SpectraMax Plus 384 multiplate reader. Results were expressed as nanomolars per milligram protein.

AMPK concentrations were measured in 20 μL of each sample (with working dilution of 1:5) by the Rat AMPK ELISA Kit. Absorbance was read at 450 nm, in a SpectraMax Plus 384 multiplate reader. Results were expressed as pictograms per milligram protein.

4.3.10 - Western Blot Analyses

Samples containing denatured brain cortical homogenates (50 μg per lane) were subjected to sodium dodecyl sulfate (SDS)/polyacrylamide gel electrophoresis (SDS/PAGE) (8–15%) and transferred onto PVDF membranes. Then, membranes were blocked for 1 h at room temperature in Tris-buffered saline (TBS, pH 7.4) plus 1 or 5% nonfat dry milk or BSA, plus 0.05% Tween 20. Membranes were then incubated overnight at 4 °C with rabbit IRβ (4B8) (1:1000), mouse Phospho-Akt (Ser473) (1:1000), rabbit PI3K p110 (1:1000), rabbit Phospho-GSK-3β (Tyr 216) (1:500), rabbit Phospho-CREB (Ser133) (1:1000), rabbit Phospho-ERK1/2 (Thr202/Tyr204) (1:1000), rabbit Phospho-IRS-2 (Ser731) (1:1000), mouse Phospho-JNK (Thr183/Tyr185) (1:2000), rabbit GLP-1R (1:1000), rabbit IGF-1Rβ (1:1000), rabbit Phospho-mTOR (Ser2448) (1:1000), rabbit p62 (1:1000), rabbit LC3 (1:1000), rabbit PI3K class III (1:1000), mouse Beclin (1:1000), rabbit Atg7 (1:1000), rabbit LAMP-1 (1:1000), mouse Parkin (1:1000), rabbit Caspase-12 (1:1000), rabbit Bcl2 (1:1000), rabbit Bax (1:1000), mouse Cytochrome c (1:1000), rabbit RIP1 (1:400), and rabbit RIP3 (1:1000) primary antibodies. Membranes were then incubated with the respective anti-rabbit, anti-mouse, or anti-goat secondary IgG antibodies (1:10,000), for 2 h at room temperature, and developed using ECF. Immunoreactive bands were visualized by the VersaDoc Imaging System (Bio-Rad, Hercules, CA, USA). Fluorescence signal was analyzed using the QuantityOne software and the results given as INT per square millimeter. Of note, membranes were then reprobed with the corresponding mouse Total Akt (1:1000), mouse Total GSK-3β (1:500), rabbit Total CREB (1:1000), rabbit Total ERK (1:1000), goat Total IRS-2 (1:1000), rabbit Total JNK (1:1000), mouse Total mTOR (1:1000), mouse β-actin (1:5000), or rabbit TOM20 (1:200) primary antibodies. Results were presented as phosphorylated protein/total protein or protein levels (corresponding to the ratio of each protein vs. β-actin or TOM20).

4.3.11 - Colorimetic Evaluation of Caspase-Like Activities

Caspase-1-, caspase-2-, caspase-3-, caspase-8-, and caspase- 9-like activities were colorimetrically determined using a previously described method (Cregan *et al.*, 1999), with some modifications (Gil *et al.*, 2003). Briefly, 25 μg (for caspase-3- and -8-like), 40 μg (for caspase- 1- and -2-like), and 65 μg (for -9-like) were incubated at 37 °C, for 2 h, in a reaction medium buffer, containing the following: 25 mM HEPES, 10% (m/v) sucrose and 0.1 % (m/v) CHAPS (pH 7.5), supplemented with 10 mM DTT and 100 μM of each specific colorimetric caspase-like substrate (Ac-YVAD-pNA, Ac-VDAV-pNA, Ac-IETD-pNA, Ac-LEDH-pNA, and Ac-DEVD-pNA). Then, each caspase-like activity was given by the formation of pNA at 405 nm, in a SpectraMax Plus 384 multiplate reader. Results were expressed as arbitrary units.

4.3.12 - Statistical Analysis

Results were presented as scatter plot with bar (mean ± SEM) of the indicated number of rats. Statistical analysis and graphic artwork were obtained using the GraphPad Prism 6.0 software. After the identification of outliers with the ROUT test and after the Kolmogorov-Smirnov normality test, statistical significance was determined using the one-way ANOVA test with protected Fisher's LSD post-test for multiple comparisons (for a Gaussian distribution). A P value <0.05 was considered statistically significant.

4.4 - RESULTS

4.4.1 - Chronic Peripheral Administration of Ex-4 Rescued Peripheral Hallmarks of T2D

GK rats are a well-known non-obese and spontaneously T2D model, presenting mild insulin resistance and glucose dysmetabolism (Vahtola *et al.*, 2008). In accordance with our previous studies (Candeias *et al.*, 2017; Duarte *et al.*, 2004; Duarte *et al.*, 2000), middle-aged T2D GK rats used herein had significantly lower (by 14%; P = 0.0002) body weight and higher fasting and occasional blood glucose and plasma insulin levels (increased by 190, 299, and 73%, with P < 0.0001, P = 0.0003, and P = 0.0003, respectively) than their age-matched Wistar cohorts (Table 4.I). These were

accompanied by a massive increase in ipGTT, HbA1c levels, and HOMA-IR index (by 147, 96, and 434%, with $P < 0.0001$ for all cases, respectively) in GK rats, whereas their HOMA-β index was significantly lower (by 82%, $P < 0.0001$) (Table 4.I). Despite no significant alterations in blood cholesterol levels, T2D rats also had significantly higher blood triglycerides and heart rate (by 72 and 21%, with $P = 0.0159$ and $P = 0.0054$, respectively) than controls (Table 4.I). Remarkably, most of these T2D-associated peripheral alterations were rescued (or at least attenuated) by a chronic s.c. Ex-4: it lowered fasted and occasional glucose levels (by 31 and 15%, with $P = 0.0050$ and $P = 0.3662$, respectively), HOMA-IR (by 42%, $P = 0.0006$), HbA1c levels (by 17%, $P = 0.0001$), ipGTT (by 21%, $P = 0.0008$), triglycerides (by 19%, $P = 0.1547$), and heart rate (by 17%, $P = 0.0055$), increasing also HOMA-β by 111% ($P = 0.2111$) (Table 4.I). Surprisingly, despite no significant changes in brain weight between Wistar and GK rats, Ex-4 exposure significantly increased (by 5%, $P = 0.0256$) GK rat brain weight (Table 4.I).

Table 4.I- Effect of Ex-4 on physical and biochemical characteristics of middle-aged Wistar and GK rats.

	Wistar	GK	GK+Ex-4
Body weight (g)	480.2 ± 15.66	414.3 ± 4.61 ***	427.3 ± 2.47
Brain weight (g)	2.17 ± 0.02	2.1 ± 0.03	2.2 ± 0.04 #
Occasional glycemia (mg glucose/dL blood)	83.17 ± 4.22	331.8 ± 56.14 ***	282.5 ± 32.19
Fasting glycemia (mg glucose/dL blood)	77.33 ± 1.54	224.3 ± 23.8 ****	155.5 ± 9.49 ##
ipGTT (area under the curve)	18742 ± 2071	46372 ± 1778 ****	36602 ± 841.9 ###
HbA_{1c} (%)	4.37 ± 0.08	8.58 ± 0.22 ****	7.15 ± 0.25 ###

Fasting insulin levels (ng/mL plasma)	0.15 ± 0.02	0.26 ± 0.02 ***	0.23 ± 0.01
HOMA-IR	0.67 ± 0.07	3.58 ± 0.36 ****	2.06 ± 0.22 ###
HOMA-β	74.93 ± 12.98	13.13 ± 0.96 ****	27.67 ± 4.0
Blood cholesterol (mg/dL blood)	157.5 ± 2.09	162.7 ± 1.82	157.2 ± 2.77
Blood triglycerides (mg/dL blodd)	156.8 ± 23.07	270.3 ± 30.67 **	219.4 ± 7.95
Heart rate (beats/min)	505.8 ± 17.02	610.7 ± 31.55 **	506.2 ± 16.53 ##

Data are mean ± SEM of six rats/group. Statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 vs. saline-treated Wistar rats; #P < 0.05, ##P < 0.01 vs. saline-treated GK rats, by one-way ANOVA test, with protected Fisher LSD post-test. HbA1c: glycated hemoglobin A1c, HOMA-IR: homeostatic model assessment for insulin resistance, HOMA-β: homeostatic model assessment for β-cell function, ipGTT: intraperitoneal glucose tolerance test.

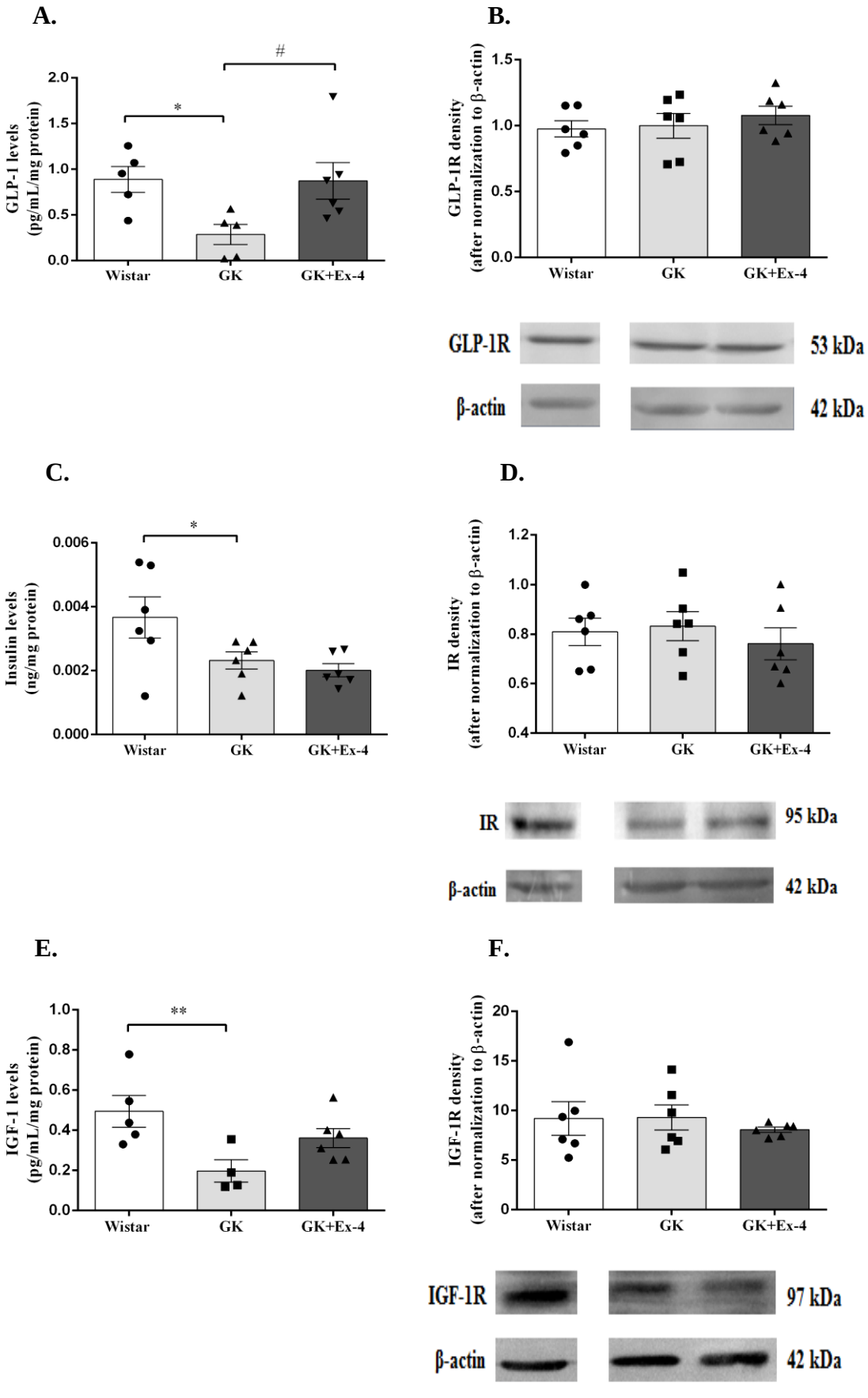
4.4.2 - Peripheral Ex-4 Exposure Stimulated Brain GLP-1/IGF-1-Mediated Signaling Cascades upon T2D

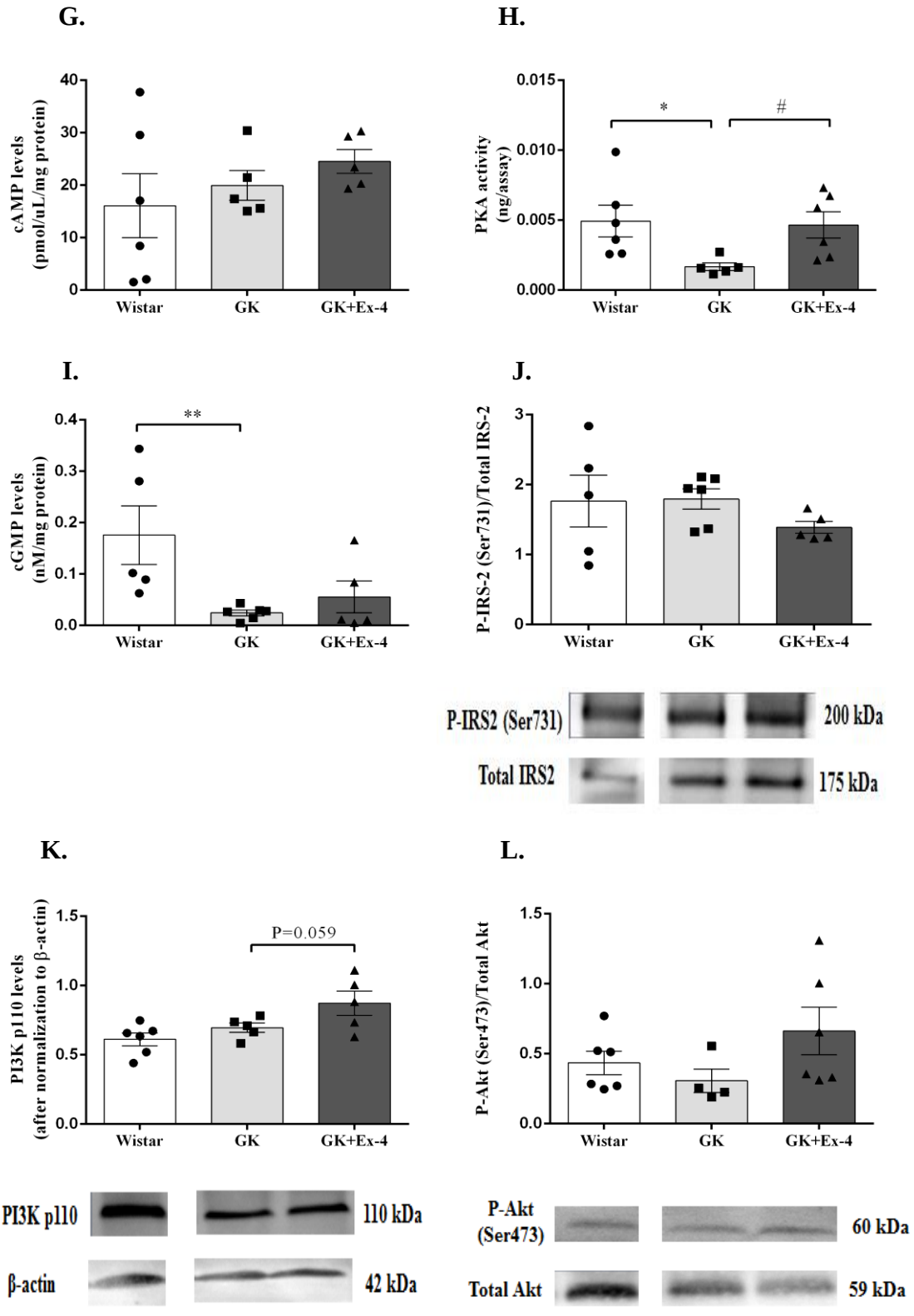
Despite the well-recognized impact of T2D on the decrement of brain insulin levels and action, more recent evidence also focused on the impact of glucagon and incretin hormones in central nervous system (CNS) upon T2D (Broichhagen *et al.*, 2015; van der Klauw and Wolffenbuttel, 2012). In line with its increasingly known peripheral and brain insulinotropic effects (Hamilton *et al.*, 2011), Ex-4 reversed (by 205%) the significantly lower brain cortical GLP-1 levels in T2D rats (Fig. 4.2A). Despite no changes in brain GLP-1R, IR or IGF-1R densities (Fig. 4.2B, D, and F), and the apparent inability of Ex-4 to recover from the significant decrease (by 37%, P = 0.0381) in brain cortical insulin levels in GK rats (Fig. 4.2C), it increased their brain IGF-1 levels by 83% (though non-statistically significant, P = 0.0927) (Fig. 4.2E).

Traditionally, Ex-4-induced activation of GLP-1R may upregulate cAMP/PKA and/or PI3K/Akt signaling pathways which, besides mediating its neuroprotective effects, may also overlap with IR/IGF-1R targets in CNS (He *et al.*, 2016; Wei *et al.*, 2016; Doyle and Egan, 2007). Although no significant differences were found in brain cAMP levels among groups (Fig. 4.2G), Ex-4 significantly restored (by 177%, P = 0.0396) the activity of PKA in GK rats (Fig. 4.2H). This was paralleled by a partial (by 127%, though non-significant; P = 0.5370) recovery of brain cGMP levels in Ex-4-treated T2D rats (Fig. 4.2I). Additionally, we observed that the tendentiously lower (by 23%, P = 0.2198) phosphorylated levels of IRS2 at Ser731 (a known negative regulator of IR activation upon pathological conditions) (Liang *et al.*, 2015; Rector *et al.*, 2013) induced by Ex-4 in GK rat brains (Fig. 4.2J) were followed by tendentiously higher (by 25%, P = 0.059) levels of the p110 (catalytic) subunit of the PI3K protein (Fig. 4.2K), and in the subsequently phosphorylated (and activated) Akt at Ser473 (by 116%, P = 0.0881) (Fig. 4.2L). Accordingly, and given the known interaction between active Akt and PKA to inactivate GSK-3β (a pivotal kinase in hyperphosphorylation of Tau protein upon T2D or AD) (Lei *et al.*, 2011; Fang *et al.*, 2000), s.c. Ex-4 significantly lowered (by 45%, P = 0.0138) GSK-3β activation by phosphorylation at Tyr216 in GK rat brains (Fig. 4.2M). No significant alterations occurred in brain cortical levels of P-ERK1/2, suggesting that this signaling pathway may not be involved herein (Fig. 4.2N).

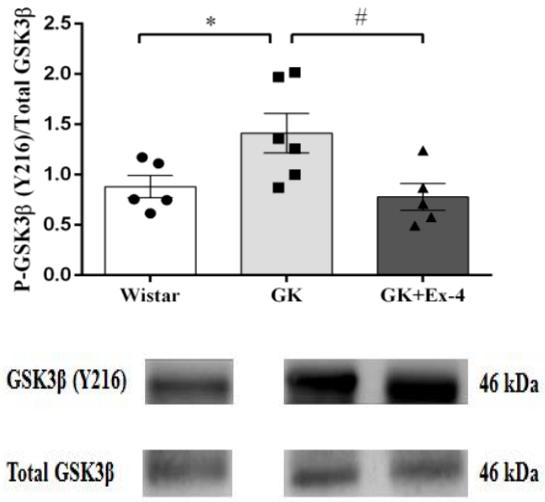
Treatment with Ex-4 also partially counteracted the tendentiously higher (by 42%, P = 0.2185) JNK activation and the significantly lower (by 86%, P = 0.3286) AMPK levels in brains from saline-treated GK rats (Fig. 4.2O, P) - two well-known modulators of intracellular stress signaling, energy sensing, and autophagy kinases that are affected by T2D and neurodegenerative diseases (as AD) (Jiang *et al.*, 2014; Li and Yu, 2013; Beeler *et al.*, 2009). Finally, no significant changes were observed on the levels of P-CREB (a transcription factor involved in the GLP-1 anti-apoptotic and long-term memory restoration effects) (Neves *et al.*, 2018; Jhala *et al.*, 2003) (Fig. 4.2Q).

This suggests that, by restoring GLP-1 (and possibly also IGF-1) brain cortical levels in T2D rats, Ex-4 may activate the downstream PKA-, PI3K/Akt-, and AMPK-mediated signaling cascades. This, together with the possible inhibition of the JNK- and GSK3β-related signaling may ultimately affect the brain cortical autophagic and cell death mechanisms upon such conditions.

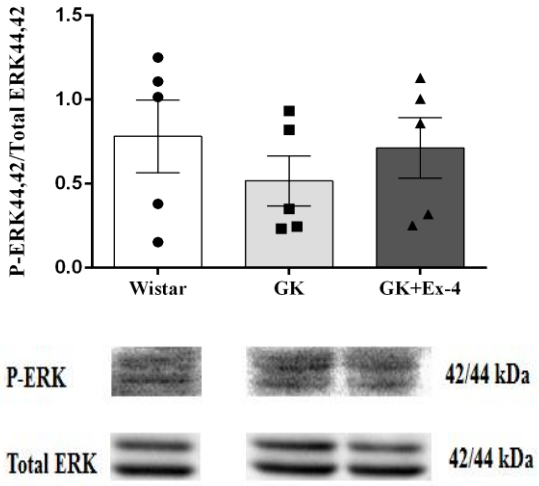

A.
B.
C.
D.
E.
F.
GLP-1 levels (pg/mL/mg protein)
GLP-1R density (after normalization to β-actin)
Insulin levels (ng/mg protein)
IR density (after normalization to β-actin)
IGF-1 levels (pg/mL/mg protein)
IGF-1R density (after normalization to β-actin)
Wistar
GK
GK+Ex-4
GLP-1R
53 kDa
β-actin
42 kDa
IR
95 kDa
IGF-1R
97 kDa


G.
cAMP levels (pmol/uL/mg protein)
40
30
20
10
0
Wistar
GK
GK+Ex-4
H.
PKA activity (ng/assay)
0.015
0.010
0.005
0.000
*
#
Wistar
GK
GK+Ex-4
I.
cGMP levels (nM/mg protein)
0.4
0.3
0.2
0.1
0.0
**
Wistar
GK
GK+Ex-4
J.
P-IRS-2 (Ser731)/Total IRS-2
3
2
1
0
Wistar
GK
GK+Ex-4
P-IRS2 (Ser731)
200 kDa
Total IRS2
175 kDa
K.
PI3K p110 levels (after normalization to β-actin)
1.5
1.0
0.5
0.0
P=0.059
Wistar
GK
GK+Ex-4
PI3K p110
110 kDa
β-actin
42 kDa
L.
P-Akt (Ser473)/Total Akt
1.5
1.0
0.5
0.0
Wistar
GK
GK+Ex-4
P-Akt (Ser473)
60 kDa
Total Akt
59 kDa

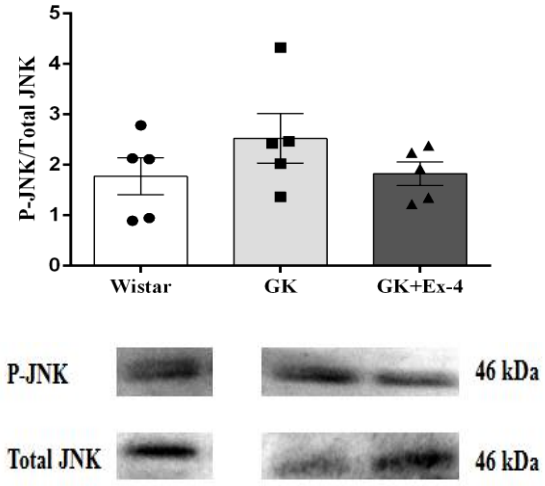
M.


P-GSK3β (Y216)/Total GSK3β
2.5
2.0
1.5
1.0
0.5
0.0
*
#
Wistar
GK
GK+Ex-4
GSK3β (Y216)
46 kDa
Total GSK3β
46 kDa

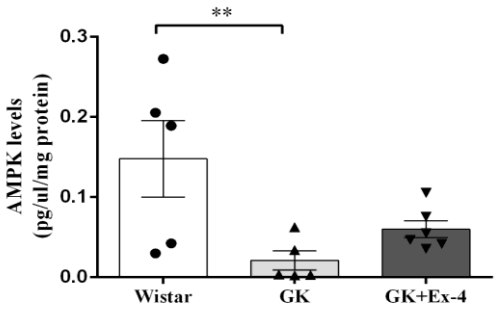
N.


P-ERK44,42/Total ERK44,42
1.5
1.0
0.5
0.0
Wistar
GK
GK+Ex-4
P-ERK
42/44 kDa
Total ERK
42/44 kDa

O.


P-JNK/Total JNK
5
4
3
2
1
0
Wistar
GK
GK+Ex-4
P-JNK
46 kDa
Total JNK
46 kDa

P.


AMPK levels
(pg/ul/mg protein)
0.3
0.2
0.1
0.0
**
Wistar
GK
GK+Ex-4

Q.

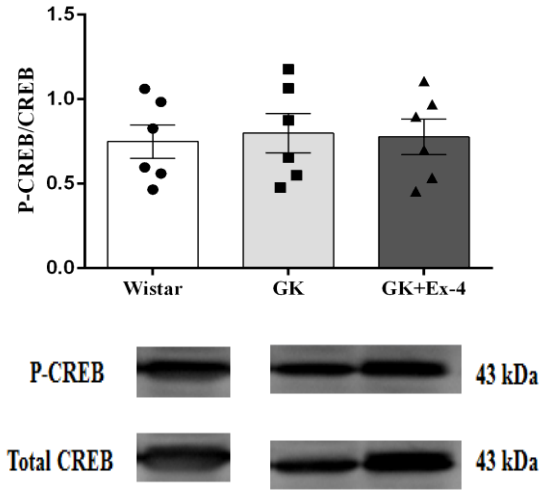

P-CREB/CREB
1.5
1.0
0.5
0.0
Wistar
GK
GK+Ex-4
P-CREB
43 kDa
Total CREB
43 kDa

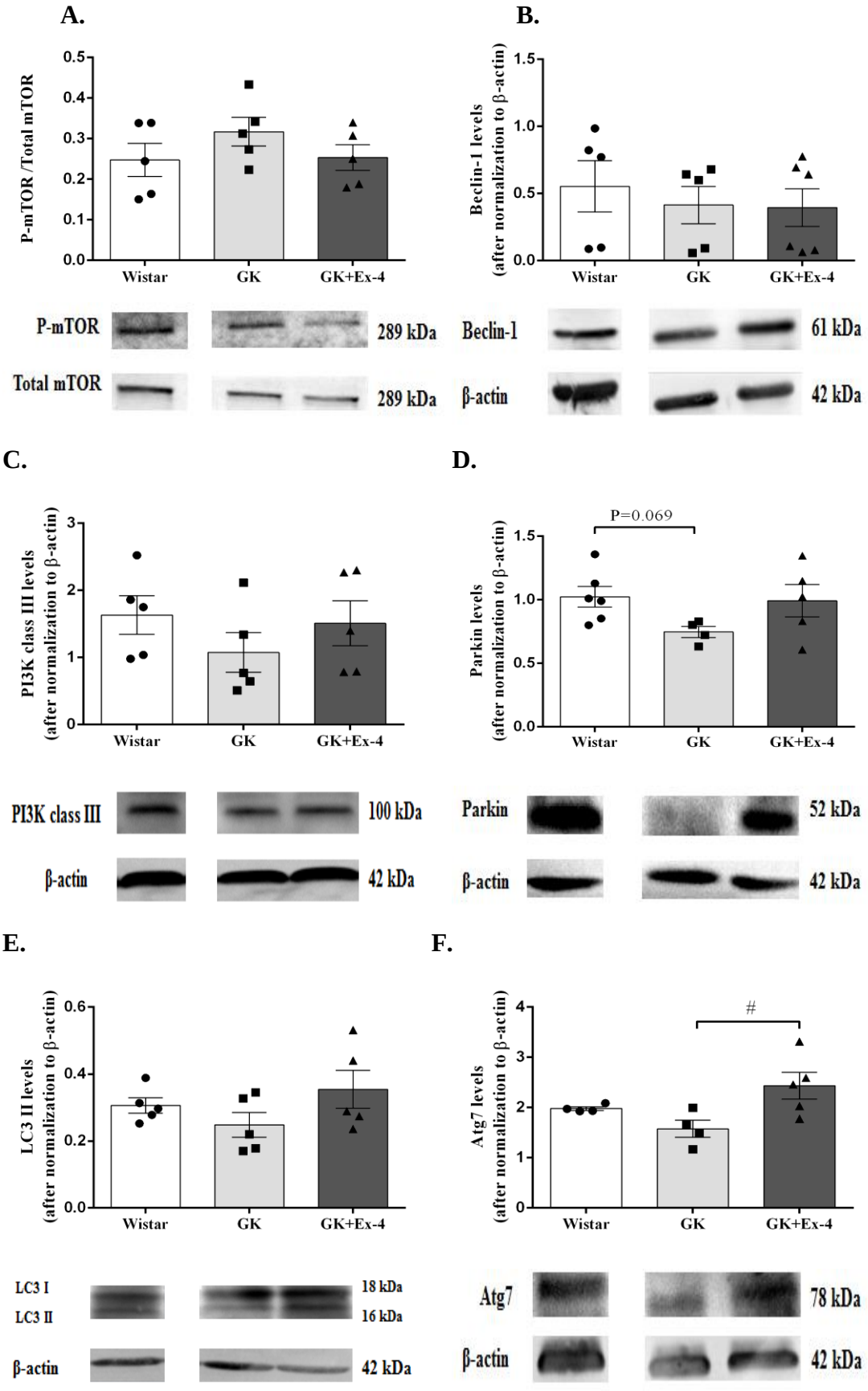
Figure 4.2 - Effect of peripheral Ex-4 administration in middle-aged T2D rat brain cortical GLP-1, insulin, and IGF-1 levels and downstream signaling pathways. GLP-1 levels (A), GLP-1R density (B), insulin levels (C), IR density (D), IGF-1 levels (E), IGF-1R density (F), cAMP levels (G), PKA activity (H), cGMP levels (I), inactivated IRS-2 (Phospho-Ser731) (J), PI3K p110 protein levels (K), activated Akt (L), activated GSK3β (Phospho-Y216) (M), activated ERK1,2 (N), activated JNK (O), AMPK levels (P), and activated CREB (Q). Data are mean ± SEM of the indicated number of rats. Statistical significance: **P* < 0.05, ***P* < 0.01 vs.Wistar rats; #*P* < 0.05 vs. GK rats, by one-way ANOVA, with protected Fisher LSD post-test.

4.4.3 - Peripheral Ex-4 Treatment Promoted Brain Cortical Autophagy upon T2D

Previous reports from our group demonstrated an impairment in autophagic mechanisms in both T2D and AD models (Santos *et al.*, 2014b; Carvalho *et al.*, 2015; Santos *et al.*, 2014a).

We observed that, although non-significant, Ex-4 treatment partially attenuated (by 20%, *P* = 0.2361) the slightly higher GK rat brain cortical levels of P-mTOR, a negative regulator of autophagy (Hands *et al.*, 2009) (Fig. 4.3A). Despite no significant changes in Beclin-1 (involved in autophagosome nucleation and maturation (Funderburk *et al.*, 2010)) (Fig. 4.3B), s.c. therapy with Ex-4 tendentiously increased (by 40%, *P* = 0.3356) the protein levels of PI3K class III (involved in autophagosome formation (Moreira *et al.*, 2010)) (Fig. 4.3C). In accordance with a potential Ex-4-induced brain cortical autophagy in T2D rats, we also found a tendentiously higher (by 33%, *P* = 0.1140) level of brain Parkin expression (a marker for the autophagic nucleation during mitophagic removal of dysfunctional mitochondria (Yamano *et al.*, 2016; Scarffe *et al.*, 2014)) upon Ex-4 administration to T2D rats (Fig. 4.3D). This was followed by 43% (though non-significant, *P* = 0.0920) and 54% higher (*P* = 0.0125) brain LC3-II and Atg7 levels (two markers for autophagosome formation and membrane elongation (Chen and Karantza-Wadsworth, 2009; Nixon, 2007)) in Ex-4-treated T2D rats compared to the saline-treated ones (Fig. 4.3E, F). Regarding p62 (a receptor involved in cargo recognition and selective clearance into autophagosomes (Katsuragi *et al.*, 2015; Bitto *et al.*, 2014)), despite non-statistically significant, Ex-4 lowered p62 protein expression in T2D brains by 26% (*P* = 0.3466) (Fig. 4.3G). In line with this, we observed a slight increase (by 42%, *P* = 0.0882) in glycosylated LAMP-1

levels (a receptor of lysossomal membranes that marks for autophagy maturation (Eskelinen, 2006)) in Ex-4-treated GK rat brains (Fig. 4.3H).



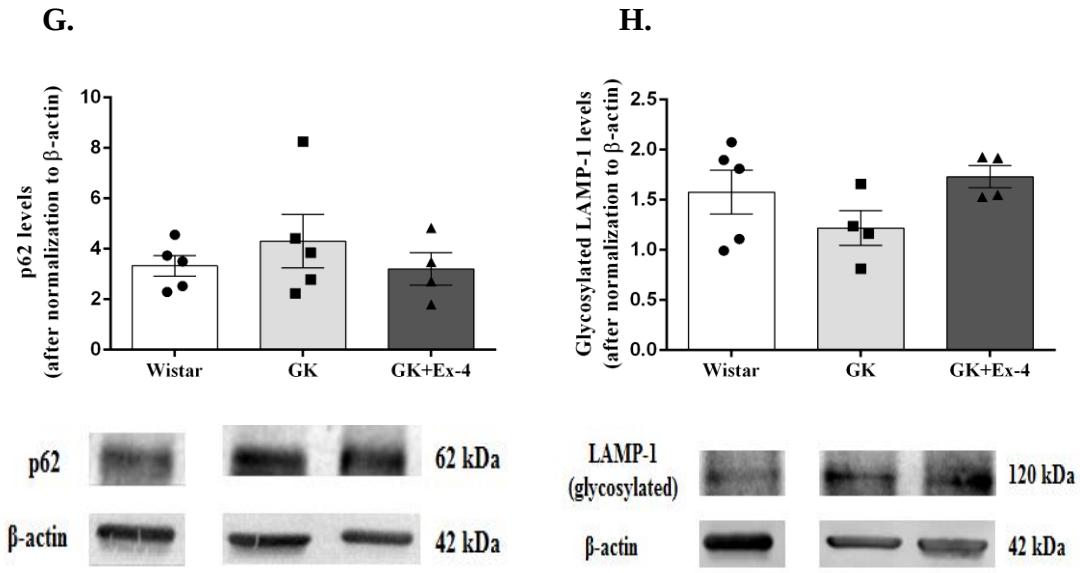


Figure 4.3 - Effect of peripheral Ex-4 administration in rat brain cortical autophagic mechanisms upon T2D. mTOR activation (A), Beclin-1 protein levels (B), PI3K class III protein levels (C), Parkin protein levels (D), LC3 II protein levels (E), Atg7 protein levels (F), p62 protein levels (G), and glycosylated LAMP-1 protein levels (H). Data are mean ± SEM of the indicated number of rats. Statistical significance: #*P* < 0.05 vs. GK rats, by one-way ANOVA, with protected Fisher LSD post-test.

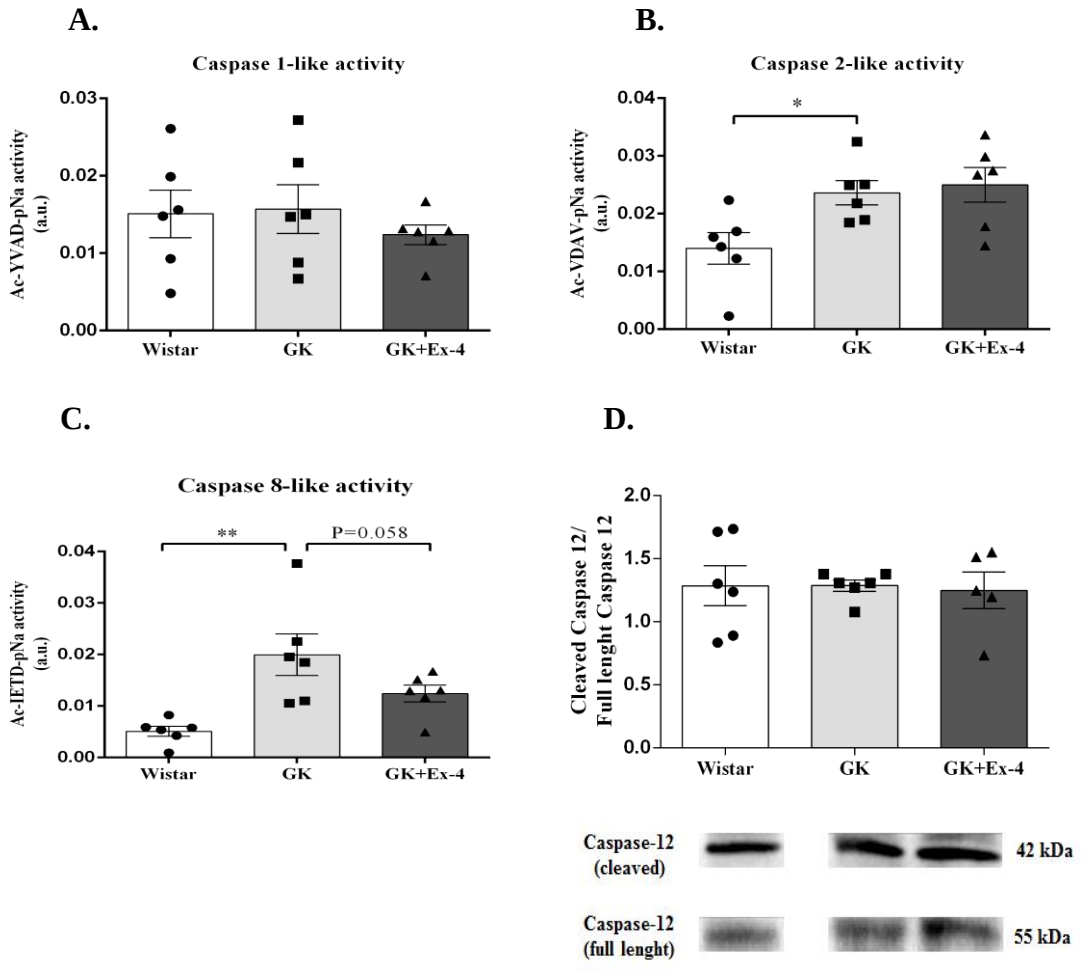
4.4.4 - Peripheral Exposure to Ex-4 Protected Against Apoptotic Cell Death upon T2D

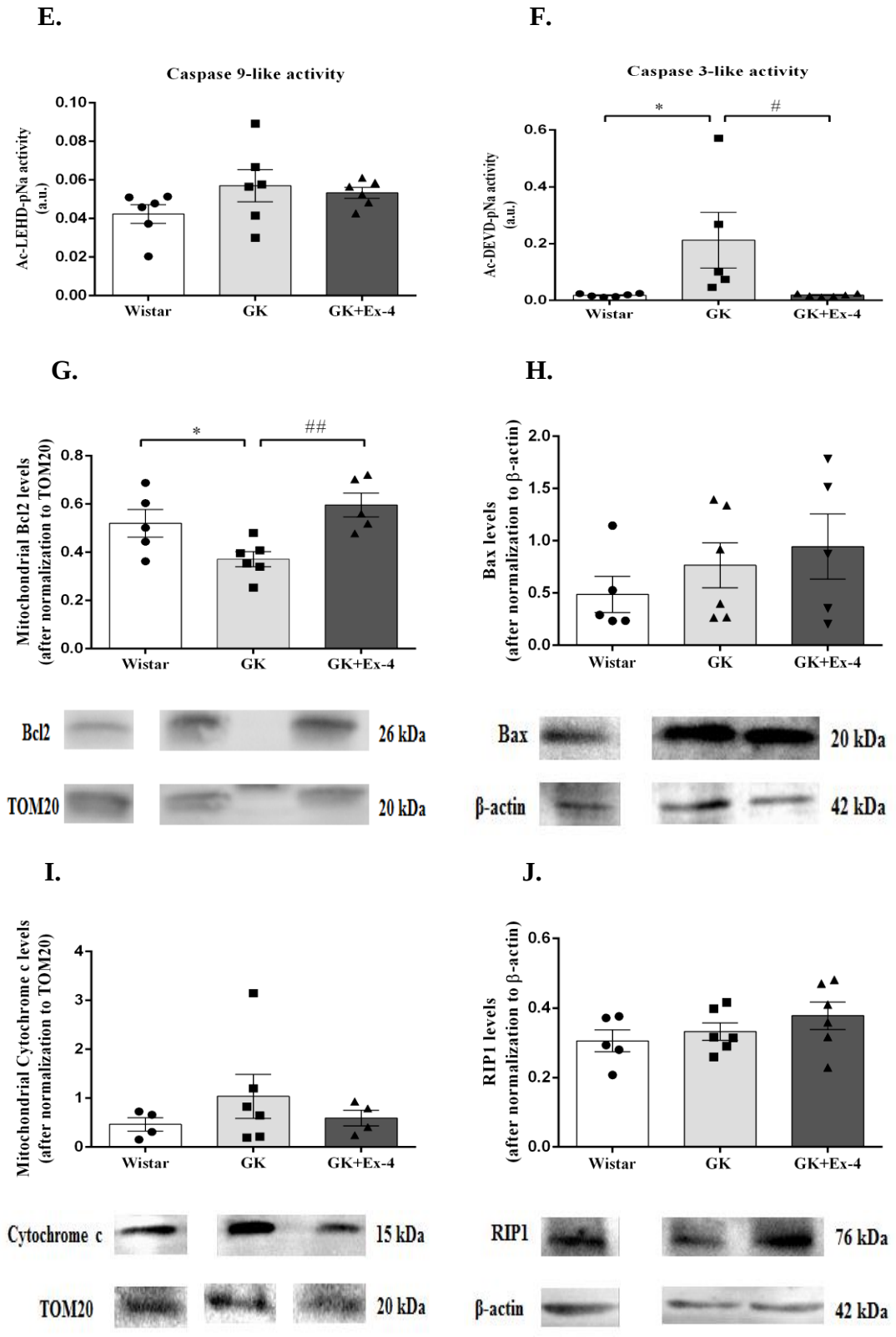
Given the potential anti-apoptotic role of Ex-4 described previously (Derosa and Maffioli, 2012), in this study, we also analyzed the effect of s.c.-administered Ex-4 on T2D brain cortical apoptotic activation. Despite no significant differences on caspase-1-like activation between saline-treated Wistar and GK rat brains, Ex-4 treatment slightly decreased its activity in GK rats (by 21%, *P* = 0.3920) (Fig. 4.4A). On the other hand, Ex-4 did not rescue the significantly higher (by 68%, *P* = 0.0211) activity of caspase-2-like in saline-treated GK rat brains (Fig. 4.4B), while it almost significantly counteracted (by 38%, *P* = 0.0577) the 292% higher activation (*P* = 0.0010) of caspase-8-like (a marker for the extrinsic apoptotic pathway (Boatright and Salvesen, 2003)), in saline-treated GK rat brains (Fig. 4.4C). No significant changes were found in the activation of the endoplasmic reticulum (ER) stress-induced apoptosis marker, caspase-12 (Fig. 4.4D), nor in caspase-9-like activity (Fig. 4.4E). However, peripheral administration of Ex-4 significantly reduced the activity of the effector caspase-3-like by 91% (*P* = 0.0163) in GK rat brains, to values nearly those of Wistar rats (Fig. 4.4F).

This was accompanied by an Ex-4-induced reversal (by 60%, *P* = 0.0037) of mitochondrial Bcl2 protein expression (an anti-apoptotic protein that inhibits the translocation of Bax from cytosol to mitochondria and the formation of toxic Bax homodimers (Murphy *et al.*, 2000)) in GK rat brain cortices (Fig. 4.4G) that, nonetheless, was followed by slightly higher levels (by 24%, *P* = 0.5975) of the pro-apoptotic protein Bax in Ex-4-treated GK rats (Fig. 4.4H). Additionally, Ex-4 induced a non-significant attenuation (by 43%, *P* = 0.3948) in mitochondrial cytochrome c protein expression in GK rats than in the saline-treated ones (Fig. 4.4I).

Our analysis of the necrotic markers RIP1 and RIP3 did not demonstrate any significant differences between groups regarding these protein levels (Fig. 4.4J, K).

In sum, our results suggest that chronic peripheral exposure to Ex-4 may counteract the T2D-related peripheral metabolic dysfunction in middle-aged rats, and exert neuroprotective effects by activating brain cortical GLP-1/IGF-1 signaling, promoting autophagy and inhibiting apoptosis under T2D.




E.
Caspase 9-like activity
Ac-LEHD-pNa activity (a.u.)
0.10
0.08
0.06
0.04
0.02
0.00
Wistar
GK
GK+Ex-4
F.
Caspase 3-like activity
Ac-DEVD-pNa activity (a.u.)
*
#
0.6
0.4
0.2
0.0
Wistar
GK
GK+Ex-4
G.
Mitochondrial Bcl2 levels (after normalization to TOM20)
*
##
0.8
0.6
0.4
0.2
0.0
Wistar
GK
GK+Ex-4
Bcl2
26 kDa
TOM20
20 kDa
H.
Bax levels (after normalization to β-actin)
2.0
1.5
1.0
0.5
0.0
Wistar
GK
GK+Ex-4
Bax
20 kDa
β-actin
42 kDa
I.
Mitochondrial Cytochrome c levels (after normalization to TOM20)
4
3
2
1
0
Wistar
GK
GK+Ex-4
Cytochrome c
15 kDa
TOM20
20 kDa
J.
RIP1 levels (after normalization to β-actin)
0.6
0.4
0.2
0.0
Wistar
GK
GK+Ex-4
RIP1
76 kDa
β-actin
42 kDa

K.

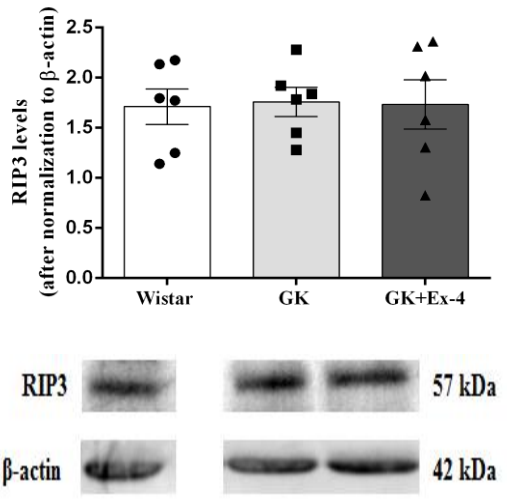


Figure 4.4 - Effect of peripheral Ex-4 administration in rat brain cortical apoptotic and necroptotic pathways upon T2D. Caspase-1-like activity (A), caspase-2-like activity (B), caspase-8-like activity (C), caspase-12 activation (D), caspase-9-like activity (E), caspase-3-like activity (F), mitochondrial Bcl2 protein levels (G), Bax protein levels (H), mitochondrial Cytochrome c protein levels (I), RIP1 protein levels (J), and RIP3 protein levels (K). Data are mean ± SEM of the indicated number of rats. Statistical significance: **P* < 0.05, ***P* < 0.01 vs. Wistar rats; #*P* < 0.05, ##*P* < 0.01 vs. GK rats, by one-way ANOVA, with protected Fisher LSD post-test.

4.5 - DISCUSSION

The potent effects of Ex-4 in the CNS, particularly in T2D or AD, have been increasingly studied in the recent years (Holscher, 2014a; Seufert and Gallwitz, 2014). To our knowledge, this is the first study on the role of peripherally administered Ex-4 in brain cortical alterations associated with T2D *per se*, particularly in autophagic and apoptotic pathways.

We found that chronic, continuous s.c. therapy with Ex-4 may initiate an insulinotropic response in middle-aged T2D GK rats, thereby attenuating their peripheral insulin resistance and abnormal glucose regulation, in line (at least partially) with previous studies in humans and Zucker rats (Derosa *et al.*, 2011; Gedulin *et al.*, 2005). Although both authors reported that Ex-4 rescues fasting hyperinsulinemia, the time of treatment used was longer than in the present study. Interestingly, recent studies suggested that Ex-4 efficacy may be also insulin-independent (Smits *et al.*, 2015;

Gastaldelli *et al.*, 2014; Dhanesha *et al.*, 2012b). Our observation of the possible benefits of Ex-4 in vascular and cardiovascular function (given by the lower triglycerides and heart rates) was also in accordance with Simó *et al.* (Simo *et al.*, 2015) and Zhou *et al.* (Zhou *et al.*, 2015b). Although T2D and/or Ex-4 treatment were recently found to affect blood cholesterol levels in rodent models (Wu *et al.*, 2016; Wang *et al.*, 2014b), we did not observe significant effects in our experimental conditions.

Recent studies suggested that the benefits of Ex-4 against peripheral features of T2D may also impact the CNS (Garcia-Casares *et al.*, 2014; Pintana *et al.*, 2013; Ryan *et al.*, 2006). This was supported by an increase in brain weight after Ex-4 administration in GK rats that may also arise from its stimulation of neuroprotective mechanisms (Solmaz *et al.*, 2015), the rescue of brain vasculature (Li *et al.*, 2016b) and/or adult neurogenesis (Bertilsson *et al.*, 2008) upon T2D. Accordingly, we found that s.c. Ex-4 protected GK rat brain cortices against apoptosis, probably due to the increase in brain GLP-1 and IGF-1 levels. Conversely, the lower brain GLP-1 levels observed in saline-treated GK rats than in Wistar ones may render them more prone to cell death, as given by the (at least partially) decrement in their PKA and Akt activation (two main downstream kinases involved in GLP-1R signaling), and increased active GSK3β, JNK, and apoptosis markers. These results suggest that a decrement in circulating GLP-1 may be involved in T2D pathophysiology, whereas under physiological conditions, GLP-1 may promote cellular homeostasis, neuronal activity and survival (*e.g.*, against β-amyloid injection) (Holscher, 2010). In line with this, the increment in peripheral endogenous GLP-1 levels can be followed by its increased brain levels (Lotfy *et al.*, 2014b; Piro *et al.*, 2014), either via the blood-brain barrier (BBB) (Candeias *et al.*, 2015) and/or via the local production of GLP-1 upon the activation of the vagal nerve (Baraboi *et al.*, 2011; Kanoski *et al.*, 2011). Similar mechanisms can also underlie the increased brain IGF-1 levels upon Ex-4 administration (Mangiola *et al.*, 2015; Werner and LeRoith, 2014). This may in turn activate, *e.g.*, the PKA and IRS-2/PI3K/Akt-mediated survival signaling (Wang *et al.*, 2012b; Hayes *et al.*, 2011). Accordingly, a massive decrease in active GSK3β (phosphorylated at Tyr 216 residue) was found in brains from Ex-4-treated GK rats. The activation of this kinase has been widely related with, *e.g.*, neuronal apoptosis, and T2D and AD-like hallmarks (Takach *et al.*, 2015; Xu *et al.*, 2015a; Chen *et al.*, 2012; Kim *et al.*, 2010). Alternatively, the

increased brain levels of cGMP found in GK rats treated with Ex-4 may also protect them against apoptosis (Wang and Zhu, 2014; Ciani *et al.*, 2002).

Controversy persists on the role of Ex-4 in the MAPK/ERK1,2 protective pathway. Some authors showed its stimulation by the drug (Natalicchio *et al.*, 2016; Fan *et al.*, 2014; Liang *et al.*, 2012; Jolivalt *et al.*, 2011), whereas others failed to report alterations in ERK1,2 phosphorylation (Mukai *et al.*, 2011). Hence, the lack of significant changes in ERK1,2 activation in the present study suggests that it may not be directly involved in the beneficial effects of Ex-4 in GK rat brains. However, we cannot exclude that Ex-4 neuroprotection against T2D may also occur via the decrement in JNK phosphorylation, previously associated with the rescue of insulin signaling and apoptosis (Natalicchio *et al.*, 2013; Lietzau *et al.*, 2016; Bomfim *et al.*, 2012). This could be also relevant since IGF-1-related signaling pathways may converge with those from insulin (Duarte *et al.*, 2013; Candeias *et al.*, 2012), thereby promoting (though indirectly) brain insulin sensitivity in Ex-4-treated T2D rats (Yang *et al.*, 2016; Sandoval and Sisley, 2015; Xu *et al.*, 2015b).

AMPK is a metabolic regulator/energy sensor, which prevents neuronal apoptosis and autophagic activation, most likely via the inactivation of mTOR (Wei *et al.*, 2016; Zhou *et al.*, 2015b; XiaoTian *et al.*, 2016; Xu *et al.*, 2014). Our observation that Ex-4 partially rescued brain cortical AMPK levels in GK rats suggested an enhancement of the autophagic pathway at different steps, and possibly also in different subtypes of autophagy. More specifically, the tendentiously lower brain mTOR activation and p62 accumulation in Ex-4-treated GK rat brains were accompanied by increased PI3K class III, LC3-II (Xu *et al.*, 2015b), Atg7, and glycosylated LAMP-1 levels, suggesting an upregulation of the autophagy pathways for the Ex-4-induced removal of toxic proteins and damaged organelles upon T2D (as recently described for dysfunctional adult T2D rodent mitochondria (Santos *et al.*, 2014b; Carvalho *et al.*, 2015) and fibrils of hyperphosphorylated tau protein (Talaei *et al.*, 2014)). Importantly, among the selective autophagy pathways possibly activated by s.c. Ex-4 in GK rat brains, one can find mitophagy, as given by the rescue in the levels of Parkin that may further ameliorate mitochondrial function, ultimately protecting against apoptosis (Chang *et al.*, 2014). These results were in agreement with the effects of Ex-4 in pancreas and liver autophagy (and the subsequent amelioration of T2D pathological hallmarks in mice and humans) (Gupta *et al.*, 2014; Abe *et al.*, 2013; Sharma *et al.*,

2011), as well as in neurons from the spinal cord of Sprague Dawley rats (Li *et al.*, 2016a). Importantly, these authors also found that autophagy may be involved in Ex-4 anti-apoptotic effects, probably via the reduction of neuronal caspase-3 protein levels.

Though recent studies showed that CREB mediated the GLP-1 receptor agonists' anti-apoptotic and memory improvement effects (Gumuslu *et al.*, 2016; Shin *et al.*, 2014; Velmurugan *et al.*, 2012), we found no significant changes in its activation. However, and in line with the known anti-apoptotic effects of Ex-4 in brains from STZ-injected rats and in a neuronal model of ischemia (most likely involving the PKA and Akt pathways) (Wang *et al.*, 2012b; Chien *et al.*, 2015), we found that s.c. delivery of Ex-4 at least partially lowered the activities of caspase-1-, caspase-8, and caspase-3-like in the cerebral cortex of GK rats. This was further reinforced by their higher mitochondrial Bcl2 levels, a well-known anti-apoptotic protein (Murphy *et al.*, 2000) that has been also recently involved in autophagy. Indeed, recent evidence points towards the crosstalk between autophagy and apoptosis, with Beclin-1 and the Bcl2-family playing a pivotal role herein. More specifically, upon its interaction with Beclin-1, Bcl2/B-cell lymphoma-extra large (BclxL) may inhibit autophagy, being this Bcl-2/BclxL-Beclin-1 complex disrupted, *e.g.*, by competition with Bad and Bax for Bcl-2/Bcl-xL binding (Pedro *et al.*, 2015; Gordy and He, 2012). Moreover, besides being both mutually negatively regulated (autophagy and apoptosis), caspases and calpains may also cleave Beclin-1 and Atg5, which may in turn degrade caspase-8 (Gordy and He, 2012). Hence, this could alternatively explain not only the slight increase of Bax levels and the lower caspase-8 activity but also suggest an additional mechanism to promote autophagy and decrease the apoptotic markers observed in Ex-4-treated GK rats. Interestingly, the tendentiously lower brain mitochondrial cytochrome c levels in Ex-4-treated T2D rats may potentially constitute an anti-apoptotic mechanism via the normalization of cytochrome c levels and the reduction of its cytosolic release (Chang *et al.*, 2014; Li *et al.*, 2015a), since in its initial phases, apoptosis was shown to upregulate mitochondrial respiratory chain proteins (Chandra *et al.*, 2002; Sanchez-Alcazar *et al.*, 2000).

Necroptosis, a RIP1- and RIP3-dependent programmed cell death, has been increasingly related with hyperglycemia (LaRocca *et al.*, 2016; Xuan *et al.*, 2015). For example, Liu and coworkers (Liu *et al.*, 2015) showed that sitagliptin (a dipeptidyl peptidase-4 inhibitor) downregulated the expression of RIP3 in the hearts of diabetic

rats. Although the mechanisms remain unclear, a recent hypothesis suggested that caspase-8-related death receptor signaling may induce necroptosis (de Almagro and Vucic, 2015). Despite the above-mentioned changes in caspase-8-like activity, the absence of significant alterations in brain cortical RIP1 or 3 levels in our study suggests that necroptosis may not be involved in chronic T2D-related brain damage.

Importantly, in an apparent contrast with our observation of increased apoptosis markers in brain cortices from middle-aged, saline-treated GK rats, Hussain *et al*. (Hussain *et al.*, 2014) found that 13-month-old GK rat cortices had a lower number of neurons and increased microglia activation, suggesting an ongoing neurodegeneration that, nonetheless, was not accompanied by caspase-mediated apoptosis. This apparent discrepancy may be due to the different ages analyzed in both studies (8 vs. 13 months old) and/or to the distinct experimental techniques used to evaluate apoptotic death between both studies. However, it seems most likely that a shift in cell death processes underlying neuronal loss may be occurring over aging, ranging from apoptosis in middle-aged towards the activation of inflammatory processes (Hussain *et al.*, 2014), autophagic cell death (Liu *et al.*, 2014a; Kroemer and Levine, 2008), and/or necrosis (Liu *et al.*, 2014b; Dhungana *et al.*, 2013). Moreover, we cannot exclude the involvement of colony-dependent effects in these different studies.

In sum, either by rescuing peripheral T2D pathology and/or brain cortical GLP-1 and insulin levels in middle-aged T2D rats, chronic s.c. exposure to Ex-4 activates their brain cortical PKA and PI3K/Akt signaling pathways, thereby promoting autophagy (including mitophagy). This may allow the clearance of misfolded/unfolded proteins and damaged organelles, ultimately protecting against chronic T2D-related brain cortical injury (Fig. 4.5). In this perspective, chronic peripheral Ex-4 administration may be a promising therapeutic approach against the long-term complications of T2D, particularly those affecting the CNS (*e.g*., cognitive impairment, AD).

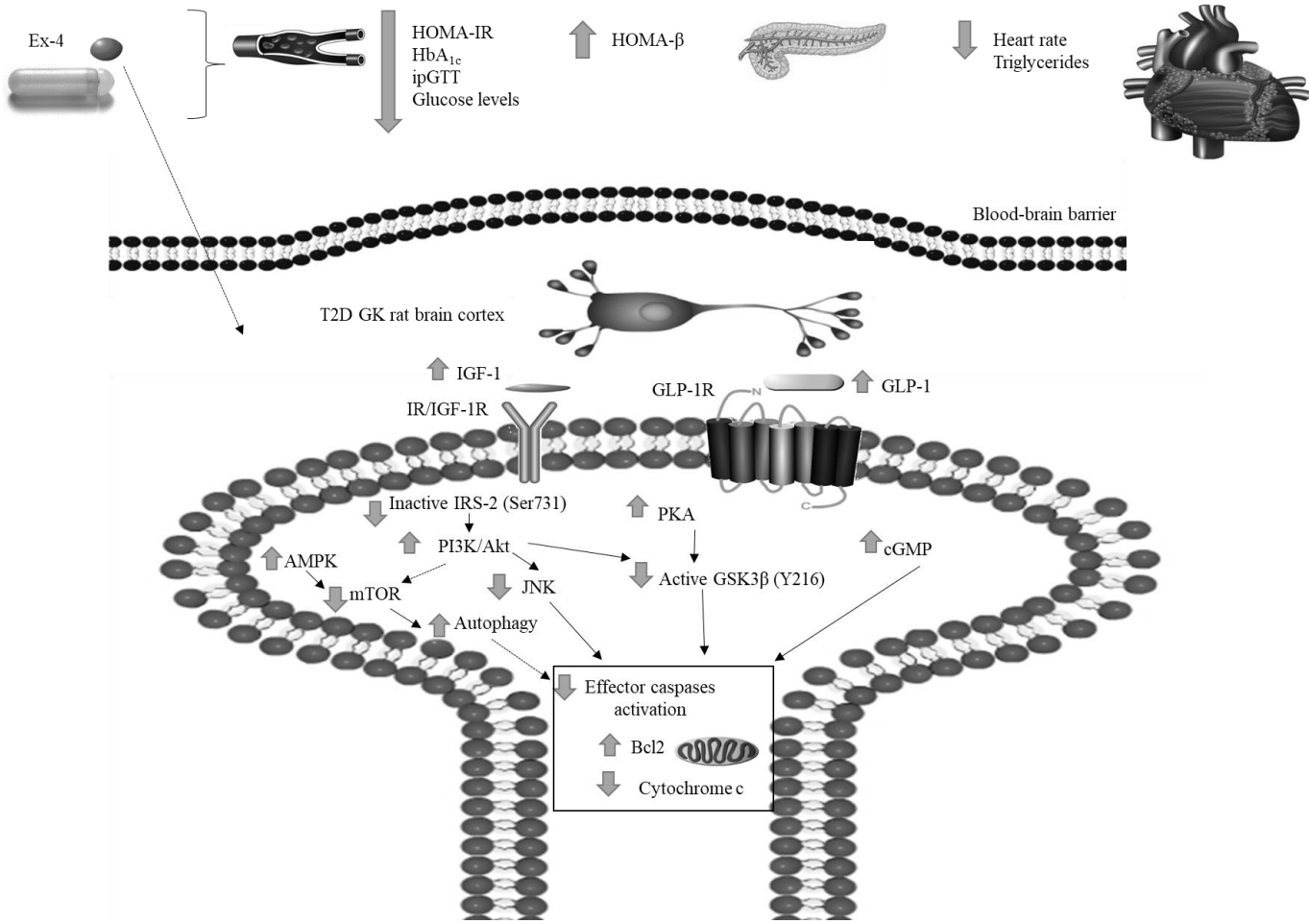


Figure 4.5 - Overview of the mechanisms underlying the effects of chronic continuous peripheral Ex-4 therapy on T2D GK rats.

Chapter 5

Exendin-4 therapy in type 2 diabetic Goto-Kakizaki rats: glucose uptake and metabolism

Exendin-4 rescues glucose transport and (energy) metabolism in type 2 diabetic rat brain

5.1 - ABSTRACT

A quarter of the world's adults have metabolic syndrome. People with this syndrome are likely to develop a cluster of dangerous conditions such as heart attack, brain abnormalities, pre-diabetes and type 2 diabetes (T2D). Insulin resistance and insulin signaling dysfunction, glucose dysmetabolism and mitochondria dysfunction are some of the features that characterize both metabolic syndrome and T2D. In this study we evaluated a continuous chronic therapy with exendin-4 (Ex-4) – a glucagon-like peptide-1 (GLP-1) receptor agonist belonging to the incretins group and approved as an anti-T2D drug – in the brains of T2D rats, focusing on the effects in glucose metabolism and mitochondria dynamics. Thus, we compared brain cortical homogenates from middle aged (8-month-old) Wistar control rats with Goto-Kakizaki (GK) rats, either treated with vehicle or Ex-4 (for 28 days). Metabolism was exhaustively analyzed through brain glucose levels, glucose uptake, GLUTs levels, activities of enzymes involved in the metabolism of glucose, the content of different basic aminoacids, levels of ketonic bodies and energy charge (ATP, ADP and AMP levels). Mitochondrial dynamics study englobed the observation of proteins involved in mitochondrial biogenesis (PGC-1α, mtFA and Nrf2), fission (Drp-1 and Fis-1) and fusion (Mfn1 and Mfn2). We showed an Ex-4-dependent effect in decreasing brain glucose levels and increasing synaptic glucose uptake, increased malate dehydrogenase activity, affected the level of different aminoacids (as glutamate, tryrosine, valine, leucine, among others), decreased the concentration of 3-hydroxybutyric acid (BOH) and improved the status of the energy charge. Regarding mitochondria results, Ex-4 appears to have no significant effect, mostly because the T2D-associated impairment is not yet present, at this age, in these GK rats. Our results revealed a neuroprotective impact of Ex-4 therapy, mainly on reverting the glucose dysmetabolism and thus restoring the homeostasis of the brain energetic status.

Keywords: type 2 diabetes, exendin-4, brain, metabolism, mitochondria

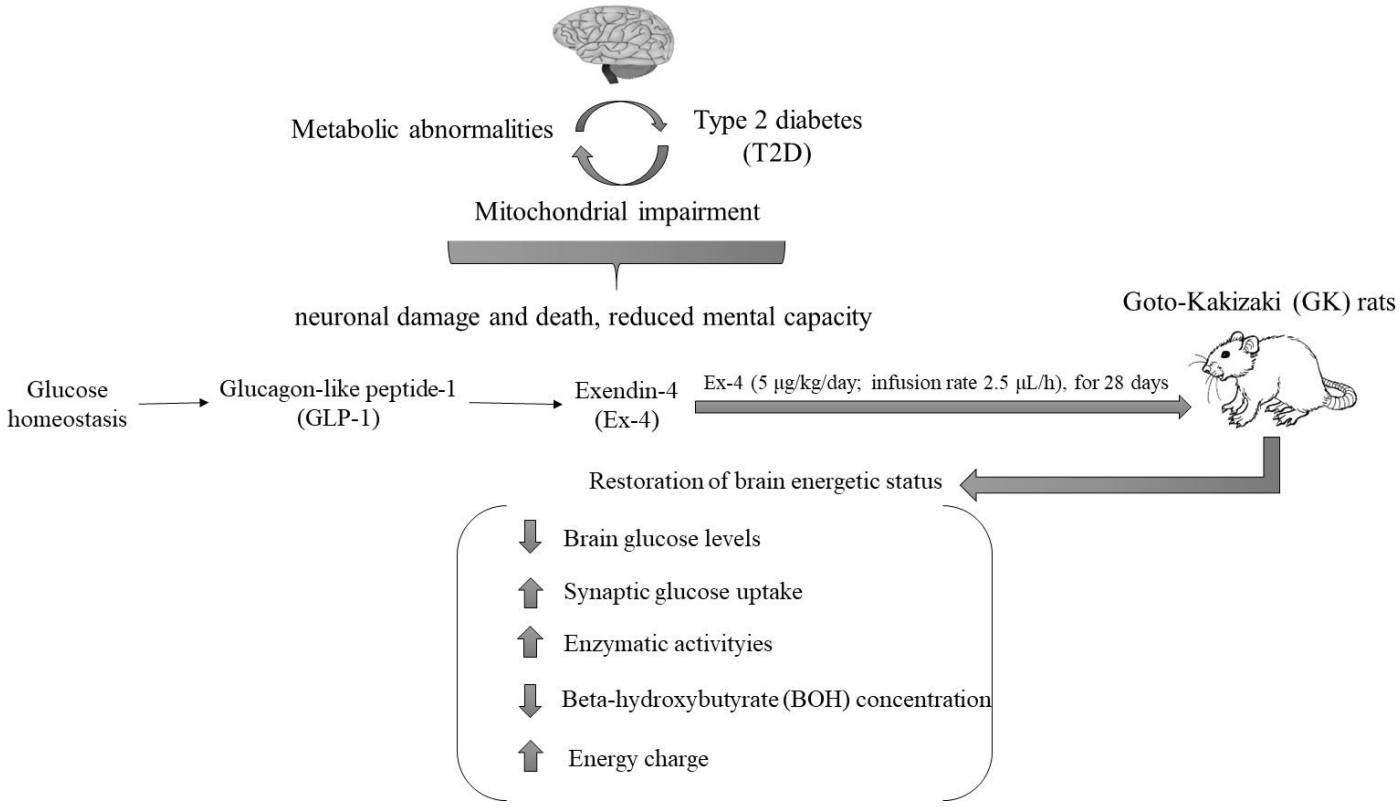


Figure 5.1– Graphical abstract.

5.2 - INTRODUCTION

Type 2 diabetes (T2D) is one of the oldest and most well-known metabolic diseases. Estimates point to more than 420 million of T2D patients worldwide and, with its steady increase, it may be the 7th leading cause of death in 2030 (Olokoba *et al.*, 2012). Although T2D features comprise hyperglycemia and insulin resistance, it is widely accepted that an extended metabolic impairment may be also a crucial pathological factor, and an early indicator of dysfunctional regulatory mechanisms that may culminate in its long-term complications affecting different organs and shortening life expectancy (Hameed *et al.*, 2015; Abdul-Ghani, 2013). Neuronal loss, brain vessels damage, microglia activation and cognitive impairment were demonstrated in the brains of T2D patients, being related with changes in their brain/peripheral metabolism (Duarte, 2015).

In mammals, brain is one of the major glucose users. It consumes ~20% of the total glucose in the human body, and strongly depends on a tight regulation of peripheral glucose homeostasis (Mergenthaler *et al.*, 2013). Physiologically, this

balance in plasma glucose levels is maintained through a tightly controlled balance between endogenous glucose production (mainly in liver, by glycogenolysis and gluconeogenesis) and its subsequent uptake⁄utilization by the cells (including skeletal muscle, adipose tissue, heart and brain) (Heijboer *et al.*, 2006). Thus, peripherally-produced glucose crosses the blood-brain barrier (BBB) and is delivered into the brain via glucose transporters (GLUTs), providing a continuous source of energy to this organ. Brain glucose transporters comprise several isoforms of GLUTs (including the most abundant GLUT1 and -3, but also GLUT4 and -8), monocarboxylate transporters (MCTs, being MCT1 and -2 the most abundant), and sodium-glucose co-transporters (SGLTs, being the most abundant SGLT1 and -6) (Shah *et al.*, 2012; Pierre and Pellerin, 2005). Despite some controversy, recent studies showed that T2D may not only impair brain glucose uptake (Garcia-Serrano and Duarte, 2020; Boersma *et al.*, 2018), but also downstream metabolic pathways (including the inhibition of glycolysis, tricarboxylic acid (TCA) and glycine-glutamate/γ-aminobutyric acid (GABA) cycles in T2D models), which could be linked with the cognitive decline upon such conditions (Zheng *et al.*, 2017; Sickmann *et al.*, 2012). Moreover, we and others showed that T2D or chronic hyperglycemia may affect brain mitochondrial function and energy metabolism (Pugazhenthi *et al.*, 2017; Wada and Nakatsuka, 2016; Carvalho *et al.*, 2014a; De Felice and Ferreira, 2014; Moreira *et al.*, 2005a; Moreira *et al.*, 2003), thereby emphasizing the involvement of brain mitochondrial dysfunction-related mechanisms in the pathogenesis of T2D (Moreira *et al.*, 2005a; Moreira *et al.*, 2003). Besides lowered rates of oxidative phosphorylation and dysfunctional mitochondrial biogenesis and fusion/fission, these may also include excessive reactive oxygen species (ROS) production, possibly culminating in insulin resistance (Nasrallah and Horvath, 2014; Carvalho *et al.*, 2012). Additionally, we and others recently provided some cues on the crosslinks between brain metabolic regulation and maintenance of intracellular quality control pathways (Ma *et al.*, 2017a; Mony *et al.*, 2016; Santos *et al.*, 2014a), including the potential relation between the T2D-associated impairment in autophagy and mitochondrial function, biogenesis and fusion/fission processes that may underlie synaptic damage and cognitive decline (Carvalho *et al.*, 2015; Santos *et al.*, 2014b).

Besides insulin, glucagon and, most importantly herein, the gastroinstetinal incretin hormone glucagon-like peptide-1 (GLP-1) constitute additional regulators of glucose levels and metabolism, energy expenditure and food intake (Sprague and

Arbelaez, 2011; Duarte *et al.*, 2013; Aronoff, 2004). Although GLP-1 anti-hyperglycemic properties are widely known and the potential link between the gut and the brain slowly uncovered (Candeias *et al.*, 2015), its short half-life in blood was a therapeutic limitation. Thus, alternative GLP-1 receptor (GLP-1R) agonists, like exendin-4 (Ex-4), were developed and are currently used to treat T2D (Sebastiao *et al.*, 2014). Importantly, Ex-4 ability to rapidly cross the BBB and exert neuroprotective effects under ischemia or AD rendered it also a potential therapy against, *e.g.*, AD (Darsalia *et al.*, 2012; Gault *et al.*, 2010) and were the bases for an ongoing clinical trial to evaluate its use in cognitive decline, and a recently finished one for treatment of AD, whose results are eagerly awaited [www.clinicaltrials.gov - NCT02847403 and NCT01255163, respectively]. In a recent study, we found that a chronic, continuous subcutaneous (s.c.) therapy with Ex-4 rescued the peripheral T2D-associated metabolic impairment, and activated brain cortical GLP-1/IGF-1 signaling and autophagy, protecting middle-aged non-obese, T2D rat brains against apoptosis (Candeias *et al.*, 2017). Importantly, Ex-4 may also modulate peripheral and brain metabolic pathways under pathological conditions, *e.g.*, by stimulating glycolysis (via glucokinase stimulation) in islets and hepatocytes of T2D diabetic mice, thus regulating their glucose homeostasis (Dhanesha *et al.*, 2012a). Others showed an Ex-4-associated increase in cerebral glucose metabolic rate (measured by positron emission tomography) in male subjects with mild postprandial hyperglycemia (Daniele *et al.*, 2015). Strikingly, the stimulation of brain lactate dehydrogenase (with the subsequent increase in lactate levels) in the PS1-KI mouse model for AD was accompanied by an enhancement on short- and long-term memory performance (Bomba *et al.*, 2013). However, to our knowledge, no studies extensively evaluated the impact of s.c. Ex-4 exposure on brain metabolic changes upon T2D *per se*. Hence, we hypothesized that s.c. administration of Ex-4 restores brain glucose (and energy) metabolism and mitochondrial dynamics upon T2D *per se*. Thus, taking advantage on our previous studies with the T2D Goto-Kakizaki (GK) rats (Candeias *et al.*, 2018; Candeias *et al.*, 2017; Carvalho *et al.*, 2014a; Santos *et al.*, 2014b; Duarte *et al.*, 2004; Moreira *et al.*, 2003; Santos *et al.*, 2000) and on our recent work on the effect of s.c. Ex-4 on brain intracellular signaling, quality control and death mechanisms (Candeias *et al.*, 2018), we aimed to evaluate the effect of a chronic, continuous s.c. therapy with Ex-4 on brain cortical glucose transport and subsequent (energy) metabolism in non-obese, middle-aged T2D GK rats.

5.3 - MATERIALS AND METHODS

5.3.1 - Materials

Ex-4, and rabbit polyclonal GLUT3, rabbit polyclonal voltage-dependent anion channel (VDAC) and rabbit polyclonal nuclear factor erythroid 2-related factor (Nrf2) antibodies were obtained from Abcam (Cambridge, UK). Micro-osmotic pumps (2ML4) were obtained from Alzet® (Cupertino, CA, USA). Bovine serum albumin (BSA), phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), Tween 20, monoclonal mouse β-actin antibody, *D*-glucose, 2-deoxyglucose, fasentin, *L*-ascorbic acid, indinavir sulfate salt hydrate, *D*-(-)-fructose, *D*-(+)-galactose, phloretin and α-cyano-4-hydroxycinnamic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). Polyvinylidene difluoride (PVDF) Immobilon-P membranes and rabbit polyclonal GLUT1 antibody were obtained from Millipore (Billerica, MA, USA). Commercial protease and phosphatase inhibitors cocktails were obtained from Roche Applied Science (Amadora, Portugal). Rat Insulin Enzyme Immunoassay kit was purchased to SPI-BIO, Bertin Pharma (Montigny le Bretonneux, France). QuantiChrom^{TM} Glucose Assay kit, EnzyChrom^{TM} Ketone Body assay kit were purchased to BioAssay Systems (Hayward, CA, USA). Uric Acid Colorimetric/Fluorometric assay kit, Lactate Colorimetric/Fluorometric assay kit, Pyruvate Colorimetric/Fluorometric assay kit were purchased to BioVision, Bio Portugal (Porto, Portugal). Rabbit polyclonal GLUT8, rabbit polyclonal peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), goat polyclonal mtFA (mitochondrial transcription factor A; TFAM), rabbit polyclonal mitofusin (Mfn)1, mouse monoclonal Mfn2 and rabbit polyclonal TOM20 antibodies were obtained from Santa Cruz Biotechnology (Heidelberg, Germany). Rabbit polyclonal P-Dynamin-1-like protein (DRP-1) (Ser616) and mouse monoclonal GLUT4 antibodies were obtained from Cell Signaling Technology (Leiden, The Netherlands). Rabbit polyclonal mitochondrial fission 1 protein (Fis1) antibody was obtained from Novus Biologicals (Abingdon, United Kingdom). Anti-mouse, anti-rabbit and anti-goat secondary antibodies, and enhanced chemifluorescence (ECF) reagent were purchased to Amersham Biosciences (Little Chalfont, UK). 2-deoxy-*D*-[1-^{3}H]glucose (2-[^{3}H]DG) was obtained from American Radiolabeled Chemicals (ARC) (St. Louis, MO, USA).

All other chemicals used were of the highest grade of purity commercially available.

5.3.2 - Animal housing and treatment

Following EU and Portuguese legislation (Directive 2010/63/EU; DL113/2013, August 7th), 8 month-old (middle-aged) male Wistar control and T2D GK rats (a non-obese model that spontaneously develop T2D early in life, resulting from the selective breeding of Wistar rats with high glucose levels) (Santos et al., 2000) 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. First, we followed the "3Rs" Reduction principle established by FELASA and the brain cortical GLP-1 levels in GK rats treated or not with Ex-4 described in our previous study (Candeias *et al.*, 2017) to estimate the number of animals required herein. Briefly, by using the t-test applied to the difference between those two independent means on the G-Power software (Faul *et al.*, 2007), an alpha error of 0.05 and a power of 80%, we estimated that a total of 6 rats should be used for the overall study. In line with this and aiming to increase the power of our hypothesis, we used at least n=4 rats/parameter. Wistar and GK rats were obtained from Charles River (Barcelona, Spain) and Taconic (Ejby, Denmark), respectively, maintained at our animal colony (Animal Research Center, University of Coimbra) in pairs of 2 animals 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 levels were used as selection index.

Thus, 6 middle-aged (8 month-old) male Wistar and 12 GK rats were s.c.-implanted with a micro-osmotic pump (2ML4, Alzet®), after a small incision in the skin between the scapulae, according to manufacturer's instructions. Rats were divided into three experimental groups. In one group, 6 GK rats were continuously infused with Ex-4 (5 μg/kg/day; infusion rate 2.5 μL/h), for 28 days (from the 8th to 9th month old) (Candeias *et al.*, 2017), whereas the remaining two groups (n=6 Wistar and n=6 GK rats) received saline infusion (0.9% sterile NaCl). Accuracy of micro-osmotic pumps was verified according to manufacturer's instructions and by weighing each pump before implantation and after removal from the animal. All surgical procedures were performed under anesthesia with inhalable isofluorane (4-5% during the induction of sedation and then 1.5-2% for maintenance) and local, s.c. butorphanol (2 mg/kg)

injection. Although not expected, a rapid decrease in body weight >15-20% was defined as a potential humane endpoint for the study.

5.3.3 - Body weight

Body weight was monitored once/week throughout the study. Results were expressed as body weight (g).

5.3.4 - Collection of peripheral blood and routine biochemical analyses

Rats were fasted for ~6h (starting early in the morning) and immediately after their euthanasia, blood from the caudal vein was collected either to determine fasting or occasional blood glucose levels by the glucose oxidase reaction, using a glucometer (Glucometer-Elite, Bayer SA, Portugal) and compatible stripes, and results were expressed as mg glucose/dL. Blood glycated hemoglobin (HbA_{1c}) was also measured with the Multi-Test HbA_{1c} (A1C Now^{+}, Bayer SA, Portugal) and results expressed as % blood was collected. The remaining blood was centrifuged at 572*xg* in a Sigma 2-16 PK centrifuge, for 10 min at 4ºC, and the resulting plasma was used to determine fasting insulin levels through the Rat Insulin Enzyme Immunoassay kit, according to manufacturer's instructions. Absorbance was read at 405nm in a SpectraMax Plus 384 multiplate reader, when maximum binding (B0) wells ranged from 0.2-0.8a.u. Results were expressed as ng/mL.

Homeostasis assessment model-insulin resistance (HOMA-IR) index was calculated using the formula: HOMA-IR = (fasting insulin [µU/mL] x fasting glucose [mmol/L])/22.5 (Wallace *et al.*, 2004; Matthews *et al.*, 1985).

5.3.5 - Isolation and preparation of brain cortical synaptosomes and homogenates

After rats' euthanasia by decapitation, the brains were immediately removed and cortices dissected. One cortex from each rat was snap-frozen for further homogenization, whilst the other cortex was used immediately to prepare crude synaptosomal fractions, according to a pre-established method (Hajos, 1975), with some

modifications. Briefly, cerebral cortices were rapidly homogenized in 10 mL of homogenization medium (containing 0.32 M sucrose, 10 mM HEPES, and 0.5 mM EGTA-K^+, buffered with Tris, pH 7.4). The homogenate was centrifuged at 1,000 *xg* for 5 min, at 4ºC, and the resulting supernatant (S_1) centrifuged again at 12,000 *xg* for 10 min, at 4ºC. Then, the resulting pellet (P_2) was resuspended in 10 mL of washing medium (containing 0.32 M sucrose, 10 mM HEPES, buffered at pH 7.4 with Tris) and centrifuged again at 12,000 *xg* for 10 min, at 4ºC. The white and fluffy crude synaptosomal layer without contaminant mitochondria (mitochondria-free) was then resuspended in the washing medium, at a protein concentration of 15–20 mg/mL, as determined by the biuret method (Layne, 1957). Experiments were carried out within 3 h after synaptosomal fraction preparation.

Regarding the preparation of brain cortical homogenates, the previously snap-frozen cortices were homogenized at 0-4ºC in lysis buffer, containing (in mM): 25 HEPES, 2 $MgCl_2$, 1 EDTA, 1 EGTA, (pH 7.4), supplemented with 2mM DTT, 100µM PMSF and commercially-available protease and phosphatase inhibitors cocktails. The crude homogenate was centrifuged at 17,968 *xg* for 10min, at 4ºC, in a Sigma 2-16K centrifuge to remove the nuclei, and the resulting supernatant was collected. The pellet was further resuspended in supplemented buffered solution and centrifuged again at 17,968 *xg* for 10min, at 4ºC. The supernatant was added to the previously obtained one and protein content was measured by the Sedmak method (Sedmak and Grossberg, 1977).

5.3.6 - Western blot analysis

Samples containing denatured brain cortical homogenates (50µg per lane) were subjected to sodium dodecyl sulfate (SDS)/polyacrylamide gel electrophoresis (SDS/PAGE) (10%) and transferred onto PVDF membranes. Then, membranes were blocked for 1h at room temperature in Tris-buffered saline (TBS, pH 7.4) plus 1% or 5% bovine serum albumine (BSA), plus 0.05% Tween 20. Membranes were then incubated overnight at 4ºC with rabbit polyclonal GLUT1 (1:1000), rabbit polyclonal GLUT3 (1:1000), mouse monoclonal GLUT4 (1:1000), rabbit polyclonal GLUT8 (1:1000), rabbit polyclonal VDAC (1:1000), rabbit polyclonal PCG-1α (1:1000), rabbit polyclonal Nrf2 (1:1000), goat mtFA (1:1000), rabbit polyclonal P-DRP-1 (Ser616)

(1:1000), rabbit polyclonal Fis1 (1:750), rabbit polyclonal Mfn1 (1:1000), mouse monoclonal Mfn2 (1:1000) primary antibodies. Membranes were then incubated with the respective anti-rabbit, -mouse or –goat secondary IgG antibodies (1:10000), for 2h, at room temperature, and developed using ECF. Immunoreactive bands were visualized by the VersaDoc Imaging System (Bio-Rad, Hercules, CA, USA). Fluorescence signal was analyzed using the QuantityOne software and the results given as INT/mm2.

Of note, membranes were then reprobed with the corresponding mouse monoclonal β-actin (1:5000) or rabbit polyclonal TOM20 (1:200) primary antibodies. Results were presented as protein levels (corresponding to the ratio of each protein *vs.* β-actin or TOM20).

5.3.7 - Analysis of 2-deoxy-D-[1-^{3}H]glucose uptake

Glucose transport was analyzed by measuring the uptake of 1 μCi/ml (12.0 Ci/mmol) of 2-[^{3}H]DG, a non-metabolizable analogue of glucose, according to a previously described method (Pellerin and Magistretti, 1994), with some modifications. Briefly, after 15 min of pre-incubation with each pharmacological inhibitor of the different glucose transporter isoforms (0.5 mM fasentin for GLUT1 (Wood *et al.*, 2008), 1 mM ascorbate for GLUT3 (Beltran *et al.*, 2011), 0.1 mM indinavir for GLUT4 (Rudich *et al.*, 2003), 250 mM *D*-fructose and *D*-galactose for GLUT8 (Ibberson *et al.*, 2000), 0.5 mM phloretin for SGLT1 and -2 (Bissonnette *et al.*, 1996), and 5 mM α-cyano-4-hydroxycinnamate (CHC) for MCT-1 and -2 (Sonveaux *et al.*, 2008), freshly-isolated brain cortical synaptosomes (1 mg/mL) were washed with sodium saline solution containing 6 mM *D*-glucose, and then washed again with glucose-free sodium solution. Then, the synaptosomal fractions were further incubated with glucose-free solution, containing 2-[^{3}H]DG (1 μCi/ml) and non-tritiated 2-Deoxyglucose (1 mM), for 10 min, at 37°C. The 2-[^{3}H]DG uptake was stopped by rinsing synaptosomes with ice-cold sodium solution. All experiments were performed in the absence of glucose. After solubilization with cold 1 M NaOH, the radioactivity was counted in a Packard Tri-Carb 2500 TR liquid scintillation analyzer. Results were expressed as pmol 2-[^{3}H]DG/mg protein.

5.3.8 - Assessment of brain glucose levels

Brain glucose levels were determined by the QuantiChrom™ Glucose Assay kit, according to manufacturer's instructions, in 5µL of each brain cortical homogenate. Absorbance was read at 630 nm in a SpectraMax Plus 384 multiplate reader. Results were expressed as mg/mL/mg protein.

5.3.9 - Determination of brain markers for glycolysis and pentose phosphate pathway

For the study of the glycolytic metabolism we determined the activity of hexokinase (the rate-limiting step of the glycolytic pathway), the rate of formation of its downstream product - glucose-6-phosphate (G6P), the levels of pyruvate and lactate, and the activity of lactate dehydrogenase (LDH) in rat brain cortical lysates (Fig. 5.3).

Hexokinase activity was determined colorimetrically at 340 nm, according to a previously described method (Crabtree and Newsholme, 1972). Briefly, 5 µL of each brain cortical homogenate were added to the 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 2U/mL glucose-6-phosphate dehydrogenase (G6PDH)) in an UV microplate, and absorbance was continuously read at 340 nm, for 2 min, with 20 s intervals, in a SpectraMax Plus 384 microplate reader, at 37ºC. The reaction was initiated by the addition of 216 mM *D*-glucose and absorbance was read again for 200 s, with intervals of 20 s, at 37ºC. Hexokinase activity was calculated using a $\varepsilon 340nm=6220\ M^{-1}cm^{-1}$. Results were expressed as µM/min/mg protein.

The rate of G6P production was determined by an adaptation of the colorimetric method described by Lamprecht and colleagues (Lamprecht and Trautschold, 1974). Briefly, 5 µL of each brain cortical homogenate were incubated in a 96-well UV plate with triethanolamine (TEA) buffer, containing (in mM): 50 TEA-hydrochloride and 22 NaOH, pH 7.5, supplemented with 0.2 mM β-$NADP^+$ sodium salt and 8.35 mM $MgCl_2$. Absorbance was continuously read for 8 min, at 339 nm, 37ºC, with 2 min intervals, in a Victor x3 plate reader. Then, the reaction was initiated by the addition of 700 U/L G6PDH from baker's yeast (*S. cerevisæ*), type VII, ammonium sulphate suspension, and the absorbance continuously read at 339 nm, for 15 min, at 37°C, in a Victor x3 plate

reader, with 2 min intervals. The rate of G6P formation was calculated by using a molar extinction coefficient of 1 $mol^{-1}mm^{-1}$, and the results expressed as nmol/min/mg protein.

Pyruvate levels were determined by the Pyruvate Colorimetric/Fluorometric assay kit, according to manufacturer's instructions, in 5 µL of brain cortical lysate (working dilution 1:10). Absorbance was read at 570 nm, in a SpectraMax Plus 384 microplate reader. Results were expressed as nmol/mg/mg protein.

Lactate levels were determined by the Lactate Colorimetric/Fluorometric assay kit, according to manufacturer's instructions, in 5 µL of each brain cortical homogenate (working dilution 1:10). Absorbance was read at 570 nm, in a SpectraMax Plus 384 microplate reader. Results were expressed as nmol/mg/mg protein.

LDH activity was determined according to the method of Bergmeyer and Bernt (Bergmeyer and Bernt, 1974). Briefly, 5 µL of each brain cortical homogenate were incubated in Tris-NaCl buffer (composed by 81.3 mM Tris and 203.3 mM NaCl, pH 7.2) supplemented with 1.5mM monosodic pyruvate. Absorbance was continuously read at 340 nm, for 3 min, with 20 s intervals, in a SpectraMax Plus 384 microplate reader, at 37ºC. The reaction was initiated by the addition of 1.2mM NADH, and the absorbance continuously read at 340 nm, for 5 min, at 37°C, in a SpectraMax Plus 384 microplate reader with 20 s intervals. LDH activity was calculated using a ε= 0.63 $mmol^{-1}mm^{-1}$. Results were expressed as µM/min/mg protein.

For the study of the pentose phosphate pathway (PPP), we determined the activity of the G6PDH, the first enzyme of this metabolic pathway, that catalyzes the conversion of G6P into 6-phosphogluconolactone. G6PDH activity was measured based on the reduction of $NADP^+$ in NADPH, according to a previously described method (Garcia-Nogales *et al.*, 1999). Briefly, 5 µL of each brain cortical lysate were incubated in a reaction buffer containing 50 mM Tris-HCl (pH 7.5), and supplemented with 50 µM $MgCl_2$ and 7.2 µM $NADP^+$. Absorbance was continuously read for 1 min, at 340 nm, 37ºC, with 20 s intervals, in a SpectraMax Plus 384 microplate reader. Then, the reaction was initiated by the addition of 0.5 mM G6P, and the absorbance continuously read for 150 s, with 20 s intervals. G6PDH activity was calculated using a ε340nm=6220 $M^{-1}cm^{-1}$, and expressed as µM/s/mg protein.

5.3.10 - Determination of brain markers for TCA cycle and the alternative formation of amino acid precursors

Citrate synthase activity was determined by a previously-described method (Coore *et al.*, 1971), with some modifications. Briefly, 20 µL of each brain cortical lysate were added to buffer A (composed by 200 mM Tris, pH=8.0, 10 mM acetyl-CoA, 10 mM DTNB), and absorbance was continuously read at 412 nm, at 37ºC, for 3 min, with 20 s intervals, in a SpectraMax Plus 384 microplate reader. Then, the reaction was initiated by the addition of 200 µM oxaloacetate, and the absorbance read again for 6 min, with 20 s intervals. Finally, a negative control was performed upon the addition of 1% Triton X-100, and the absorbance read again for more 6 min, with 20 s intervals. Citrate synthase activity was calculated using a ε=13.6 mM^{-1} cm^{-1}, and expressed as nmol/min/mg protein.

α-ketoglutarate dehydrogenase activity was determined by the conversion of $NADP^+$ in NADPH, by the method of Starkov *et al.* (Starkov *et al.*, 2004), with some modifications. Briefly, 10 µL of each brain cortical homogenate were incubated in a reaction medium containing (in mM): 25 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, 0.2 mM nicotinamide adenine dinucleotide (NAD^+), 10 mM $CaCl_2$, 0.3 mM thiamine pyrophosphate (TPP), 0.13 mM coenzyme A and 1 mM cysteine. Basal absorbance was continuously read at 340 nm, at 37ºC, during 2 min, with 20 s intervals, in a SpectraMax Plus 384 microplate reader. The reaction was initiated upon the addition of 5 mM α-ketoglutarate and absorbance read again for 2 min, with 20s intervals. α-ketoglutarate dehydrogenase activity was calculated using an ε=6220 M^{-1} cm^{-1}, and expressed as µM/min/mg protein.

Malate dehydrogenase activity was determined according to the procedure described by Nulton-Persson and Szweda (Nulton-Persson and Szweda, 2001), with some modifications. Briefly, 5 µL of each brain cortical lysate were incubated in reaction buffer, containing: 10 µM rotenone, 5 mM $MgCl_2$, 25 mM malate, 1 U/mL citrate synthase, 0.3 mM acetyl-CoA, 10 mM NAD^+. Volume was adjusted with lysis buffer, containing: 25 KH_2PO_4 (pH=7.25), 0.5 EDTA, 0.01% Triton X-100. Absorbance was continuously read at 340 nm, for 20 min, with 20 s interval, at 37ºC, in a SpectraMax Plus 384 microplate reader. Malate dehydrogenase activity was calculated using an ε340nm=6220 $M^{-1}cm^{-1}$, and the results expressed as µM/min/mg protein.

Amino acids (aspartate, glutamate, glycine, threonine, alanine, taurine, GABA, tyrosine, valine, methionine, tryptophan, phenylalanine, isoleucine, leucine, ornithine and lysine) were detected as fluorescence derivatives after pre-column derivatization with *o*-phthaldialdehyde/2-mercaptoethanol, as described by Sitges *et al.* (Sitges *et al.*, 2000), with some modifications. Briefly, amino acids were separated by reverse-phase Gilson-ASTED high performance liquid chromatography (HPLC) system, composed of a Spherisorb ODS column (particle size, 5 μm; 150 mm long; 4.6 mm i.d.) at 25°C and a Gilson model 121 fluorescence detector set, at 340 nm (excitation wavelength) and at 410 nm (emission wavelength). A linear gradient elution program carried out over 45 min was applied for amino acid elution: eluent A (30 mM sodium acetate buffer, pH 6.8) from 100% to 50%, and eluent B (methanol) from 0% to 50%, with a flow rate of 2.5 ml/min. The integration of the amino acid peak area and further calculations were carried out by Gilson system software, and quantification was allowed by running standard amino acids solutions under the same conditions. The results were expressed as pmol/mL/mg protein.

5.3.11 - Determination of brain cortical mitochondrial respiratory chain complexes I-IV activities

Complex I (NADH-ubiquinone oxidoreductase) activity was determined by a method previously described by Long *et al.* (Long *et al.*, 2009), with some modifications. Briefly, 25 μg of each brain cortical homogenate were diluted in reaction buffer containing (in mM): 25 KH_2PO_4 (pH 7.5), 5 $MgCl_2$, 0.3 KCN, 0.246 antimycin A, supplemented with 3 mg/mL BSA, 60 μM coenzyme Q_1 and 160 μM 2,6-dichlorophenolindophenol (DCPIP). Complex I activity was continuously measured for 15 min with 30s intervals, at 600 nm, in a SpectraMax Plus 384 microplate reader, by following the decrease in absorbance of DCPIP at 37ºC, upon addition of 100 μM of freshly-prepared NADH. Enzyme activity was calculated through the mean of 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). A molar extinction coefficient of ε600 = 19.1 $mM^{-1}.cm^{-1}$, and normalization to protein amount and citrate synthase activities were applied. Complex I activity was expressed as nmol DCPIP/min/mg protein.

Complex II/III (succinate-cytochrome c reductase) activity was determined by a modification of the method previously described by Tisdale (Tisdale, 1967). Briefly, 25 μg of each brain cortical lysate were preincubated for 5 min, at 37 °C, in 200 μL of phosphate buffer (composed by 166 mM KH_2PO_4, 166 mM K_2HPO_4, pH 7.4), supplemented with 1 mM KCN and 20 mM sodium succinate. The reaction was initiated by the addition of 120 μL of phosphate buffer supplemented with 0.1 mM oxidized cytochrome c plus 0.3 mM EDTA-dipotassium. Enzyme activity was measured by following the increased absorbance associated with the reduction of cytochrome c, at 550 nm, for 5 min with 30s intervals, using a VICTOR 269 X3 plate reader. Mitochondrial complex II/III was calculated through the mean of slopes obtained during the linear phase. Mitochondrial complex II/III specific activity was determined as the difference between basal activity in the absence and presence of 40 μM antimycin A (specific inhibitor of complex III). An $\varepsilon 550 = 19.1\ mM^{-1} \cdot cm^{-1}$, and normalization to protein amount and citrate synthase activities were applied. Mitochondrial complex II/III activity was expressed as nmol oxidized cytochrome c/min/mg protein.

Complex III (cytochrome c reductase) activity was determined by the method previously described by Luo *et al.* (Luo *et al.*, 2008), with some modifications. Briefly, 25 μg of each brain cortical lysate were incubated in reaction buffer containing: 25 mM KH_2PO_4 (pH 7.5), 4 μM rotenone, 0.025% Tween-20, 100 μM freshly-prepared decylubiquinone, at 37 °C, and enzymatic activity was followed by the increase in absorbance of oxidized cytochrome c at 550 nm, upon the addition of 75 μM oxidized cytochrome c, in a VICTOR X3 plate reader, for 5 min with 30s intervals. Complex III activity was calculated through the mean of 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 mM antimycin A (specific inhibitor of complex III). An $\varepsilon 550 = 19.1\ mM^{-1} \cdot cm^{-1}$, and normalization to protein amount and citrate synthase activities were applied. Mitochondrial complex III activity was expressed as nmol oxidized cytochrome c/min/mg protein.

Complex IV (cytochrome c oxidase) activity was determined by a method previously described (Brautigan *et al.*, 1978) with some modifications. Briefly, 25 μg of each brain cortical lysate were incubated at 37 °C, in reaction buffer containing 50 mM KH_2PO_4 (pH 7.0), 4 μM antimycin A, 0.05% n-dodecyl-β-D-maltoside. Enzymatic

activity was followed by a decrease in absorbance of reduced cytochrome c at 550 nm, upon addition of 57 µM of freshly-prepared reduced cytochrome c in a VICTOR X3 plate reader, for 15 min with 30s intervals. Complex IV activity was calculated through the mean of 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 of KCN (specific inhibitor of complex IV). An ε550 = 19.1 $mM^{-1} \cdot cm^{-1}$ and normalization to protein amount and citrate synthase activities were applied. Mitochondrial complex IV activity was expressed as nmol reduced cytochrome c/min/mg protein.

5.3.12 - Determination of adenine nucleotide, phosphocreatine, adenosine metabolites and uric acid levels

Brain cortical homogenates (5 µL/sample, further diluted into 25 µL supplemented lysis buffer) were assayed for adenine nucleotides (ATP, ADP and AMP) by separation in a reverse-phase HPLC, as described by Stocchi *et al.* (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. The required time for each analysis was 6 min. Adenine nucleotides (ATP, ADP and AMP) were identified by their chromatographic behavior (retention time, absorption spectra, and correlation with standards). Results were presented as nmol or pmol/mg protein. Adenylate energy charge (AEC) was determinate according the following formula: ATP + 0.5 ADP/(ATP + ADP + AMP).

The rate of phosphocreatine formation was determined by a similar procedure to the above mentioned colorimetric method for G6P formation, as described by Lamprecht and colleagues (Lamprecht and Trautschold, 1974). Briefly, 5 µL of each brain homogenate were incubated in a 96-well plate with TEA buffer containing (in mM): 50 TEA-hydrochloride and 22 NaOH, pH 7.5, supplemented with 0.2 mM β-$NADP^+$ sodium salt, 8.35 mM $MgCl_2$, 1.7 uL 21.1 mM ADP disodium salt, 700 U/L G6PDH from baker's yeast (*S. cerevisæ*), type VII, ammonium sulphate suspension, and

1.7 µL of 70 kU/L hexokinase from baker's yeast (*S. cerevisæ*), type F-300, sulfate-free. Absorbance was continuously read for 15min at 339nm, 37ºC, with 2 min intervals, in a Victor X3 plate reader. Then, the reaction was started with the addition of 3.3 µL of freshly prepared 1900 kU/L creatine phosphokinase, from rabbit muscle, and the absorbance read for 15 min, with 2 min intervals, against a blank prepared in the absence of protein. The rate of phosphocreatine formation was calculated by using a molar extinction coefficient of 1 $mol^{-1}.mm^{-1}$, from the extrapolation of absorbance, according the formula $A_2 - A_1 = \Delta A$, where A_2 was the reading after the addition of creatine kinase and A_1 was the basal reading. Results were expressed as nmol/min/mg protein.

Similar to adenine nucleotides, adenosine metabolites (adenosine, inosine and hypoxanthine) were determined in brain cortical homogenates (5 µL/sample, further diluted into 25 µL supplemented lysis buffer) by reverse-phase HPLC, as previously described (Stocchi *et al.*, 1985). Briefly, a mobile phase containing 10 mM NaH_2PO_4 (pH 6.0) and 16% methanol was used at a flow rate of 1.5 mL/min, in the above mentioned Beckman System Gold apparatus. The required time for each analysis was 5 min. Adenosine metabolites (adenosine, inosine and hypoxanthine) were identified by their chromatographic behavior (retention time, absorption spectra, and correlation with standards). Peak identity was determined by following the retention time of standards. The results were presented as nmol/mg protein.

Uric acid levels were determined by the Uric Acid Colorimetric/Fluorometric assay kit, according to manufacturer's instructions, in 5 µL of each brain cortical homogenate (working dilution 1:10). Absorbance was read at 570 nm, in a SpectraMax Plus 384 microplate reader. Results were expressed as nmol/mL/mg protein.

5.3.13 - Determination of ion ATPases (Na^+/K^+-, Ca^{2+}- and Mg^{2+}ATPases) activities

Na^+/K^+-, Ca^{2+}- and Mg^{2+}ATPase activities were determined upon the quantification of the phosphate formation, according to Taussky and Shorr (Taussky and Shorr, 1953), with some modifications. Briefly, 4 µL of each brain cortical homogenate were pre-incubated for 15 min, at 37ºC, in a 96-well plate with 32.5 µL reaction medium containing (in mM): 100 NaCl, 25 KCl, 2 $MgCl_2$, 0.1 EGTA, 10

Hepes-Tris (pH 7.4), in the absence (total ATPases) or presence of 2 mM ouabain, and 2 mM ouabain plus 0.1 mM $CaCl_2$. The reaction started by the addition of 10 mM ATP-Mg^{2+} and, after 8 min incubation, at 37ºC, it was stopped by the addition of ice-cold 5% TCA. The mix was centrifuged at 1123 *xg*, for 10 min, at 4ºC in a Sigma 2-16K centrifuge. Then, 50 µL of the resulting supernatant were mixed with an equal volume of molibdate reagent (composed by 10% (w/v) ammonium molibdate and 10 N H_2SO_4) and the absorbance measured at 660 nm, against a blank prepared in the absence of protein, in a SpectraMax Plus 384 microplate reader. Determination of the P_i released from the hydrolysis of ATP was made by comparison with known concentrations of KH_2PO_4. Na^+/K^+ATPase activity was given by the difference between the total ATPases activity and the activity measured upon ouabain incubation. Ca^{2+}ATPase activity resulted from the difference between the activity measured in the presence of ouabain and the one measured in the presence of ouabain plus $CaCl_2$. Mg^{2+}ATPase activity was given by the activity measured in the presence of ouabain. Results were expressed as nmol P_i/min/mg protein or pmol P_i/min/mg protein.

5.3.14 - Measurement of ketone bodies levels

Acetoacetic acid (AcAc) and 3-hydroxybutyric acid (BOH) concentrations were determined by the EnzyChrom™ Ketone Body assay kit, according to manufacturer instructions, in 2.5 µL of each brain cortical homogenate (working dilution 1:2). Absorbance was read in a 96-well plate, at 340 nm, in a SpectraMax Plus 384 multiplate reader. Results were expressed as nM/mg protein.

5.3.15 - Statistical analysis

Results were presented as scatter plot with bar (mean ± SEM) 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 Kolmogorov-Smirnov normality test, statistical significance was determined using the one-way ANOVA test with protected Fisher's LSD post-test for multiple

comparisons (for a Gaussian distribution) or the Kruskal-Walis test, with Dunn post-test (non-Gaussian distribution). A *P*-value <0.05 was considered statistically significant.

5.4 - RESULTS

5.4.1 - Chronic Ex-4 therapy attenuated the T2D-associated peripheral hallmarks

In accordance with our previous studies (Candeias *et al.*, 2018; Candeias *et al.*, 2017), the middle-aged GK rat males used herein displayed a significant loss of body weight (by 13%), higher fasting and occasional glycemia (by 162% and 353%, respectively), HbA_{1c} levels (by 92%) and insulin resistance (by 404%, as given by the HOMA-IR index) compared to the Wistar cohort (Table 5.I). The chronic peripheral administration of Ex-4 significantly decreased both fasting and occasional glycemia (by 35%), HbA_{1c} levels (by 20%) and HOMA-IR index (by 46%) (Table 5.I). These results suggested that the continuous s.c. treatment with Ex-4 may attenuate the peripheral features of T2D in middle-aged male GK rats.

Table 5.I – Effect of Ex-4 on T2D-related physical and biochemical features of middle-aged Wistar and GK rats.

	Wistar	GK	GK+Ex-4
Body weight (g)	474.4 ± 10.41	410.8 ± 5.46****	417.2 ± 2.9
Occasional glycemia (mg glucose/dL blood)	83.33 ± 4.3	377.5 ± 37.68****	246.3 ± 37.17##
Fasting glycemia (mg glucose/dL blood)	78.5 ± 0.76	205.3 ± 20.8****	144.3 ± 12.23##
HbA_{1c} (%)	4.40 ± 0.04	8.43 ± 0.15****	6.77 ± 0.37###
HOMA-IR	0.69 ± 0.08	3.48 ± 0.40****	1.87 ± 0.30##

Data are mean ± SEM of 6 rats/group. Statistical significance: ****P<0.0001 *vs.* Wistar rats; ##P<0.01, ###P<0.001 *vs.* GK rats, by one-way ANOVA test, with protected Fisher LSD post-

test. HbA_{1c}: glycated haemoglobin A_{1c}; HOMA-IR: homeostatic model assessment for insulin resistance.

5.4.2 - Peripheral treatment with Ex-4 promoted brain glucose uptake in T2D rats

Hyperglycemia and impaired glucose utilization are known to decrease the efficiency of brain networks, ultimately inducing cognitive dysfunction (Xiang *et al.*, 2015; Morris *et al.*, 2014). Though Ex-4 was shown to attenuate high glucose-related damage, either in brain or peripheral organs (Younce *et al.*, 2013; Huang *et al.*, 2012), to our knowledge there are no studies on the effect of chronic s.c. Ex-4 treatment on brain glucose transport and metabolism upon T2D *per se*. We observed that peripheral Ex-4 therapy recovered (by 162%) the massive decrement (by 71%) of synaptosomal 2-[^{3}H]DG uptake in T2D GK rats (Table 5.II). Despite the non-significant decrement in GLUT-3 (the main neuronal GLUT isoform), -4 and -8 isoforms' (insulin-responsive GLUT isoforms that transport glucose across intracellular membranes) (Jurcovicova, 2014) densities (by 23%, 25% and 28%, respectively) in brain cortices from GK rats, and the apparent inability of s.c. Ex-4 to significantly increase their levels (Fig. 5.2A-D), when analyzing their functioning (as given by the transport of 2-[^{3}H]DG; Table 5.II) in the presence of the different glucose transporter isoforms inhibitors, we observed that Ex-4 attenuated (at least partially) the massive inhibition of all GLUT, SGLT and MCT isoforms evaluated in GK rats (Table 5.II). More specifically, s.c. Ex-4 significantly stimulated the GLUT8-, and MCT1- and 2-mediated (that transport brain lactate, pyruvate and ketone bodies (Perez-Escuredo *et al.*, 2016)) synaptosomal 2-[^{3}H]DG uptake (by 123% and 142%, respectively) in GK rats, whilst the increased transport via GLUT1 (the main endothelial and astrocyte isoform), GLUT3, GLUT4 and SGLT1 and -2 (involved in neuroprotection against, *e.g.*, ischemia (Patching, 2017)) did not reach statistical significance (Table 5.II). These results pointed towards an overall inhibition in the transport of glucose into brain upon T2D that was ameliorated by the chronic, continuous s.c. Ex-4 administration.

Table 5.II - Effect of peripheral Ex-4 treatment on GLUTs, MCTs and SGLTs-mediated uptake of 2-[^{3}H]DG in T2D rat brain cortical synaptosomes.

	Wistar	GK	GK+Ex-4
No inhibitor (pmol/mg protein)	4393 ± 325.8 n=6	1257 ± 73.27*** n=6	3295 ± 552.2## n=6
Fasentin (pmol/mg protein) (GLUT1)	4014 ± 308.6 n=6	1071 ± 139.4**** n=6	2081 ± 518.3 P=0.064 n=6
Ascorbate (pmol/mg protein) (GLUT3)	3926 ± 791.7 n=6	1261 ± 134.8* n=6	3288 ± 863.8 P=0.053 n=6
Indinavir (pmol/mg protein) (GLUT4)	3543 ± 620.8 n=6	1306 ± 178.9* n=6	2564 ± 863.1 n=5
Fructose + Galactose (pmol/mg protein) (GLUT8)	4323 ± 291.9 n=6	1433 ± 274*** n=6	3189 ± 662.1# n=6
Phloretin (pmol/mg protein) (SGLT1 and 2)	4071 ± 637.6 n=5	1376 ± 566.2** n=5	2831 ± 614.7 P=0.080 n=6
CHC (pmol/mg protein) (MCT1 and 2)	4094 ± 578.2 n=6	1385 ± 279.5** n=6	3353 ± 561.3# n=6

Glucose uptake was analyzed by measuring the uptake of 2-[^{3}H]DG, a non-metabolizable analogue of glucose. Pharmacological inhibitors: fasentin for GLUT1, ascorbate for GLUT3, indinavir for GLUT4, fructose and galactose for GLUT8 and phloretin for SGLT1. Uptake by MCT-1 and -2 (involved in transport of lactate, pyruvate and ketone bodies; CHC used as inhibitor) was also analyzed. Data are mean (SD) of the indicated number of rats/group. Statistical significance: *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 *vs.* Wistar rats; #P<0.05, ##P<0.01 *vs*. GK rats, by one-way ANOVA test, with protected Fisher LSD post-test. SGLT: sodium-glucose linked transporter, CHC: α-cyano-4-hydroxycinnamate, MCT: monocarboxylate transporter.

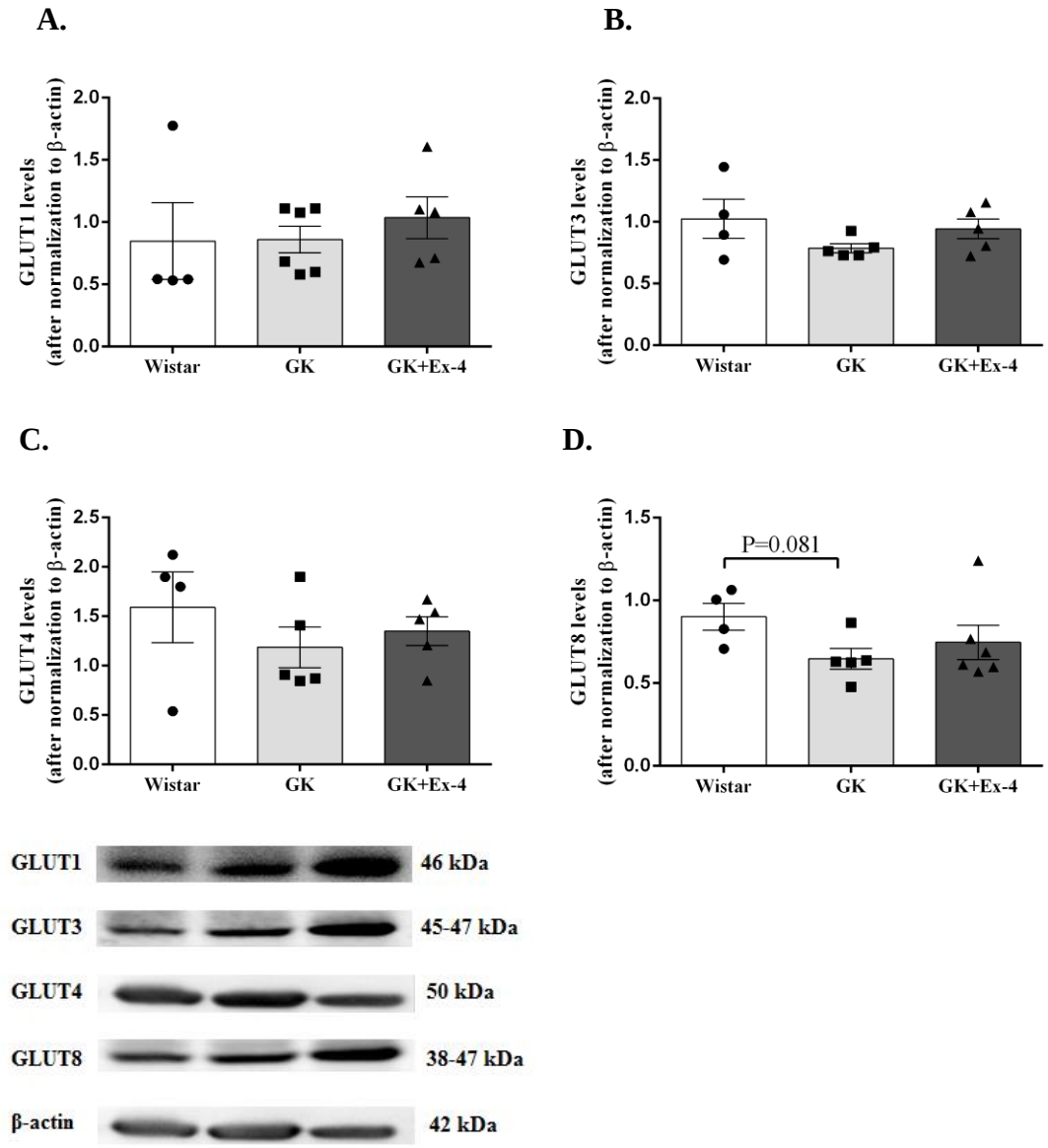


Figure 5.2 – Effect of peripheral Ex-4 treatment on T2D rat brain cortical GLUTs protein levels. GLUT 1 protein levels (A), GLUT3 protein levels (B), GLUT4 protein levels (C), GLUT8 protein levels (D). Data are mean ± SEM of the indicated number of rats/group. Statistical significance: by one-way ANOVA, with protected Fisher LSD post-test. GLUT: glucose transporter.

5.4.3 - Effect of peripheral Ex-4 administration on T2D rat brain glycolysis and pentose phosphate pathway

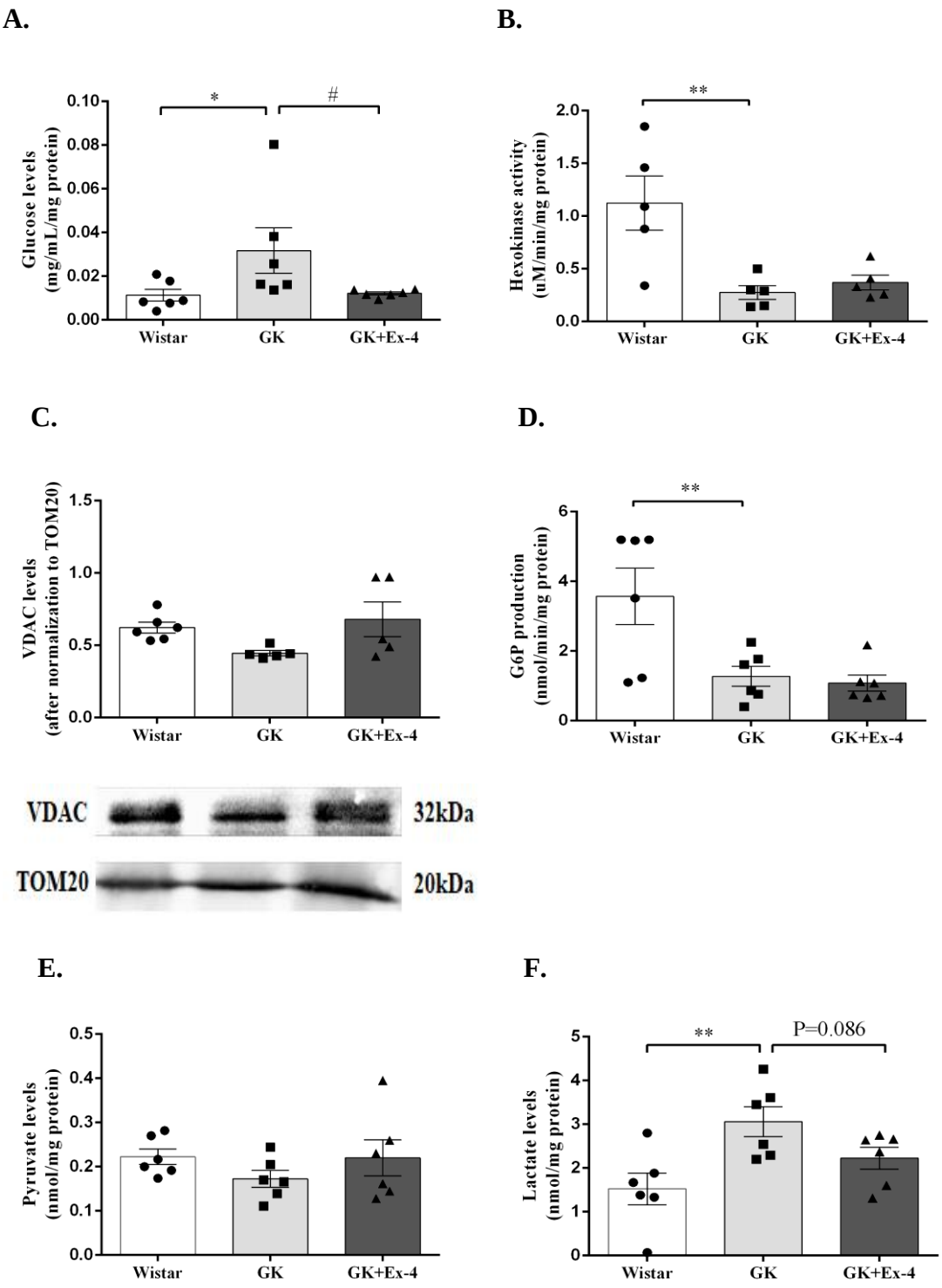
Despite the above-mentioned stimulation of brain glucose uptake induced by s.c. Ex-4 in GK rats, we observed that the drug significantly reversed the 182% higher

glucose content in GK rat brains (Fig. 5.3A). This suggested that, besides promoting brain glucose transport, Ex-4 may also accelerate its metabolism by the cells. Indeed, under physiological conditions, once inside neurons/astrocytes, glucose is immediately and efficiently metabolized, mainly via glycolysis and subsequent TCA cycle and oxidative metabolism, to produce the energy that fuels brain function (Falkowska *et al.*, 2015).

Since hexokinase constitutes the first limiting enzyme of the glycolytic pathway, involving the phosphorylation of glucose to G6P at the expense of ATP (Wilson, 2003), we next evaluated hexokinase activity. Though there was a major drop (by 75%) in hexokinase activity in brain cortices from GK rats, peripheral therapy with Ex-4 did not rescue this effect (Fig. 5.3B). This was partially mirrored by the levels of plasma membrane and mitochondrial outer membrane (MOM) protein, VDAC, recently described to interact with hexokinase to form a complex at MOM that protects against its permeabilization and apoptosis (Boulbrima *et al.*, 2016; Rosa and Cesar, 2016). Accordingly, we observed that the levels of mitochondrial VDAC were tendentiously decreased in GK rats (by 28%), being reestablished with Ex-4 exposure (by 53%) (Fig. 5.3C).

As these results suggested that peripheral Ex-4 may overcome the glycolysis inhibition in T2D diabetic rat brains, we next evaluated the rate of G6P formation resulting from the reaction catalyzed by hexokinase. Interestingly, we found that while G6P production significantly decreased (by 64%) in T2D brains, Ex-4 therapy had no effect herein (Fig. 5.3D). Although no significant changes on brain cortical pyruvate levels (a downstream product of glycolysis) were observed between the three experimental groups (Fig. 5.3E), Ex-4 partially attenuated (by 27%) the significant increase (by 101%) in GK rat brain cortical lactate levels (Fig. 5.3F). Since pyruvate may be converted into lactate in a process catalyzed by the reversible enzyme LDH (anaerobic glycolysis) or enter the TCA cycle via oxidation to acetyl coenzyme A (acetyl CoA), these results, together with the massive drop (by 82%) in brain LDH activity in GK rats and the inability of Ex-4 to counteract it (Fig. 5.3G), suggested that T2D may induce an overall inhibition of brain glycolysis. This appeared to be only slightly attenuated by the chronic s.c. Ex-4 treatment. However, we cannot exclude a stimulatory role for Ex-4 immediately at the level of PPP, since we found an Ex-4-dependent increase (although not statistically significant) in the activity of G6PDH (Fig.

5.3H), the enzyme that catalyzes the formation of 6-phosphogluconolactone from G6P, generating also NADPH from $NADP^+$.



G. **H.**

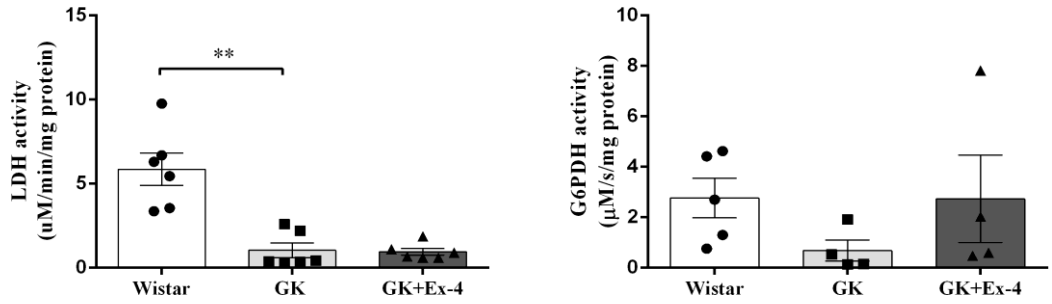


Figure 5.3 – Effect of peripheral Ex-4 treatment on T2D rat brain cortical glycolysis and pentose phosphate pathway. Glucose levels in brain cortex (A), Hexokinase activity (B), VDAC protein levels (C), G6P production (D), Pyruvate levels (E), Lactate levels (F), LDH activity (G), G6PDH activity (H). Data are mean ± SEM of the indicated number of rats/group. Statistical significance: **P*<0.05, ***P*<0.01 *vs.* Wistar rats; #*P*<0.05 *vs.* GK rats, by one-way ANOVA, with protected Fisher LSD post-test or Kruskal-Walis test, with Dunn post-test (G). LDH: lactate dehydrogenase; VDAC: Voltage-dependent anion channel; G6P: Glucose-6-phosphate. G6PDH: Glucose-6-phosphate-dehydrogenase.

5.4.4 - Peripheral Ex-4 administration rescued T2D-related impairment in brain TCA cycle and the alternative formation of amino acid precursors

Given the above-mentioned profile of brain cortical pyruvate levels upon Ex-4 treatment in GK rats and its potential metabolism through the TCA cycle, we next evaluated the activities of three main enzymes: citrate synthase (that is also a marker for functional mitochondria (Larsen *et al.*, 2012)), α-ketoglutarate dehydrogenase and malate dehydrogenase (Fig. 5.4A-C). In line with the previous results, T2D massively decreased the activities of all the three enzymes (by 54, 72 and 90%, respectively) that, nonetheless, were only partially attenuated by Ex-4 in the case of citrate synthase (by 39%) and malate dehydrogenase (non-statistically significant) (Fig. 5.4A-C).

Besides its most explored energetic and redox homeostasis outcomes, glucose metabolism is also pivotal in the biosynthesis of essential brain (and neurotransmission) components, including the amino acid neurotransmitters glutamate, aspartate, GABA and glycine, and acetylcholine (Hoyer, 1990)). Besides their involvement in neurotransmission, most of the amino acids can be further metabolized, *e.g.* by entering at several steps of the TCA cycle (Akram, 2014). This, together with our studies on the

changes induced by oxidative stress in brain GK rat synaptosomal GABA and glutamate levels (Duarte *et al.*, 2004), led us to determine brain cortical levels of the most abundant amino acids (Table 5.III).

Despite the tendentiously lower (by 88%) brain cortical glycine levels in Ex-4-treated vs. saline-treated GK rats, the drug reversed the non-statistically significant 80% and 52% decrease in both threonine and alanine levels (Table 5.III), thus possibly contributing (although indirectly) to the slight increase in T2D rat brain pyruvate levels upon s.c. Ex-4 treatment (Fig. 5.3E). Of note, despite the known T2D-mediated lowering of taurine levels in patients' platelets (De Luca *et al.*, 2001) and the ability of this amino acid to promote glycolysis in rats (Kim *et al.*, 2007), taurine levels were 64% higher in GK rat brain cortices than in Wistar ones, being only slightly increased (by 6%) upon Ex-4 administration (Table 5.III).

These observations were followed by a tendentious increase in levels of aspartate (a precursor of oxaloacetate and fumarate) in GK rat brains upon Ex-4 therapy compared to saline-treated ones (Table 5.III). This, together with the tendentiously higher levels of the acetyl CoA precursor leucine, may account for the stimulation of citrate synthase activity upon Ex-4 administration (Fig. 5.4A). However, the drug did not recover the levels of another precursor of acetyl CoA, tryptophan, but decreased (by 68%) the 215% higher levels of isoleucine (Table 5.III).

Interestingly, the T2D-induced decrement on brain cortical levels of tyrosine, tryptophan, phenylalanine and lysine (by 41%, 71%, 79% and 56%, respectively) (Table 5.III) may not only indirectly affect the TCA cycle (by generating less acetoacetyl-CoA, a precursor for acetyl CoA synthesis at mitochondria) (Berg *et al.*, 2002), but also the brain cortical cholesterol formation from acetyl CoA (Berg *et al.*, 2002), as given by the 50% decrement in GK rat brain cholesterol levels (Fig. 5.4D). On the other hand, peripheral Ex-4 may account to a higher formation of acetoacetyl-CoA (and, ultimately, to the TCA cycle) in GK rat brains, mainly due to their increased levels of tyrosine (by 334%) and leucine (by 208%) (Table 5.III) that, nonetheless, was not accompanied by the rescue in their cholesterol content (Fig. 5.4D).

Similar to the above brain cortical tyrosine, the T2D-induced decrease in the levels of aspartate (by 98%) (Table 5.III) – both are precursors of fumarate - may possibly contribute (via malate formation) to the tendentiously lower activity of malate

dehydrogenase (Fig. 5.4C). Conversely, the Ex-4-induced massive increase in both tyrosine and aspartate levels in GK rat brains (Table 5.III) may partially stimulate malate dehydrogenase (Fig. 5.4C).

Notably, the inhibition of brain cortical α-ketoglutarate dehydrogenase upon T2D (Fig. 5.4B) appeared to correlate with the tendentiously lower (by 69%) glutamate levels (Table 5.III) (another precursor of α-ketoglutarate), further contributing to the general inhibition in TCA cycle. This was followed by the Ex-4-related restoration of T2D rat brain glutamate levels to those found in Wistar rats (Table 5.III) (though this may not be enough to significantly stimulate α-ketoglutarate dehydrogenase (Fig. 5.4B)), and by the massive decrease (by 67%) in the levels of GABA (Table 5.III). This GABA can then be used as an inhibitory neurotransmitter and/or further metabolized in mitochondria, leading to the formation of succinate (Purves *et al.*, 2001), thus reinforcing the hypothesis that Ex-4 stimulates TCA cycle upon T2D. Since GABA can also originate from ornithine (via its decarboxylation into putrescine) (Yoon and Lee, 2014), the massively decreased (by 80%) brain ornithine levels upon T2D (Table 5.III) suggested that it may be alternatively converted into GABA under these conditions. However, we cannot exclude that ornithine can be also metabolized via the urea cycle towards citrulline, a precursor of arginine either directly (by the arginine-citrulline cycle, with the activation of nitric oxide synthase and the formation of nitric oxide) or indirectly (via the formation of argininosuccinate, and ultimately fumarate) (Hansmannel *et al.*, 2010; Kornberg, 2000) (Table 5.III).

At another level of this crosslink between the metabolism of amino acids and the TCA cycle, the formation of succinylCoA, a slight decrement in both valine and methionine (by 61% and 31%, respectively) was found in saline-treated GK rats (Table 5.III), again emphasizing that T2D impairs brain TCA cycle at multiple levels. However, Ex-4 therapy only improved (though non-statistically significant) the levels of valine in GK rat brains (Table 5.III). Surprisingly, the opposite pattern was observed for another amino acid precursor of succinylCoA, isoleucine, being its increased levels in GK rats normalised by Ex-4 treatment (Table 5.III).

These results suggested that chronic T2D *per se* not only impaired pivotal enzymes from the TCA cycle in brain cortices, but may also hamper the alternative formation of its amino acid precursors. Nevertheless, s.c. therapy with Ex-4 rescued, at least partially, these dysfunctional metabolic pathways in GK rat brains.

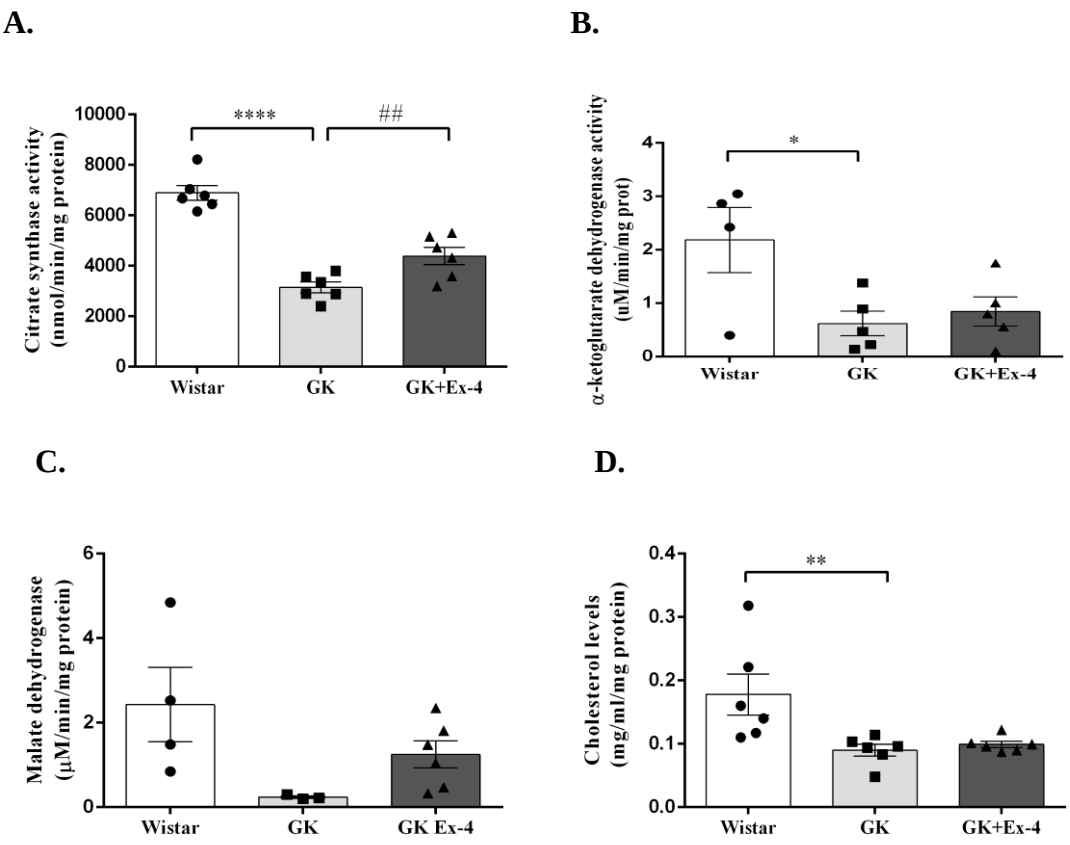


Figure 5.4 - Effect of peripheral Ex-4 treatment on T2D rat brain cortical TCA cycle. Citrate synthase activity (A), α-ketoglutarate activity; (B) Malate dehydrogenase activity (C) Cholesterol levels (D). Data are mean ± SEM of the indicated number of rats/group. Statistical significance: **P*<0.05, ***P*<0.01, *****P*<0.0001 *vs.* Wistar rats; ##*P*<0.01 *vs*. GK rats, by one-way ANOVA, with protected Fisher LSD post-test.

Table 5.III – Effect of peripheral Ex-4 treatment on rat brain cortical levels of aminoacids upon T2D.

	Wistar	GK	GK+Ex-4
Aspartate (pmol/ml/mg protein)	0.5267 ± 0.1415 n=3	0.0102 ± 0.0002* n=3	0.0467 ± 0.0233 n=6
Glutamate (pmol/ml/mg protein)	0.2217 ± 0.1192 n=6	0.0683 ± 0.0111 n=6	0.2617 ± 0.11 n=6
Glycine (pmol/ml/mg protein)	0.0967 ± 0.0633 n=3	0.1833 ± 0.0689 n=3	0.0225 ± 0.0025 n=5

Threonine (pmol/ml/mg protein)	0.1133 ± 0.0318 n=3	0.0225 ± 0.0048 n=3	0.1380 ± 0.0562 P=0.082 n=5
Alanine (pmol/ml/mg protein)	0.5933 ± 0.1519 n=3	0.2840 ± 0.1456 n=5	0.6560 ± 0.2512 n=5
Taurine (pmol/ml/mg protein)	0.4933 ± 0.0731 n=3	0.8100 ± 0.0467* n=6	0.8550 ± 0.0823 n=6
GABA (pmol/ml/mg protein)	0.5133 ± 0.0677 n=6	0.6100 ± 0.2238 n=6	0.1983 ± 0.0836 P=0.06 n=6
Tyrosine (pmol/ml/mg protein)	0.2467 ± 0.0325 n=6	0.1467 ± 0.0527 n=6	0.6380 ± 0.13### n=5
Valine (pmol/ml/mg protein)	0.2567 ± 0.0715 n=6	0.1000 ± 0.0113 n=6	0.366 ± 0.1668 P=0.072 n=5
Methionine (pmol/ml/mg protein)	0.3783 ± 0.0938 n=6	0.2600 ± 0.0421 n=6	0.2183 ± 0.0514 n=6
Tryptophan (pmol/ml/mg protein)	2.010 ± 0.2855 n=6	0.5850 ± 0.1236**** n=6	0.4817 ± 0.0244 n=6
Phenylalanine (pmol/ml/mg protein)	0.9267 ± 0.1098 n=6	0.1917 ± 0.0445**** n=6	0.2117 ± 0.0545 n=6
Isoleucine (pmol/ml/mg protein)	0.0483 ± 0.0083 n=6	0.1520 ± 0.0431** n=5	0.0483 ± 0.0130## n=6
Leucine (pmol/ml/mg protein)	0.0767 ± 0.0123 n=6	0.0800 ± 0.0146 n=6	0.2467 ± 0.0655## n=6
Ornithine (pmol/ml/mg protein)	0.8100 ± 0.2518 n=6	0.1633 ± 0.0327** n=6	0.1317 ± 0.0309 n=6
Lysine (pmol/ml/mg protein)	0.770 ± 0.1321 n=6	0.3350 ± 0.0661** n=6	0.2483 ± 0.0232 n=6

Data are mean ± SEM of the indicated number of rats/group. Statistical significance: *P<0.05, **P<0.01, ****P<0.0001 *vs.* Wistar rats; ##P<0.01, ###P<0.001 *vs.* GK rats, by one-way

ANOVA test, with protected Fisher LSD post-test or Kruskal-Walis test, with Dunn post-test (Aspartate).

5.4.5 - Peripheral Ex-4 administration improved mitochondrial respiratory chain activity and energy production in GK rat brains

Given the above observations on peripheral Ex-4 stimulation of the TCA cycle in GK rat brain cortices (Table 5.III and Fig. 5.4A-C), and the partial increment in citrate synthase activity (Fig. 5.4A) (indicating either an increased functional mitochondria or even an increase in mitochondria number), we next evaluated the role of s.c. Ex-4 administration on markers for GK rat brain mitochondrial function (Fig. 5.5, 5.6).

Despite no significant changes in the activities of the mitochondrial respiratory chain complexes I-IV between saline-treated Wistar and GK rat brain cortical homogenates (Fig. 5.5A-D), a 46% and 51% lower ATP/ADP and energy charge were observed in saline-treated T2D than in control rats (Fig. 5.6A, B). Following the significantly higher rate (by 140%) of brain phosphocreatine formation (a molecule that, together with creatine, acts as a buffer for ATP levels to maintain brain energy homeostasis (Rae, 2014)) in T2D GK rats (Fig. 5.6C), brain cortical ATP levels were significantly diminished (by 49%) upon T2D (Fig. 5.6D), whereas both ADP and AMP levels were significantly increased (by 47% and 147%, respectively) (Fig. 5.6E, F). Conversely, peripheral Ex-4 significantly increased the activities of the complexes I, II/III and III (by 59%, 103% and 243%, respectively) (Fig. 5.5A-C), and slightly stimulated (by 55%) the activity of complex IV in GK rat brain cortices (Fig. 5.5D). This was accompanied by a significant restoration in both ATP/ADP and energy charge in Ex-4-treated GK rat brain cortices to nearly normal values (Fig. 5.6A, B), and a significant reversion in their phosphocreatine levels (by 77%) (Fig. 5.6C). This Ex-4-mediated recovery from brain energy loss appeared to involve also a significant decrement in brain levels of both ADP and AMP in GK rats (by 67% in both cases) (Fig. 5.6E, F).

These results suggested that, though T2D may promote the hydrolysis of brain cortical ATP, the tissue may try to compensate by upregulating its alternative source, phosphocreatine, in a process reversed by the peripheral administration of Ex-4.

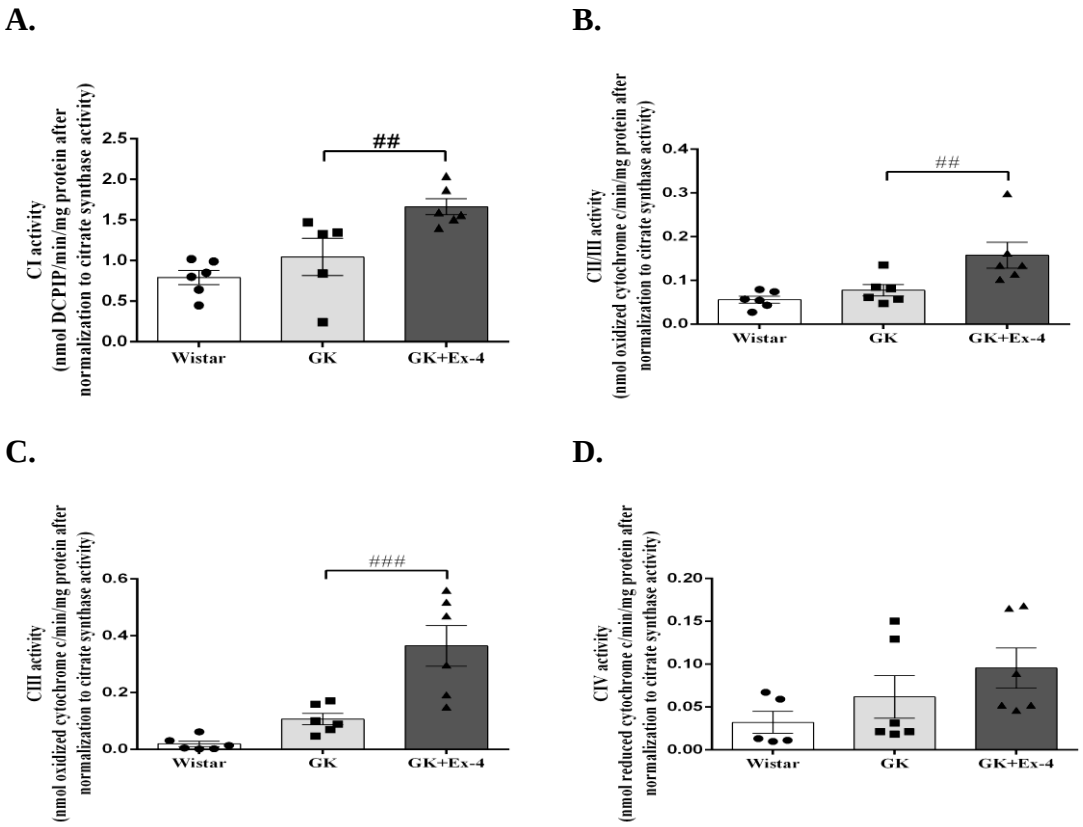
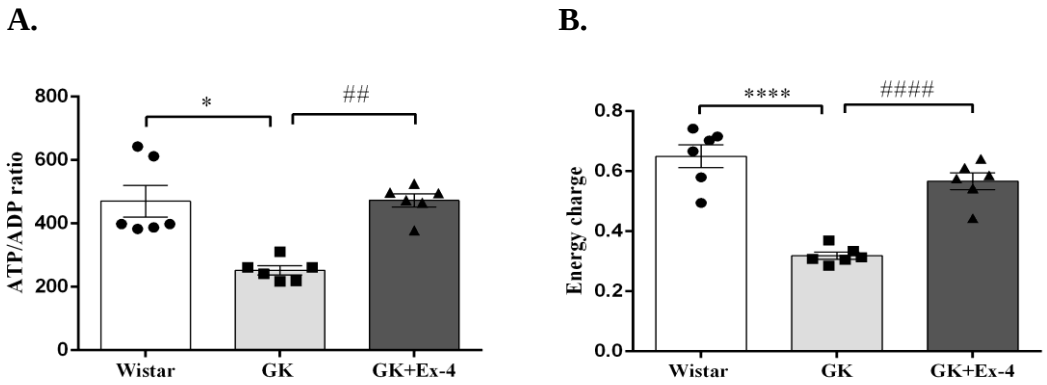


Figure 5.5 - Effect of peripheral Ex-4 treatment on T2D rat brain cortical mitochondrial complexes activities. Complex I activity (A), Complex II/III activity (B), Complex III activity (C), Complex IV activity (D). Data are mean ± SEM of the indicated number of rats/group. Statistical significance: ##*P*<0.01, ###*P*<0.001 *vs*. GK rats, by one-way ANOVA, with protected Fisher LSD post-test.



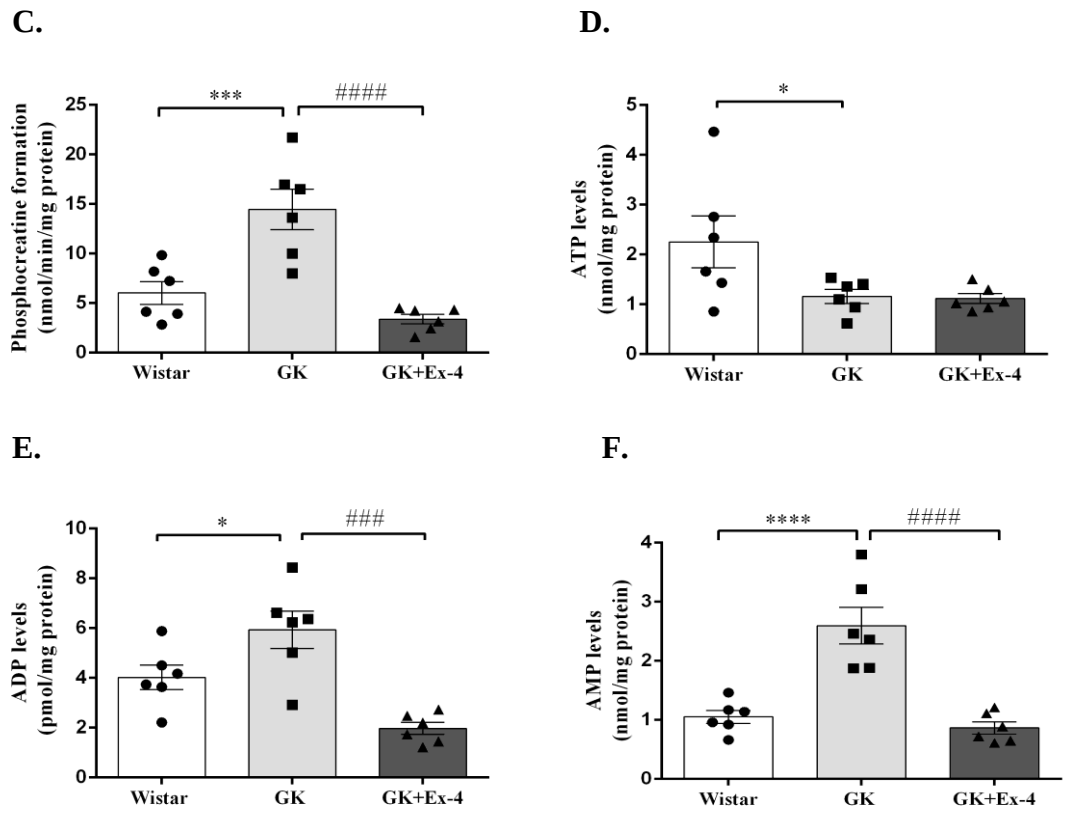


Figure 5.6 – Effect of peripheral Ex-4 treatment on T2D rat brain cortical energy production. ATP/ADP ratio (A), Energy charge (B), Phosphocreatine formation (C), ATP levels (D), ADP levels (E), AMP levels (F). Data are mean ± SEM of the indicated number of rats/group. Statistical significance: **P*<0.05, ****P*<0.001, *****P*<0.0001 *vs.* Wistar rats; ##*P*<0.01, ###*P*<0.001, ####*P*<0.0001 *vs.* GK rats, by one-way ANOVA, with protected Fisher LSD post-test or Kruskal-Walis test, with Dunn post-test (A). ATP: adenosine triphosphate; ADP: adenosine diphosphate; AMP: adenosine monophosphate.

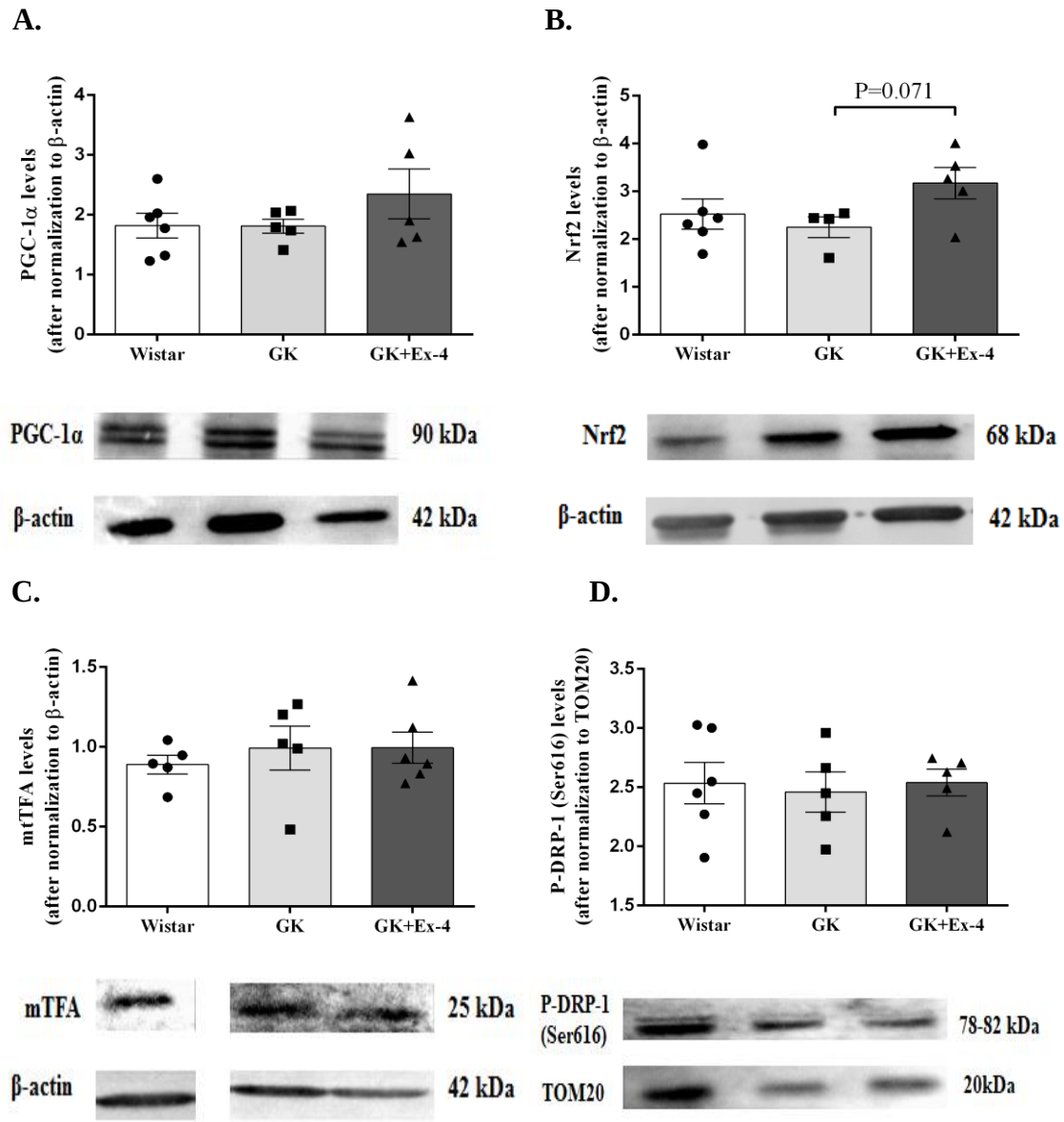
5.4.6 - Peripheral Ex-4 administration in brain cortical mitochondrial dynamics upon T2D

Since changes in brain cortical mitochondria biogenesis and fusion/fission mechanisms may influence the energy profile in middle-aged GK rats, and given our previous studies in 6-month-old GK rat brains (Santos *et al.*, 2014b), we also evaluated the effect of s.c. Ex-4 on several markers for these processes (Fig. 5.7A-G).

Despite no significant alterations in levels of the mitochondrial biogenesis markers PGC-1α, Nrf2 and mtTFA between saline-treated Wistar and GK rats (Fig. 5.7A-C), peripheral therapy with Ex-4 slightly increased (by 30% and 41%) the levels

of brain cortical PGC-1α and Nrf2. Hence, it is plausible that the maintenance of brain cortical ATP levels upon T2D may not rely primarily on an increased mitochondrial biogenesis.

Surprisingly, though no significant changes were observed in brain cortices' levels of the fission markers P-DRP-1(Ser616) and Fis1 (Fig. 5.7D, E), the tendentiously lower brain levels of the fusion markers Mfn1 and Mfn2 in saline-treated GK rats (by 29% and 26%, respectively) were slightly recovered (by 30%) upon Ex-4 treatment, but only for the Mfn1 levels (Fig. 5.7F, G). This suggested that mitochondrial fusion may, at least partially, play a role in the Ex-4-mediated effects in T2D rat brain.



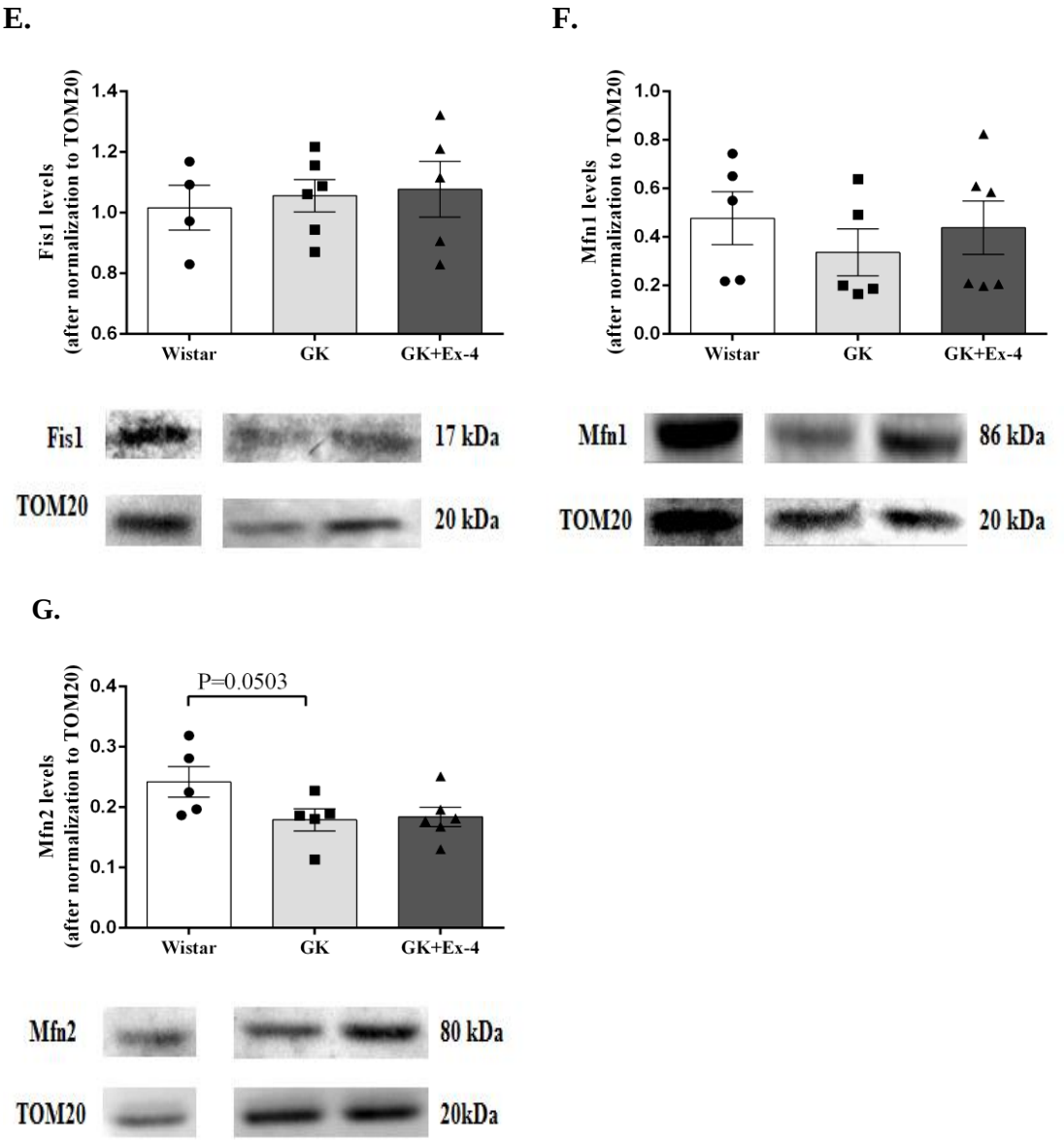


Figure 5.7 - Effect of peripheral Ex-4 treatment on T2D rat brain cortical mitochondrial fission and fusion and mitochondrial biogenesis. PGC-1α protein levels (A), Nrf2 protein levels (B), mtTFA protein levels (C), Phosphorylated DRP-1 protein levels (D), Fis1 protein levels (E), Mfn 1 protein levels (F), Mfn2 protein levels (G). Data are mean ± SEM of the indicated number of rats/group. Statistical significance: by one-way ANOVA, with protected Fisher LSD post-test. DRP-1: Dynamin-related protein 1; Fis1: Mitochondrial fission 1 protein; Mfn: mitofusin; mtTFA: Mitochondrial transcription factor A; Nrf2: Nuclear factor (erythroid-derived 2)-like; PGC-1α: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha.

5.4.7 - Peripheral Ex-4 administration partially normalized the levels of purine metabolites in T2D rat brain

AMP can then undergo the purine metabolic cycle, being dephosphorylated into adenosine that is subsequently deaminated into inosine, and then converted to hypoxanthine and xanthine, in a reaction catalyzed by xanthine oxidase. Though this last reaction may also boost superoxide generation, the subsequent metabolism of xanthine to uric acid may constitute an alternative antioxidant mechanism in brain (Jinnah *et al.*, 2013; Fredholm *et al.*, 2005). Thus, we next evaluated the levels of these products of purine metabolism (Fig. 5.8A-D). In line with the general energy depletion in GK rat brain cortex, we observed a decrement in their adenosine, inosine, hypoxanthine and uric acid content (by 53%, 36%, 48 and 75%, respectively) (Fig. 5.8A-D). On the other hand, peripheral Ex-4 treatment only partially attenuated this effect, by significantly rescuing the levels of adenosine (by 170%) and inosine (by 53%) in T2D rat brains (Fig. 5.8A, B).

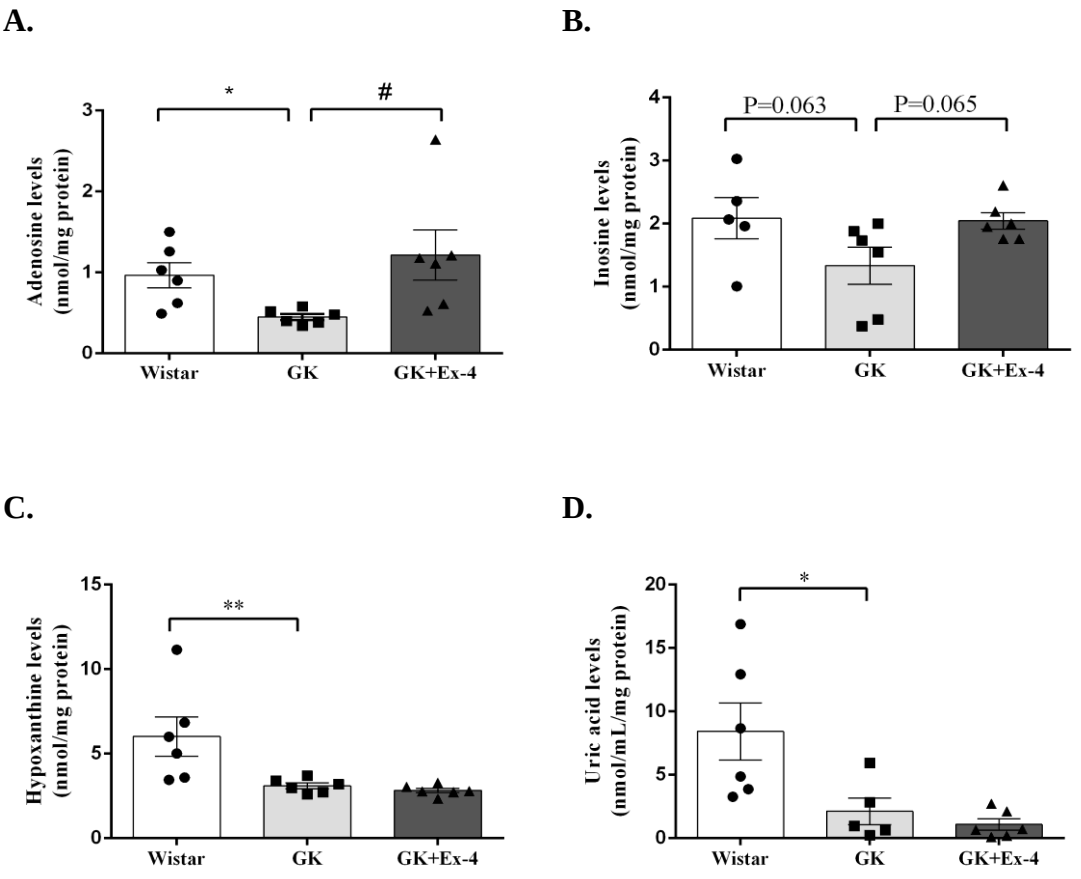
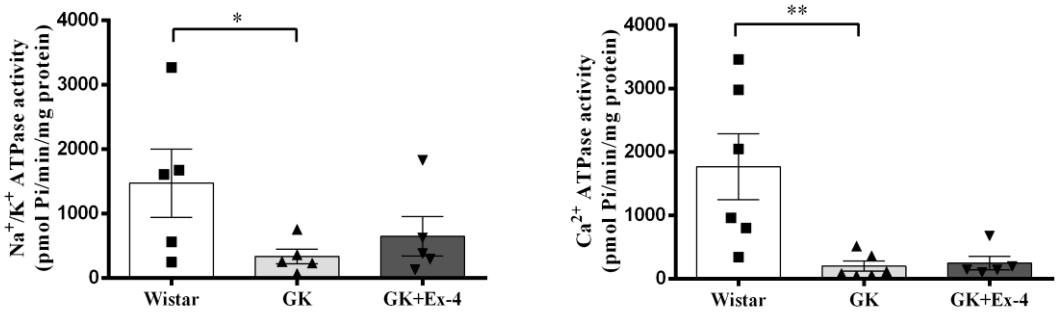


Figure 5.8 - Effect of peripheral Ex-4 treatment on T2D rat brain cortical purines metabolism. Adenosine levels (A), Inosine levels (B), Hypoxanthine levels (C), Uric acid levels (D). Data are mean ± SEM of the indicated number of rats/group. Statistical significance: **P*<0.05, ***P*<0.01 *vs.* Wistar rats; #*P*<0.05 vs. GK rats, by one-way ANOVA, with protected Fisher LSD post-test (B, C, D) or Kruskal-Walis test, with Dunn post-test (A).

5.4.8 - Peripheral Ex-4 administration only partially rescued the activity of brain cortical Na^+/K^+ATPase upon T2D

Given the involvement of cation ATPases in pivotal intracellular mechanisms (*e.g.* neuronal excitability, Ca^{2+} homeostasis, intra and intercellular transport) at the expense of energy (de Lores Arnaiz and Ordieres, 2014; Zaidi, 2010; Sanui and Rubin, 1982), we next determined the activities of the main cation ATPases in the brain: Na^+/K^+, Ca^{2+} and Mg^{2+}ATPases (Fig. 5.9A-C). Although T2D significantly decreased the activities of the three brain cortical ATPases (by 77%, 91 and 88%, respectively) (Fig. 5.9A-C), s.c. Ex-4 only tendentiously increased the activity of Na^+/K^+ ATPase in GK rats (Fig. 5.9A). This suggested that T2D inhibits brain cortical cation ATPases, which may in turn affect not only synaptic transmission, but also the overall cellular homeostasis.

A. **B.**



C.

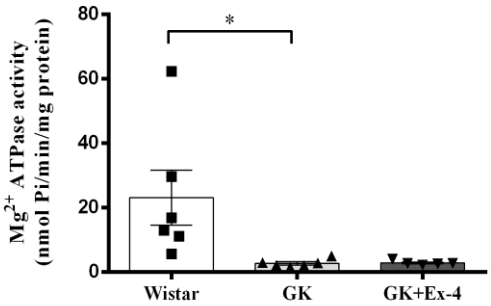


Figure 5.9 - Effect of peripheral Ex-4 treatment on T2D rat brain cortical Na^+/K^+-, Ca^{2+}- and Mg^{2+}-ATPase activities. Na^+/K^+ ATPase activity (A), Ca^{2+} ATPase activity (B), Mg^{2+} ATPase activity (C). Data are mean ± SEM of the indicated number of rats/group. Statistical significance: **P*<0.05, ***P*<0.01 *vs.* Wistar rats, by one-way ANOVA, with protected Fisher LSD post-test (A, C) or Kruskal-Walis test, with Dunn post-test (B).

5.4.9 - Peripheral Ex-4 administration rescued the T2D-induced shift to ketone bodies' metabolism in rat brain

Several decades ago, Owen *et al.* (Owen *et al.*, 1967) found that ketone bodies can be used as an alternative source for cerebral energy generation. More recently, others have also shown that the decline in glucose transport and metabolism in mouse brain is accompanied by a shift to a ketogenic phenotype (less efficient bioenergetic fuel), particularly in aging and AD (Ding *et al.*, 2013). Thus, given the above-mentioned changes in the amino acid precursors for acetoacetylCoA and its alternative involvement in the mitochondrial formation of the two main ketone bodies, AcAc and BOH (Laffel, 1999), we next analyzed the effect of s.c. Ex-4 on their brain levels in GK rats (Fig. 5.10A-B).

Though our previous observations indirectly suggested that less acetoacetylCoA might be synthesized in GK rat brains and no significant changes were found in their AcAc levels compared to Wistar rats (Fig. 5.10A), the levels of BOH were significantly increased (by 151%) upon T2D (Fig. 5.10B). Conversely, Ex-4 therapy significantly lowered both ketone bodies levels in GK rat brains (by 47 and 44% in AcAc and BOH, respectively) (Fig. 5.10A,B). This was in line with the partial increment in the amino acid precursors for acetoacetylCoA – tyrosine and leucine (Table 5.III). These results

suggested that peripheral Ex-4 administration counteracts the chronic T2D-associated increase in brain ketone bodies, protecting against their highly deleterious effects.

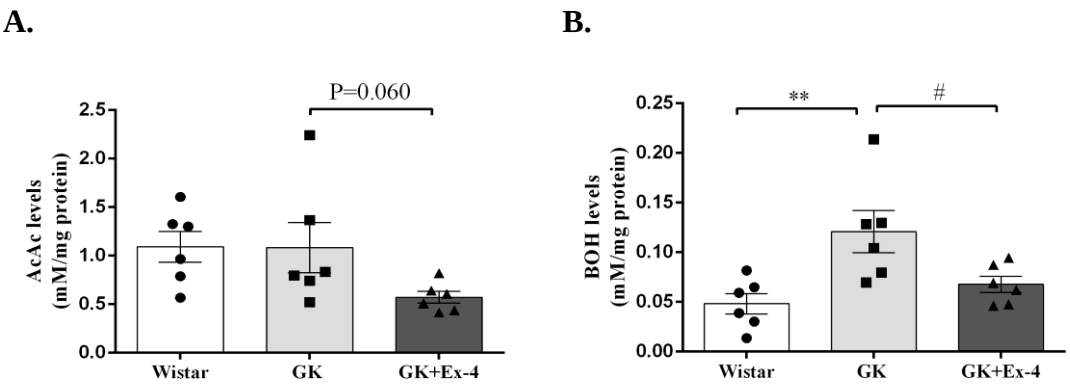


Figure 5.10 – Effect of peripheral Ex-4 treatment on T2D rat brain cortical ketone bodies levels. AcAc levels (A), BOH levels (B). Data are mean ± SEM of the indicated number of rats/group. Statistical significance: ***P*<0.01 *vs.* Wistar rats; #*P*<0.05 *vs.* GK rats, by one-way ANOVA, with protected Fisher LSD post-test. AcAc: acetoacetate; BOH: beta-hydroxybutyrate.

Taken together, our results suggested an Ex-4-induced attenuation of the peripheral features of T2D in middle-aged GK rats that may (directly or indirectly) rescue their brain cortical glucose transport and glycolytic metabolism (at least partially). Ex-4 appeared also to stimulate brain PPP and TCA cycle, as well as the alternative formation of amino acidic precursors that fuel the TCA cycle and constitute pivotal neurotransmitters. Conversely, the drug counteracted the chronic T2D-induced adaptive formation of brain ketone bodies. These putative benefits of peripheral Ex-4 therapy in GK rat brains may also involve the rescue in mitochondrial function, reversing the energy depletion and partially restoring adenosine and inosine levels, rather than a significant improvement in brain cation ATPases activities, mitochondrial biogenesis or dynamics profiles.

5.5. - DISCUSSION

The anti-T2D drugs from the class of GLP-1R agonists have increasingly shown some neuroprotective effects (Erbil *et al.*, 2019; Holscher, 2018). As far as we know,

this is the first study on the effects of peripheral Ex-4 in brain cortical glucose (energy) metabolism upon T2D *per se*.

As in our previous studies, s.c. Ex-4 improved peripheral hyperglycemia and insulin resistance/sensitivity in middle-aged GK rats (Candeias *et al.*, 2018). This was accompanied by the rescue of their brain cortical glucose uptake and metabolism. Although human brain glucose content appears to mirror that from plasma (Roberts *et al.*, 2014; Nigrovic *et al.*, 2012; de Graaf *et al.*, 2001), the impact of hyperglycemia on GLUTs from BBB, neurons and glia remains conflicting. Some authors observed that chronic hyperglycemia in brain downregulates GLUTs expression and activity (Hou *et al.*, 2007; Duelli *et al.*, 2000; Pardridge *et al.*, 1990), while others found no changes (Jacob *et al.*, 2002; Hasselbalch *et al.*, 2001; Simpson *et al.*, 1999). In particular, in the study by Sahin *et al.* (Sahin *et al.*, 2011) it was observed that feeding HFD to male Wistar rats caused significant reductions in both GLUT-1 and GLUT-3 expressions in brain tissue, whereas there was a dysregulation in hippocampal glycometabolism and memory function in a rat model of type 2 diabetes (OLEF rats), with these animals expressing lower levels of MCT-2 than controls (Shima *et al.*, 2017). Accordingly, despite the apparent inability of s.c. Ex-4 to overcome the slight decrease in T2D brain densities of GLUT3, -4 and 8, it recovered their function, as well as that from GLUT1, SGLT1 and -2, and MCT1 and -2. This may allow the recovery of synaptosomal 2-[^{3}H]DG uptake in Ex-4-treated T2D rats. Similar effects of Ex-4 on GLUT2 and -4 expression were described in liver and adipose tissue from streptozotocin (STZ)-induced T2D rats, in GLUT1 expression in human myocardium, and in glucose uptake across rat muscle and adipose tissue, most likely via PI3K, PKA or AMPK signaling (Andreozzi *et al.*, 2016; Wallner *et al.*, 2015; Moreno *et al.*, 2012). This appeared to agree with the rescue of such brain cortical signaling pathways in s.c. Ex-4-treated GK rats (Candeias *et al.*, 2018).

Notably, the stimulation of GK rat brain glucose uptake upon s.c. Ex-4 therapy was followed by lower brain cortical glucose levels, suggesting that glucose entering the brain may be immediately metabolized. In fact, a positron emission tomography (PET) study showed that GLP-1 infusion decreased intracerebral glucose content and raised its metabolic rate in hyperglycemic humans, probably by stimulating GLUT1 and hexokinase (Gejl *et al.*, 2012). Additionally, intracerebroventricular delivery of Ex-4 stimulated glycolysis in a hypothalamic cell line (GT1-7) (Burmeister *et al.*, 2013), and

intraperitoneal administration of Ex-4 increases glucokinase activity (hexokinase isozyme) in hepatocytes isolated from *db/db* mice (Dhanesha *et al.*, 2012b). Despite the apparent inability of s.c. Ex-4 to recover the massive inhibition of GK rat brain hexokinase, it slightly increased their VDAC levels, suggesting a possible indirect control of their brain glycolysis to protect against apoptosis (Pastorino and Hoek, 2008), as seen in our previous study (Candeias *et al.*, 2018) . This effect of s.c. Ex-4 on GK rat brain VDAC levels may also recruit Parkin to defective mitochondria to promote mitophagy (Sun *et al.*, 2012), in line with our previous findings (Candeias *et al.*, 2018).

Though the tendentious decrease in T2D rat brain threonine levels (an amino acid precursor for pyruvate or succinyl CoA) agreed with the negative correlation between threonine content, hyperglycemia and insulin resistance described in T2D patients (Drabkova *et al.*, 2015; Yamada *et al.*, 2015), the s.c. Ex-4-mediated increase in GK rat brain threonine did not change their pyruvate content. Analogously, the tendentiously lower brain alanine levels in T2D rats pointed to an alanine (and ultimately glucose) dysmetabolism that was reversed by Ex-4. However, this was contradicted by their unchanged pyruvate levels. Interestingly, obesity and T2D were found to increase brain alanine/glutamate ratio and impair glucose metabolism (Sickmann *et al.*, 2010), whereas others reported a dysfunctional alanine cycle (probably involving an increased serum alanine aminotransaminase and an abnormal synthesis of pyruvate) in liver pathology, insulin resistance and T2D (Qian *et al.*, 2015; Sattar *et al.*, 2004). Concerning taurine levels, our findings that s.c. Ex-4 therapy did not overcome its higher levels in GK rat brains suggest this may not play a role in brain glycolytic effects of Ex-4. This appears to be in contrast with the lower taurine levels in platelets of T2D patients (De Luca *et al.*, 2001), the stimulated glycolysis and insulinotropic properties of taurine supplementation (Kim *et al.*, 2007), and anti-neuropathic features in STZ-induced diabetic rats (Agca *et al.*, 2014). Interestingly, our observation of a tendentious decrease in lactate levels upon s.c. Ex-4 suggested a stimulation of an alternative lactate-mediated metabolism (other than the formation of pyruvate via LDH) to maintain brain energy homeostasis in Ex-4-treated GK rats, in line with their stimulated MCT1 and -2 transporters (known to facilitate neuronal lactate uptake and use as an energy source, and/or to export the excess of brain lactate to the periphery (Bergersen, 2015)). Indeed, lactate is another major fuel for neuronal energy metabolism that ‘buffers’ glycolysis and oxidative metabolism through the “astrocyte-

neuron lactate shuttle" (Perez-Escuredo *et al.*, 2016; Dienel, 2012), especially in hyperglycemia (Jacob *et al.*, 2002; Combs *et al.*, 1990). Accordingly, enhanced brain MCTs levels were found in hyperglycemic or obese animals (Canis *et al.*, 2009; Pierre *et al.*, 2007). High lactate levels also occurred in plasma from insulin resistant, T2D and obese patients, being associated to lactic acidosis, impaired MCTs and in lactate transport (Metz *et al.*, 2005; English and Williams, 2004; Py *et al.*, 2001). Thus, the lower brain lactate in s.c. Ex-4-treated GK rats may be also due to a normalization of its plasma levels. However, Ex-4 was also found to increase brain lactate and LDH activity in a mouse model for neuronal dysfunction (Bomba *et al.*, 2013).

Alternatively, s.c. Ex-4 may activate the brain pentose phosphate pathway (via the slight induction of G6PDH) in GK rats, thus contributing (at least partially) to their lower brain glucose levels. This appears to be in line with the idea that G6PDH deficiency is a risk factor for T2D (Heymann *et al.*, 2012), and that boosting the PPP might represent a target for protection the cardiac progenitor cells (CPCs) in T1D, since it was observed a restoration of the redox state and the promotion of survival pathways (Katare *et al.*, 2013). On the other hand, others hypothesized that a pro-inflammatory stimulus in both cultured human aortic smooth muscle cells (HASMC) and isolated rat mesenteric microvessels with a background of excessive glucose increased the glucose uptake, which was subsequently diverted into the PPP, resulting in the overactivation of prooxidant and death mechanisms (Peiro *et al.*, 2016). Besides PPP stimulation in T2D brains, s.c. Ex-4 may also stimulate their TCA cycle, since increased (at least tendentiously) activities of citrate synthase and malate dehydrogenase occurred in these conditions. Despite the lack of data on Ex-4 effects in brain citrate synthase activity, Prasad *et al*. (Prasad *et al.*, 2016) showed its impairment in diabetes, with the activity of citrate synthase being significantly reduced in the cortex and striatum of a STZ diabetic rat model. Moreover, Takada *et al. (Takada et al., 2016)* found that a DPP-4 inhibitor (MK-0626) normalized it in mice with heart failure, in an effect abolished by the GLP-1 antagonist, Exendin-(9-39). Besides this direct amelioration of brain TCA cycle enzymes in GK rats, one cannot exclude an additional role for amino acid precursors herein. Indeed, the slightly higher brain levels of aspartate and leucine in s.c. Ex-4-treated T2D rats may promote glucose homeostasis (Lynch and Adams, 2014), with leucine being also known to stimulate insulin secretion after an ingestion of a high glucose concentration by healthy subjects (Kalogeropoulou *et al.*, 2008) and, thus, may

possibly restore (brain) insulin signaling and glucose metabolism in our conditions. Furthermore, aspartate is a known precursor of oxaloacetate and fumarate, and an excitatory amino acid neurotransmitter that, together with glutamate, was decreased in brains of T2D Zucker Diabetic Fatty (ZDF) rats (as in our saline-treated GK rat brains) and pivotal in their balance between excitatory and inhibitory neurotransmission (Sickmann *et al.*, 2012). Thus, the slight increase in brain aspartate and glutamate in s.c. Ex-4-treated GK rats may account for their homeostasis and, indirectly, also to stimulate their citrate synthase and malate dehydrogenase. However, this higher glutamate may not be enough to promote α-ketoglutarate synthesis and α-ketoglutarate dehydrogenase activity in T2D rats treated with Ex-4.

The tendentiously decreased brain GABA and glycine levels in s.c. Ex-4-treated GK rats suggested an additional neuroprotection, since higher brain levels of GABA were recently associated with accelerated cognitive decline in T2D patients (van Bussel *et al.*, 2016), whereas higher glycine levels occurred in GK rat synaptosomes upon amyloid-β-induced toxicity (Pereira *et al.*, 2000) and in *db/db* mouse brain (Makar *et al.*, 1995). Additionally, we cannot exclude herein the involvement of the mitochondrial metabolism of GABA in succinate synthesis (Purves *et al.*, 2001) and/or of the observed lower levels of its precursor ornithine (Yoon and Lee, 2014) - this may be also neuroprotective, since T2D-induced nitric oxide dysmetabolism and vascular deficits were correlated with high levels of ornithine, ornithine/citrulline, ornithine/arginine ratio in both animal models and human patients (Kovamees *et al.*, 2016b; Huang *et al.*, 2014; Kashyap *et al.*, 2008). This was accompanied by an upregulation of arginase activity, with subsequent deficits in endothelial nitric oxide synthase and nitric oxide synthesis, and free radicals' overproduction (Kovamees *et al.*, 2016a; Tessari *et al.*, 2011). Besides ornithine, methionine metabolism is also regulated by insulin, being their changes associated with endothelial dysfunction and vascular disease in T2D (Tessari *et al.*, 2011). Despite the possible relation between high levels of methionine and its byproduct homocysteine with, *e.g.*, T2D, schizophrenia and dementia (Tapia-Rojas *et al.*, 2015; Shimomura *et al.*, 2011), our saline-treated GK rats had slightly lower brain methionine levels, that were further decreased by s.c. Ex-4.

At the level of acetyl CoA synthesis from amino acid precursors, it is possible that the (at least tendentious) increase in T2D rat brain tyrosine and phenylalanine levels upon s.c. Ex-4 may indirectly stimulate citrate synthase. Together with tryptophan,

these amino acids are also precursors for the catecholaminergic and serotonergic pathways (Fernstrom and Fernstrom, 2007), being their (at least slight) decrease in GK rat brains in agreement with other studies on T2D, as well as with an increased risk for Parkinson disease (Shpakov *et al.*, 2015). Moreover, the lower levels of lysine (another precursor of acetyl CoA) in T2D rat brains were in line with its supplementation's benefits in T2D patients, namely anti-hyperglycemic, -insulin resistance, -inflammatory, and –AGEs (Mirmiranpour *et al.*, 2016; Sulochana *et al.*, 2001). However, s.c. Ex-4 did not rescue GK rat brain cortical lysine levels. Although the s.c. Ex-4-mediated increase (at least partially) in the brain branched-chain amino acid (BCAAs) precursors valine and leucine upon T2D may constitute an alternative provider of, *e.g.*, succinyl CoA, studies reported an association between elevated circulating levels of BCAAs, insulin resistance, HbA_{1c} and metabolic syndrome, suggesting their use as markers for T2D progression (Badoud *et al.*, 2014; Wang *et al.*, 2011; Fiehn *et al.*, 2010). Obesity, malnutrition and inborn errors (*e.g.* maple syrup urine disease) have been related with defects in BCAA metabolism, and high BCAAs levels, in brain and beyond (also in plasma and muscle), were linked with decreased mitochondrial function (Olson *et al.*, 2014; Amaral *et al.*, 2010) and deregulation of mTORC1-mediated autophagy (Lynch and Adams, 2014; Yan *et al.*, 2012; Sugawara *et al.*, 2009).

Other brain energy sources to maintain its function, particularly upon uncontrolled T2D, include ketone bodies (Mahendran *et al.*, 2013; Owen, 2006; Avogaro *et al.*, 1996). Ding *et al*. (Ding *et al.*, 2013) observed that the shift to ketogenic metabolism may occur early after the decline in glucose transport and metabolism in aging and early AD, while Paoli *et al*. (Paoli *et al.*, 2014) addressed the advantages of a ketogenic diet in T2D and neurodegenerative diseases. Although the increased levels of β-hydroxybutyrate in saline-treated GK rat brains appears to agree with this, their lower MCT-1- and -2-mediated uptake may be also a limitation to its metabolism. Conversely, the s.c. Ex-4-mediated decrease in their brain acetoacetate and β-hydroxybutyrate levels may prevent their shift to the less efficient ketogenic metabolism (despite the higher uptake by MCTs) that was also associated with mitochondrial dysfunction and neurodegeneration (Ding *et al.*, 2013).

Oxidative stress and mitochondrial dysfunction have been widely associated with T2D-related brain energy deficits and the subsequent vicious cycle of dysfunctional brain glucose metabolism and insulin signaling, pointing mitochondrial

damage as a possible link with, *e.g.*, AD (Moreira, 2012). In line (at least partially) with the effect on T2D brain glycolysis and TCA cycle, s.c. Ex-4 further increased the slightly stimulated mitochondrial complexes I, II/III, III and IV in GK rats, ultimately improving their ATP/ADP ratio and energy charge, as described for pancreatic islets from young GK rats upon Ex-4 (Mukai *et al.*, 2011). Although the drug potentiated their islet ATP production, in our study this was mainly due to the massive decrement of their ADP and AMP levels (possibly by blunting ATP hydrolysis), rather than a recovery from the lower brain ATP levels measured in saline-treated GK rats (also found in our studies on GK rat synaptosomes and brain mitochondria (Duarte *et al.*, 2004; Moreira *et al.*, 2003)). An interesting study by Chang and coworkers (Chang *et al.*, 2018) showed a novel mechanism of protection of cardiomyocytes by Ex-4, involving an improvement in mitochondria function. Exenatide pretreatment prior to exposure to hypoxia in H9c2 cells significantly decreased mitochondrial abnormalities by enhancing ATP synthesis, mitochondrial ATPase activity and mitochondrial membrane potential, also inhibiting the opening of the mitochondrial permeability transition pore (mPTP) and reducing mitochondrial calcium overload and cardiomyocyte apoptosis in H9c2 cells subjected to hypoxia (Chang *et al.*, 2018). Alternatively, since phosphocreatine is a reservoir for rapid ATP formation in brain (especially under pathological conditions, as ischemia) (Rae and Broer, 2015), its high levels in saline-treated GK rats may provide a rapid source to overcome the impaired brain glycolysis and ATP formation through oxidative phosphorylation. Indeed, creatine supplementation improved glycemic control in T2D patients (Gualano *et al.*, 2012) and analysis of the blood of T2D patients also revealed that they displayed higher creatine kinase activity, which positively correlated with glucose levels (Jevric-Causevic *et al.*, 2006). The activation of this enzyme suggests an attempt of the cells to protect themselves from the imbalance of the ATP/ADP/AMP ratio, ensuring amounts of ATP sufficient to activate metabolic pathways that will produce more energy. However, some controversy exists herein, since an increased hippocampal creatine content in type 2 diabetic rats and patients was also associated with cognitive impairment (Wang *et al.*, 2015b; van der Graaf *et al.*, 2004). Moreover, low phosphocreatine/creatine and high lactate/pyruvate may constitute early signs of mitochondrial defects (Erecinska and Nelson, 1994), being low ATP and phosphocreatine levels related with uncoupled oxidative phosphorylation in kainate-treated rat brain cortices (Gupta and Dettbarn, 2003) and in obese patients (Lindeboom *et al.*, 2014; Perseghin *et al.*, 2007). Despite

previous data on insulin infusion-mediated increase in phosphocreatine levels in rat hippocampus and in primary cortical and adult sensory neurons, recovering mitochondrial function and ATP synthesis (Duarte *et al.*, 2006; Huang *et al.*, 2005; Henneberg and Hoyer, 1994), here the insulinotropic Ex-4 reversed brain phosphocreatine formation in GK rats, further reinforcing its use as an alternative source of energy upon disease conditions. This appeared to be in line with an Ex-4-associated protection against hyperglycemia-induced (Cai *et al.*, 2012a) or T1D-induced (AbdElmonem Elbassuoni, 2014) cardiac injury via decreased creatine kinase levels.

Another possibility for the decreased ATP in T2D rat brains could be a lower mitochondria number and biogenesis (as given by their inhibited citrate synthase), as previously described in T2D human muscle (Mootha *et al.*, 2003; Kelley *et al.*, 2002) and in 11-month-old, T2D mice brains (Carvalho *et al.*, 2015). However, this may not be the case herein as, apart from a slight increase in Nrf2 levels upon s.c. Ex-4, no changes occurred in other markers for mitochondrial biogenesis, partially in line with the absence of changes in 6-month old GK rat brain cortical mitochondrial biogenesis and mitochondrial fission (Santos *et al.*, 2014b). Exceptionally, we observed a slight decrease in fusion markers Mfn1 and 2 in T2D rats that was only slightly recovered by s.c. Ex-4 in the case of Mfn1. This suggested a partial role for mitochondrial fusion in the drug-mediated rescue of brain metabolism under T2D, in agreement with an *in vitro* Ex-4-mediated stimulation of mitochondrial fusion in a PKA-dependent manner in vascular smooth muscle cells (Torres *et al.*, 2016) and with the enhanced mitochondrial biogenesis by Ex-4 protection in pancreatic beta cells (INS-1E cell line) from human islet amyloid polypeptide-induced cell damage (Fan *et al.*, 2010). Besides this, ATP expenditure in T2D rat brains may not involve cation ATPases, since their Na^+/K^+-, Ca^{2+}- and Mg^{2+}-ATPases were massively inhibited, in line with reports from type 2 diabetic rodent sciatic nerves (Ding *et al.*, 2014) and synaptosomal membranes (Makar *et al.*, 1995). Despite expecting a more prominent stimulation of their brain cortical ATPases upon s.c. Ex-4, it is possible that the slight stimulation of Na^+/K^+-ATPase under these circumstances may account for the increased SGLTs-mediated glucose uptake, lower phosphocreatine levels and anti-apoptotic role of s.c. Ex-4 in GK rat brains (Candeias *et al.*, 2018). Interestingly, the drugs protected cultured cardiomyocytes against high glucose damage by activating the sarco/endoplasmic reticulum Ca^{2+}-ATPase-2a, blunting endoplasmic reticulum stress (Younce *et al.*, 2013).

Finally, the lower adenine nucleotides levels in GK rat brains could arise from either the increased purines metabolites (adenosine, inosine, xanthine and uric acid), as in T2D patients with diabetic retinopathy (Xia *et al.*, 2014) and in both AD and mild cognitively impaired (MCI) subjects (Kaddurah-Daouk *et al.*, 2013), and/or from an overactivation of adenosine A_1 and A_{2A} receptors, as in T2D mice (Duarte *et al.*, 2012b). However, this may not be the case herein, since all brain purine metabolites were (at least slightly) lowered in T2D rats, being adenosine and inosine recovered by s.c. Ex-4. Notably, the slight decrease in brain uric acid in Ex-4-treated GK rats agreed with its decreased serum levels and recovered kidney function in STZ-induced diabetic rats treated with GLP-1 (Lotfy *et al.*, 2014a).

As is well known, during normal brain aging there is a decline in metabolism and in mitochondrial function, both of them being exacerbated impaired with neurodegeneration (Dodson *et al.*, 2017). Furthermore, the bioenergetics stress-related with neurodegenerative disease also associates with a decline in autophagic function, all of these culminating in accelerated cellular stress and ultimately neuronal death (Nixon, 2013). Strikingly, one can hypothesize that, in GK rat brains, Ex-4 may promote the conversion of ATP into cAMP thus stimulating PKA activity (Candeias *et al.*, 2018) that may in turn contribute (at least partially) either to the activation of mitochondrial fusion mechanisms (Fig. 5.7) and/or of respiratory chain complexes (Fig. 5.5) (Zhang *et al.*, 2016a). Moreover, Ex-4-increased autophagy (Candeias *et al.*, 2018) may mediate a feedback response leading to mitochondrial fusion (Fig. 5.7) through cAMP-PKA activation and involving Drp1 phosphorylation (Gomes *et al.*, 2011).

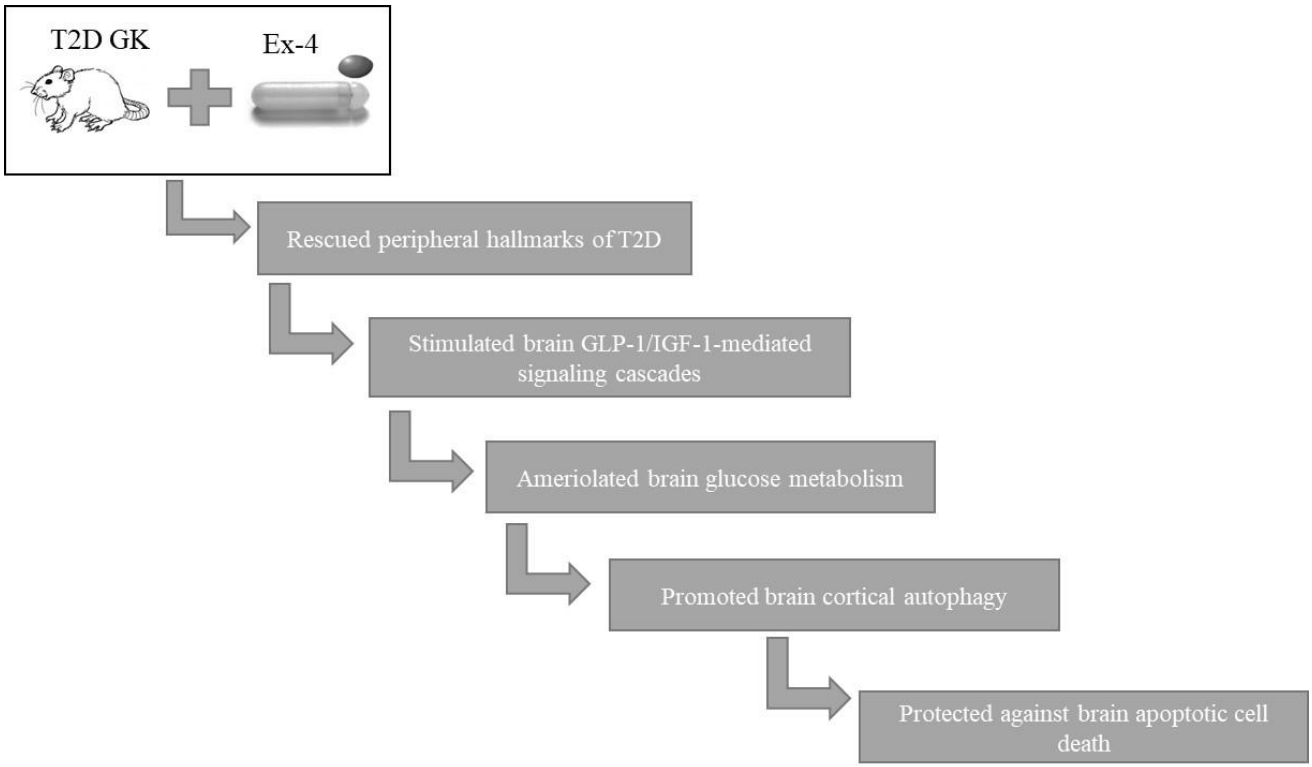


Fig. 5.11 – Summary of the main effects of Ex-4 treatment on T2D GK rats.

Chapter 6

Diabetes, obesity, aging and consequent Parkinson disease: potential for Linagliptin therapy

Adapted from: Lietzau G, Magni G, Kehr J, Yoshitake T, Candeias E, Duarte AI, Pettersson H, Skogsberg J, Abbracchio MP, Klein T, Nystrom T, Ceruti S, Darsalia V, Patrone C (2020) Dipeptidyl peptidase-4 inhibitors and sulfonylureas prevent the progressive impairment of the nigrostriatal dopaminergic system induced by diabetes during aging. *Neurobiol Aging*. doi: 10.1016/j.neurobiolaging.2020.01.004.

Chronic high fat diet consumption along with aging impairs the striatal pathway –linagliptin neuroprotective effects

6.1 - ABSTRACT

Type 2 diabets (T2D) is a progressive disease, in which long-term complications further deteriorate with aging. Impairments in the central nervous system (CNS) have been largely associated with T2D, with impacts from cognition/memory to sensorimotor functions. Indeed, several neurodegenerative diseases share common features with T2D and an increased risk to develop Alzheimer disease (AD) and Parkinson disease (PD) has been reported in T2D patients.

Furthermore, an increased number of reports have focused on the role of fast-spiking γ-aminobutyric acid (GABA)ergic interneurons positive for parvalbumin (PV) and their involvement in the development of brain diseases. In addition, it has been shown that striatal PV cells are involved in the protection of the striatal pathway.

Moreover, dipeptidyl peptidase-4 inhibitors (DPP-4is) have demonstrated appealing results in the treatment of T2D, although the mechanisms underlying their neuroprotective effects remain unclear.

Herein, we hypothesized that age-related striatal dysfunction is exacerbated by high fat diet (HFD) and the correspondent brain area becomes more easily impaired when other neurodegenerative diseases arise (*e.g.* PD), and that treatment with the DPP4i, linagliptin, may revert these pathological changes

For this purpose, we compared young (2-month-old) C57BL/6 mice with middle-aged (14-month-old) mice that received standard diet (SD) or HFD for 12 months (starting at the age of 2 months). For the linaglitin treatment study, we analyzed HFD mice in the same conditions and mice that received linagliptin (5-7 mg/kg b.w./day) for 3 months (from 11-month- to 14-month-old). PV+ interneurons, glial fibrillary acidic protein (GFAP)+ astrocytes and ionized calcium binding adaptor molecule 1 (Iba-1)+ microglia were analysed.

We observed a loss of PV+ interneurons with age, as well as an increased GFAP and Iba-1 presence. However, there was no additive effect from HFD. Linagliptin

therapy normalized the effects of the pathology in astrocytes and microglia.

Linagliptin showed a potential neuroprotective effect against aging-induced alterations, which may ultimately prevent or ameliorate sensorimotor functions with age. Therefore, these results may constitute an important overview on the pathogenic mechanisms induced by aging on striatum and on the linagliptin administration as a therapeutic approach against brain's complications during aging.

Keywords: Obesity, T2D, aging, Parkinson disease, parvalbumin, linagliptin

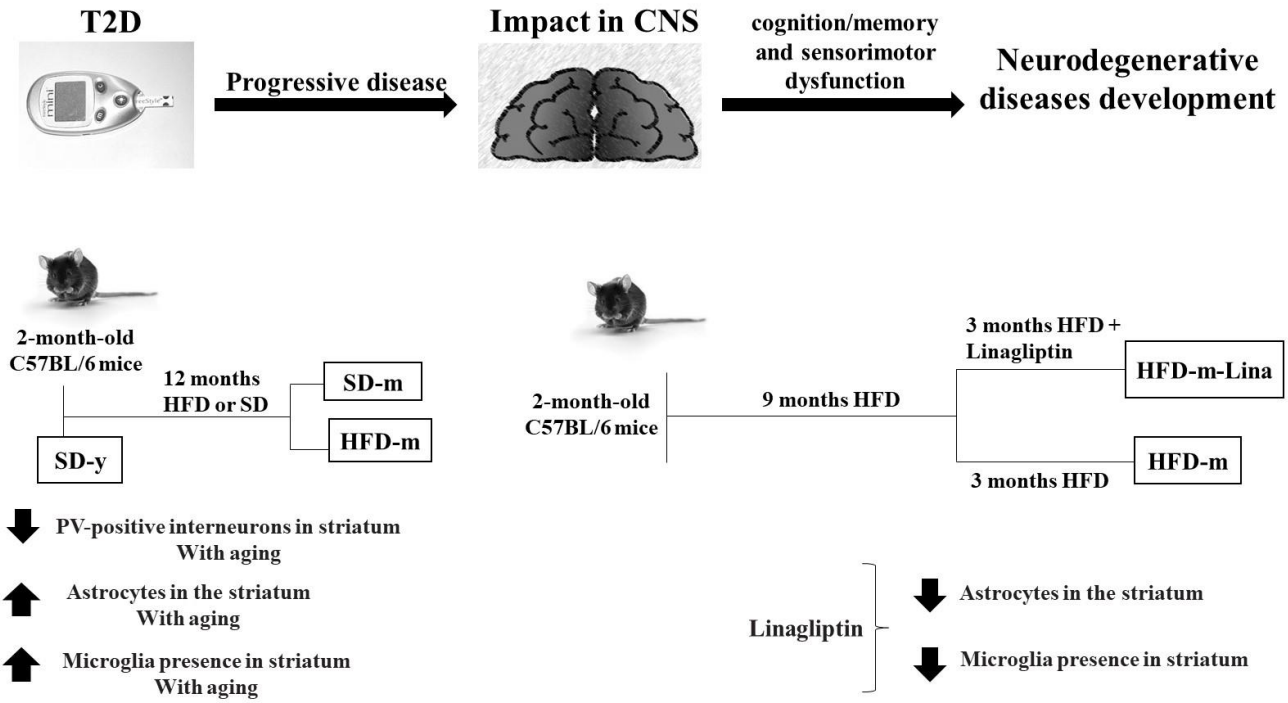


Figure 6.1 – Graphical abstract.

6.2 - INTRODUCTION

The combination of an unhealthy lifestyle and the increasing aging population are substantially rising the number of long-term complications, namely chronic diseases, where type 2 diabetes (T2D) is one of the most prevalent (Zheng *et al.*, 2018). This is further complicated by evidence that T2D patients present a broad range of cognitive/sensorimotor problems and may be at a higher risk for neurodegenerative

diseases, including Parkinson disease (PD) (Gorniak *et al.*, 2019). Indeed, T2D has been associated with impaired balance and a higher risk of developing physical disabilities (Orlando *et al.*, 2016; Timar *et al.*, 2016). Being the magnitude of the risk for PD greater in individuals who developed T2D complications. The association of both pathologies may be attributable to either a genetic predisposition and/or shared dysregulated cellular pathways (*e.g.* mitochondrial dysfunction, impaired insulin signaling, and metabolic inflammation), which pathogenic brain changes may be cumulative with aging-related decay (De Pablo-Fernandez *et al.*, 2018; Yang *et al.*, 2017). Interestingly, emerging evidence has also shed some light on the role of striatal neuronal activity and the dopaminergic signaling on the regulation of brain glucose homeostasis (Ter Horst *et al.*, 2018).

Despite the multifactorial causes of T2D, obesity may be pointed as one of its main risk factors, with the increase in obesity prevalence directly increasing the cases of T2D (Bhupathiraju and Hu, 2016). Several studies correlated high-fat feeding and neurodegeneration, particularly in the nigrostriatal dopamine (DA) signaling. Moreover, high-fat diet (HFD) may attenuate DA release in the substantia nigra and the striatum and decrease striatal DA uptake and turnover. This may occur, mainly through insulin resistance, followed by hyperglycemia and systemic oxidative stress (Morris *et al.*, 2011; Morris *et al.*, 2010).

Regarding fast-spiking, parvalbumin-positive (PV+) γ-aminobutyric acid (GABA)ergic interneurons increasing investigation uncovered their noteworthy properties that may help understand the connections between different networks in normal brain function. The basic function of these interneurons (the selectively expression of the calcium-binding protein PV and involvement in the feedback and feedforward inhibition and generation of gamma-frequency oscillations) accounts for the functioning of complex neuronal networks and information processing, and thus may represent a key therapeutic target in brain aging and in several brain diseases, such as vascular cognitive impairment, stroke, epilepsy, schizophrenia, Alzheimer disease (AD) and PD (Hu *et al.*, 2014; Lanoue *et al.*, 2013). Due to the high energy expenditure in neuronal spiking (*i.e.* the generation of action potentials) and synchronization of the activity of principal cells during fast network oscillations by rhythmic inhibition (mainly mediated by neurotransmitter, GABA), PV+ interneurons may represent a

critical threshold from cognitive function to decline during situations of metabolic and/or oxidative stress (Kann, 2016).

Interestingly, PV interneurons have recently been highlighted as protectors of the dopaminergic nigrostriatal pathway (d'Anglemont de Tassigny *et al.*, 2015). Two decades after the discovery of the glial cell line-derived neurotrophic factor (GDNF), the endogenous expression, production and release of GDNF through PV+ interneurons was identified in the rodent striatum (Hidalgo-Figueroa *et al.*, 2012). Despite the difficulties in administering the GDNF protein to human patients, alternative GDNF-based therapies still hold much potential in the treatment of PD, in light of the undeniable benefits on nigrostriatal DA neuron survival in animal models of PD (d'Anglemont de Tassigny *et al.*, 2015). It is postulated that GDNF provides trophic support to DA neurons and interacts with several neuroprotective cellular pathways, leading to the expression of pro-survival genes, inhibition of pro-apoptosis factors, modulating calcium signaling and detoxifying reactive oxygen species (ROS) (Sawada *et al.*, 2000). In this panorama, PV+ interneurons, as the main producers of GDNF in rodent striatum, appear as an appealing target for the therapeutic applicability of endogenous brain GDNF activation in PD patients.

Dipeptidyl peptidase-4 inhibitors (DPP-4is) are a recent therapeutic tool against T2D, with neuroprotective effects. DPP-4is are small, orally-administered molecules that exert their anti-diabetic effects primarily by avoiding the inactivation of native glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotrophic polypeptide (GIP), extending their half-time and increasing their levels in circulation, thus prolonging the postprandial insulin secretion (Candeias *et al.*, 2015). Currently approved DPP-4i drugs for the treatment of T2D in the USA and Europe are: linagliptin, saxagliptin, sitagliptin, vildagliptin and alogliptin (Schwartz, 2014; Stolar *et al.*, 2013). Regarding neuroprotection, reports have shown that DPP-4 inhibition may hinder mechanisms of neurodegeneration in T2D (Matteucci and Giampietro, 2015) and in AD (Wicinski *et al.*, 2018c). Moreover, a remarkable study by Nassar and coworkers has uncovered an antiparkinsonian effect of DPP-4 inhibition through preservation of substantia nigra pars compacta and striatal functions (Nassar *et al.*, 2015). Despite several peptides have been identified as DPP-4 substrates, the precise mechanisms by which DPP-4 inhibitors exert their neuroprotective effects remain unknown. Hence, a specific DPP-4-mediated signaling pathway is still undetermined (Andersen *et al.*,

2018). Regardless, our group was able to demonstrate a linagliptin-induced neuroprotection independent from both blood sugar regulation and GLP-1R (Chiazza *et al.*, 2018; Darsalia *et al.*, 2016; Darsalia *et al.*, 2013).

This study is part of a recently published paper (Lietzau *et al.*, 2020). Here, our major goals were to determine if age-related nigrostriatal dysfunction is exarcebated by obesity-induced T2D, rendering cortico-striatum more vulnerable to other neurodegenerative diseases, and whether a chronic treatment with the DPP-4i, linagliptin, reverts these pathological changes.

6.3 - MATERIAL AND METHODS

6.3.1 - Animals

C57BL/6 mice (Nova SCB Stockholm, Sweden) were housed in 12-h light/dark cycle with free access to food and water. All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by U.S. National Institute of Health and approved by the regional ethics committee for animal experimentation (ethical permits granted by Stockholm's Djurförsöksetiska Nämnd: S7–13 and N43/16).

In the first study, 2-month-old (young) mice (SD-y) were compared with 14-month-old (middle-aged) mice either fed with a standard diet (SD-m) or with a high fat diet (ssniff E15126-34, 54% calories from fat, Germany) (HFD-m). HFD was given for 12 months until mice were euthanized (Fig. 6.2A). For the second part, linagliptin (Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany) treatment (mixed in the standard rodent chow at 83 mg/kg, estimated daily intake at ≈5-7 mg/kg/bw) was initiated at 9 months of the HFD and carried out for 3 months until the end of the study at 14 months (HFD-m-Lina). Control mice for this part received HFD for 12 months (HFD-m) (Fig. 6.2B).

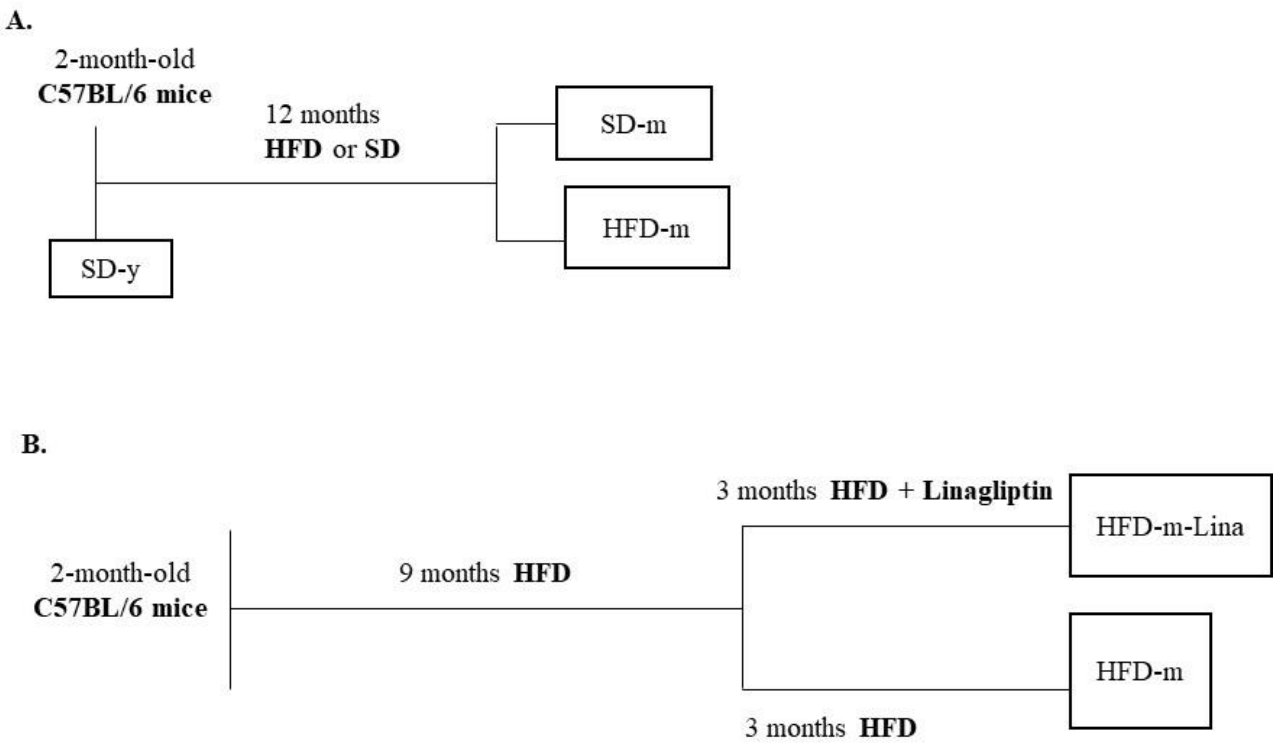


Figure 6.2 – Experimental design. Study 1: to determine potential T2D and/or aging-induced neural alterations in the brain areas of the dopaminergic system (A); Study 2: to determine the potential effect of anti-T2D treatment on neural alterations induced by either T2D or aging in the nigrostriatal dopaminergic system.

6.3.2 - Body weight, glycemia, DPP-4 activity and GLP-1 levels

Body weight was monitored in all animals before euthanasia. Glycemia was evaluated by measuring fasting blood glucose (blood was collected from the tail vein after 10 h of fasting) with a LifeScan glucometer (Milpitas, CA, USA).

Plasma DPP-4 activity and total active GLP-1 levels (blood collected in the fed state) were determined by enzyme immunoassay (EIA) and by ELISA, respectively (Meso Scale Discovery, Gaithersburg, MD, USA).

6.3.3 - Immunohistochemistry (IHC) and quantitative analyses

Mice were deeply anesthetized with sodium pentobarbital and transcardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA). The brains were then extracted and after overnight post-fixation in 4% PFA

were put in 20% sucrose solution for 3 days. Afterwards, brains were attached to platform using optimal cutting temperature compound (OCT) and frozen with dry ice, and 40 μm-thick coronal sections were cut throughout the brain using Leica SM2010 R sliding microtome (Leica, Wetzlar, Germany). Collected sections were put in a cryoprotective solution for storage at -20ºC, and later stained as free-floating sections. The following primary antibodies were used: rabbit polyclonal against parvalbumin (1:1500, Abcam, Cambridge, UK), rabbit polyclonal against glial fibrillary acidic protein (GFAP) (1:1500, Dako, Glostrup, Denmark), goat polyclonal against ionized calcium-binding adaptor molecule-1 (Iba-1) (1:750, Abcam). Antigen retrieval was performed using 1mM EDTA, in 70ºC for 35min. Sections were incubated with primary antibodies overnight at 4°C in a phosphate buffer containing 5% natural horse serum (NHS, Millipore, Burlington, MA, USA) and 0.25% Triton X-100. Primary antibodies were visualized using biotin-conjugated secondary antibodies (1:200, Vector Laboratories, Burlingame, USA) after peroxidase substrate reaction (ABC kit, Vector Laboratories). Sections were incubated with secondary antibodies for 2h at room temperature in phosphate buffer containing 5% NHS and 0.25% Triton X-100. For chromogenic visualization, the ABC kit (Vector Laboratories) and 3,3'-diaminobenzidine (DAB) (Sigma-Aldrich, St. Louis, MO, USA) were used.

PV-, GFAP- and Iba-1- positive cells were quantified using a computerized stereology toolbox equipped with Visiopharm v. 4.2.1.0 software for digital image analysis (NewCast, Denmark), connected to an Olympus BX51 epifluorescent/light microscope (Olympus, Japan). In striatum, positive cells were counted on three coronal sections per animal located at 1.50, 0.00 and -1.00 mm from Bregma. The cell density per $1x10^5 \mu m^2$ for all the IHC markers was determined. Mean volume (in μm^3) of Iba-1+ and PV+ cells was measured, using nucleator technique (Gundersen *et al.*, 1988), by Visiopharm software.

6.3.4 - Statistical Analysis

Data are presented as means ± SEM. Statistical analysis and graphic artwork were obtained using the GraphPad Prism 8.0 software. Statistical significance was determined using unpaired t-test or one-way ANOVA with Tukey's multiple comparison test. A P value <0.05 was considered statistically significant.

6.4 - RESULTS

6.4.1 - HFD induced obesity and hyperglycemia

12 months of HFD significantly increased mice body weight and fasting blood glucose levels in comparison with both age-matched SD-fed mice and young mice (Fig. 6.3A-B).

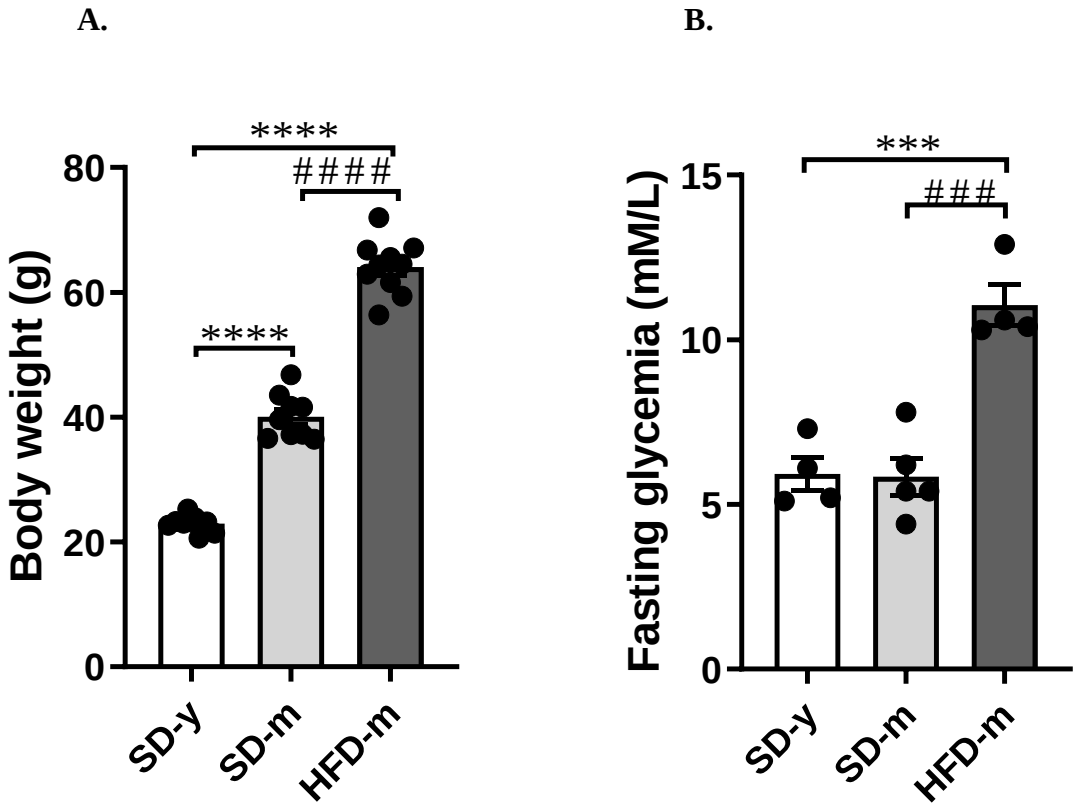


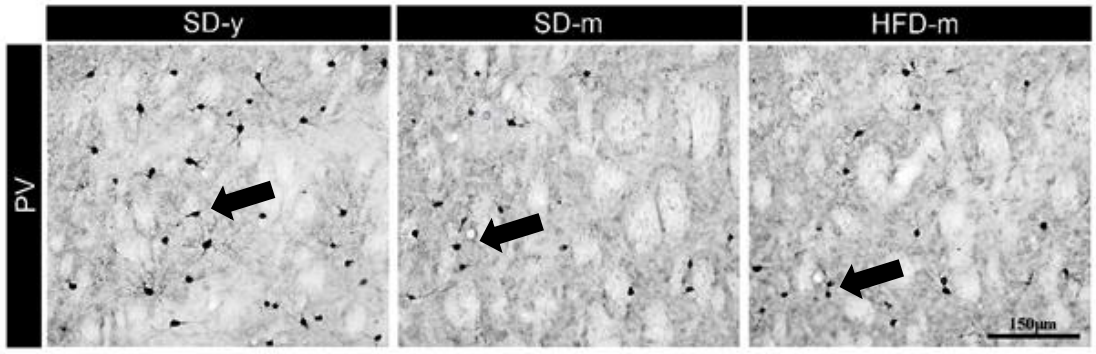
Figure 6.3 – HFD effects on weight and glycemia. Body weight (A), blood glucose concentration after 10hrs of fasting (B). Data are mean ± SEM of the indicated number of mice. Statistical significance: ****P*<0.001, *****P*<0.0001 *vs.* SD-y mice; ##*P*<0.01, ####*P*<0.0001 *vs.* SD-m mice, by one-way ANOVA, with Tukey's post-test.

6.4.2 - Aging induced a loss of PV+ interneurons in the striatum, while T2D did not significantly affect their number

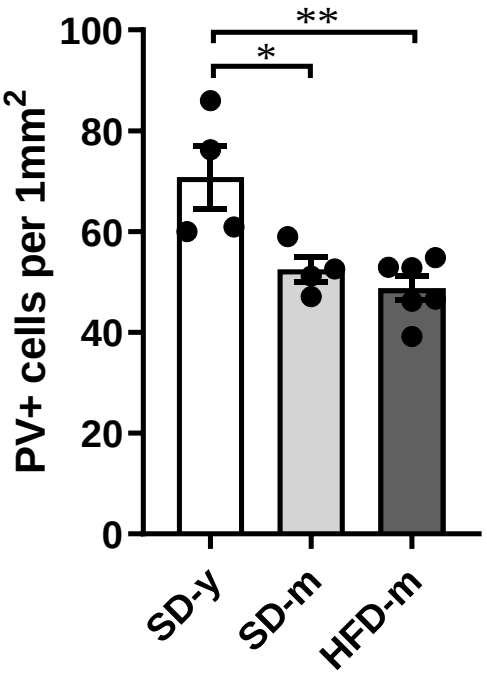
The number of PV+ striatal cells decreased massively in middle-aged mice (SD-m) (Fig. 6.4B), which also showed a significant decrease in their somatic volume

compared to 2-month-old (SD-y) mice (Fig. 6.4C). Although we expected that HFD-induced T2D aggravated both density and volume of the PV+ subpopulation of GABAergic interneurons in 14-month-old mice, our results indicate that no significant changes occurred in these parameters between 2- and 14-month-old mice (Fig. 6.4A-C).

A.



B.



C.

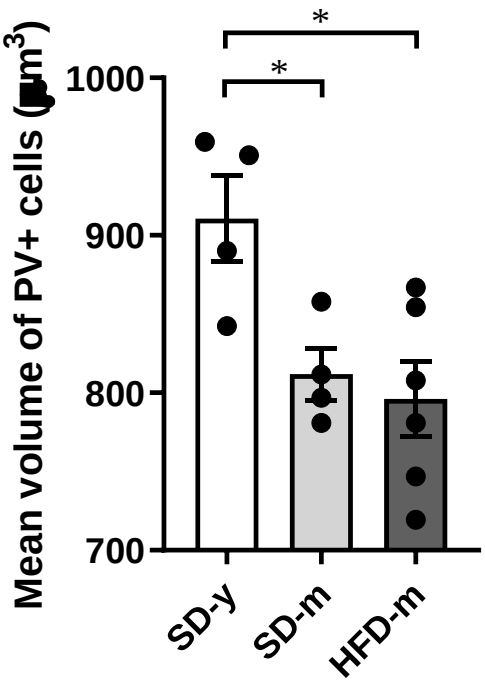


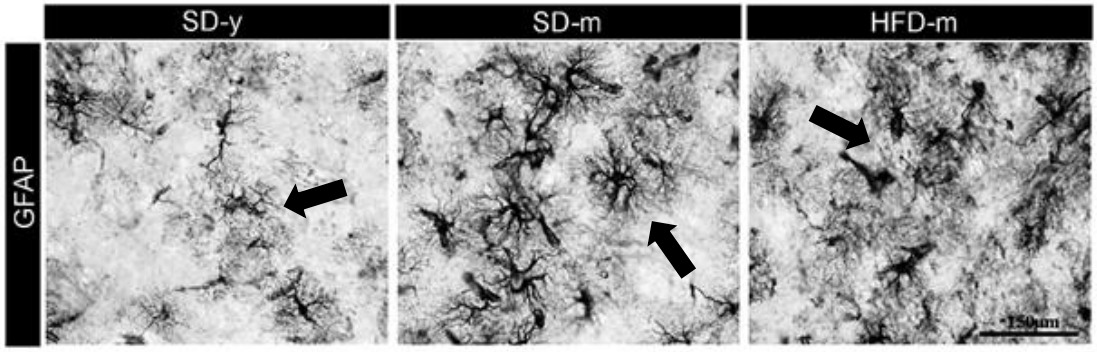
Figure 6.4 – Effect of T2D and aging on PV+ interneurons in the striatum of mice. Representative microphotographs of PV+ staining (A), and density (B) and volume of PV+ interneurons (C). Data are mean ± SEM of the indicated number of mice. Statistical

significance: *P<0.05, **P<0.01 *vs.* SD-y mice, by one-way ANOVA, with Tukey's post-test. Arrows indicate the populations of PV+ interneurons.

6.4.3 - Aging increases the number of astrocytes in the striatum

Astrocytes are key players in maintaining the homeostasis of the CNS, including the normal function of brain energy metabolism (Pekny and Pekna, 2016). Persistent astrogliosis, as a response to insults in the CNS, is associated with neurodegeneration. Moreover, the GFAP is the main component of the astrocyte intermediate filament system and its upregulation is one of the hallmarks of reactive astrocytes (Pekny and Pekna, 2016). In our study, both aging and HFD upregulated GFAP+ glial cells in comparison with 2-month-old mice (Fig. 6.5). This may indicate a reactive astrogliosis response to the pathophysiological environment.

A.



B.

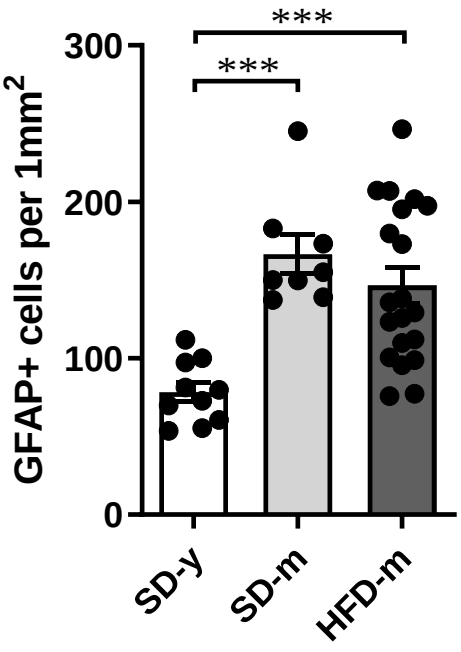
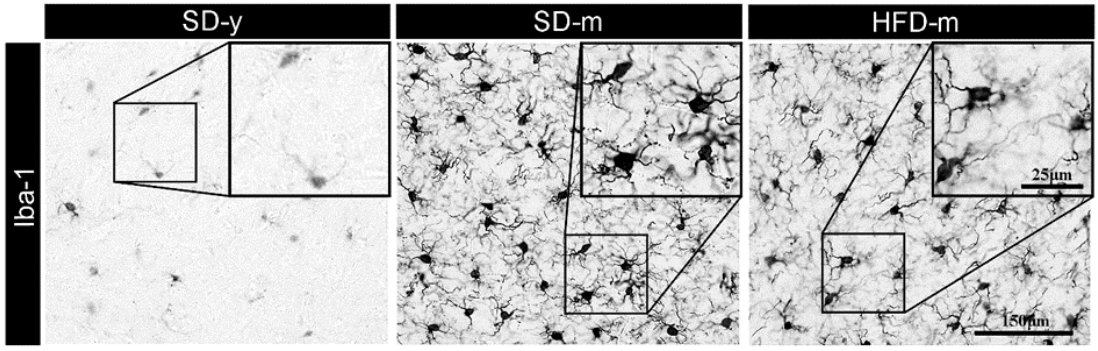


Figure 6.5 – Effect of T2D and aging on GFAP+ cells in the striatum of mice. Representative microphotographs of GFAP+ staining (A), and density of GFAP+ cells (B). Data are mean ± SEM of the indicated number of mice. Statistical significance: ****P*<0.001 *vs.* SD-y mice, by one-way ANOVA, with Tukey's post-test. Arrows are indicate the populations of GFAP+ glial cells.

6.4.4 - Aging increases neuroinflammation in striatum

Updated insights in microglia function define these main neuroimmune cells as sensing, housekeeping and defending players, whose imbalance (*e.g.* in aging or disease) may initiate or propagate neurodegeneration (Hickman *et al.*, 2018). Our observations demonstrated significant increases in Iba-1+ cells' density and volume in a similar pattern in both aging and HFD when compared to SD-y (Fig. 6.6). These results suggest an increased neuroinflammation, which may be at the basis of the aging and T2D-induced injuries to interneurons.

A.



B. **C.**

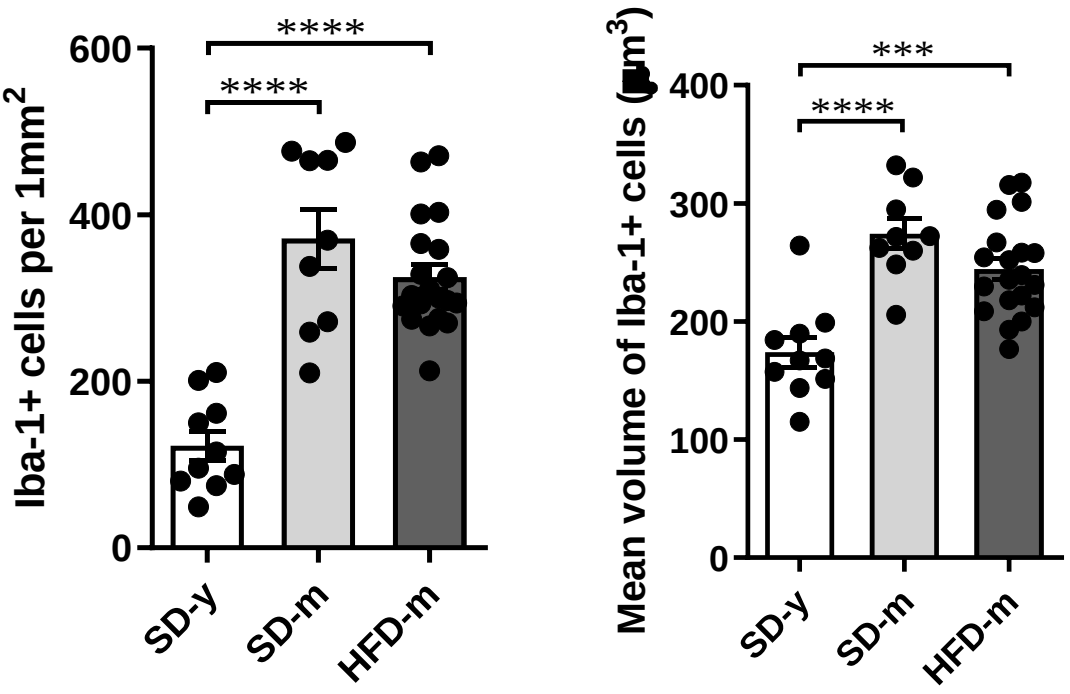
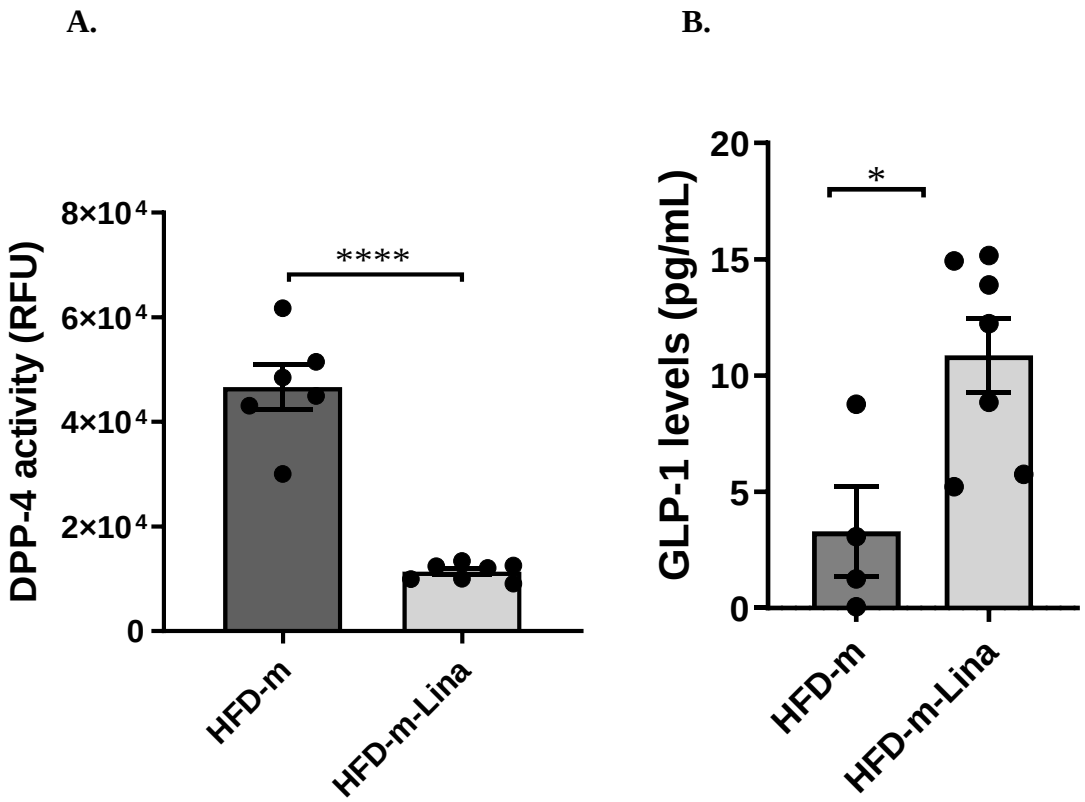


Figure 6.6 –Effect of T2D and aging on Iba-1+ cells in the striatum of mice. Representative microphotographs of Iba-1+ staining (A), and density (B) and volume of Iba-1+ cells (C). Data are mean ± SEM of the indicated number of mice. Statistical significance: ***P<0.001, ****P<0.0001 *vs.* SD-y mice, by one-way ANOVA, with Tukey's post-test.

6.4.5 - Linagliptin inhibits DPP-4 and reduces hyperglycemia

As one of the main assumptions of this study was that DPP-4i would be able to counteract the detrimental striatal effects of T2D during aging, we aimed to demonstrate

that peripheral linagliptin administration attenuates the peripheral features of aging/T2D by inhibiting the DPP-4 protein. Indeed, linagliptin dramatically reduced the peripheral activity of DPP-4 (Fig. 6.7A). Furthermore, middle-aged HFD-fed mice treated for 3 months with linagliptin showed higher plasma GLP-1 concentration (Fig. 6.7B). Chronic treatment with the DPP4i also partially rescued the hyperglycemia induced by HFD, despite no significant impact in the body weight of HFD-m-Lina animals (Fig. 6.7C-D).



C. **D.**

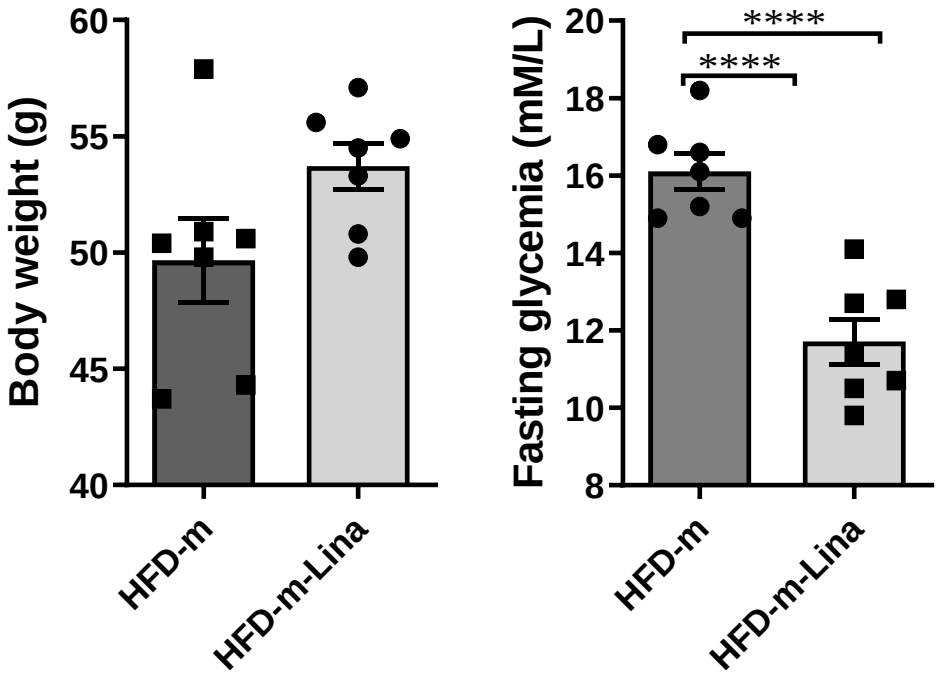
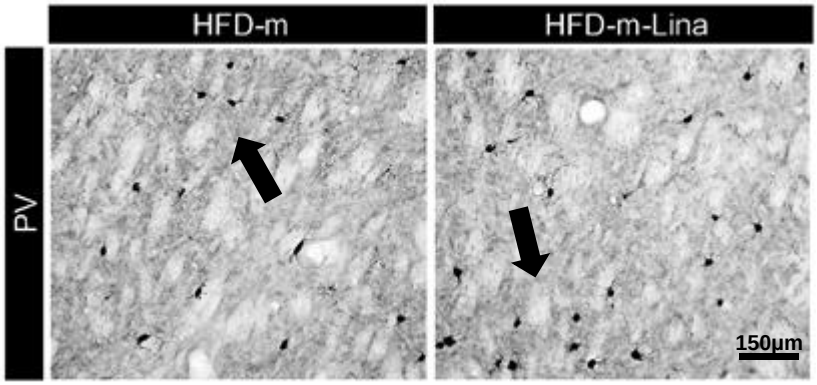


Figure 6.7 - Effect of linagliptin diet on peripheral features of T2D in middle-aged mice. Plasma dipeptidyl peptidase-4 (DPP-4) activity (A) and glucagon-like peptide-1 (GLP-1) concentration (B), body weight (C) and fasting blood glucose concentration (D). Data are mean ± SEM of the indicated number of mice. Statistical significance: **P*<0.05, *****P*<0.0001 *vs.* HFD-m mice, by unpaired t test. RFU: relative fluorescence units.

6.4.6 - Linagliptin does not affect PV+ interneurons' number or volume in striatum

Despite the similar loss of PV+ interneurons in the striatum of both SD-m and HFD-m mice described in Fig. 6.4, the chronic administration of linagliptin was not able to reverse their compromise (Fig. 6.8).

A.



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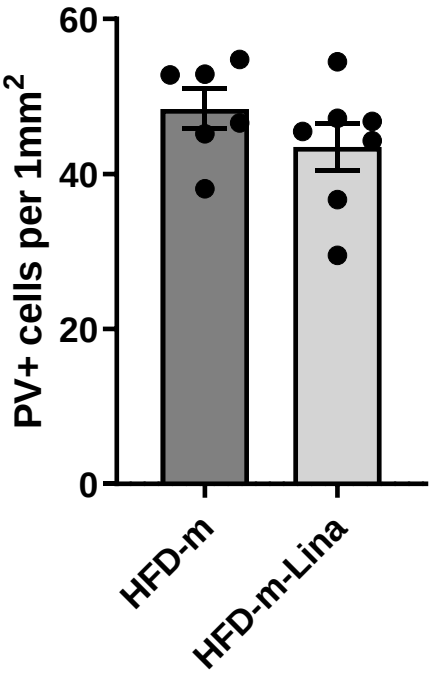

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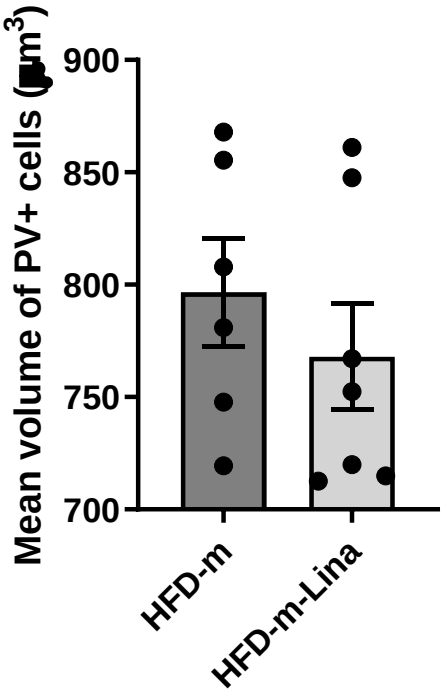
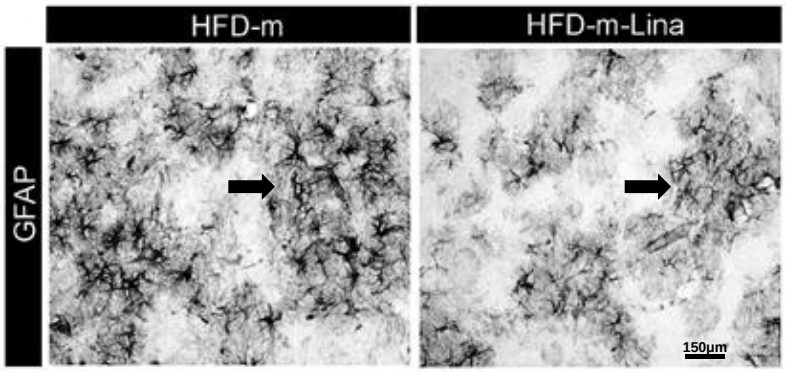


Figure 6.8 – Effect of linagliptin on PV+ interneurons in the striatum of T2D in middle-aged mice. Representative microphotographs of PV+ staining (A), and density (B) and volume of PV+ interneurons (C). Data are mean ± SEM of the indicated number of mice. Arrows indicate the populations of PV+ interneurons.

6.4.7 - Linagliptin reduced the number of GFAP+ astrocytes in middle-aged T2D mice

In the follow-up of linagliptin impact in the striatum, we evaluated its effect on T2D-dependent reactive astrogliosis. We observed a significant decrease of GFAP+ cells in middle-aged HFD-fed mice treated with linagliptin (Fig. 6.9).

A.



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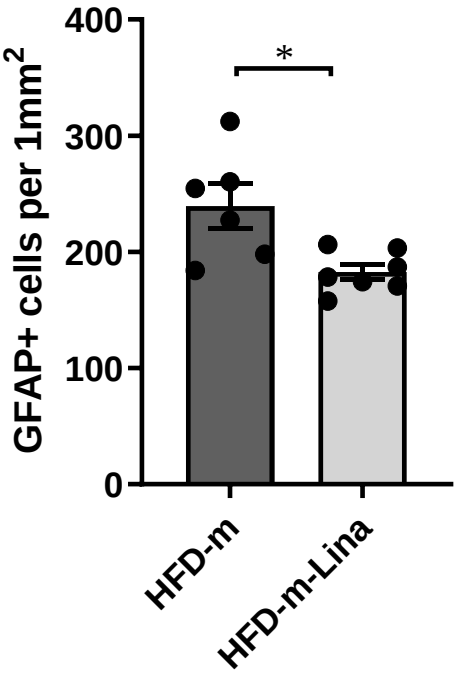


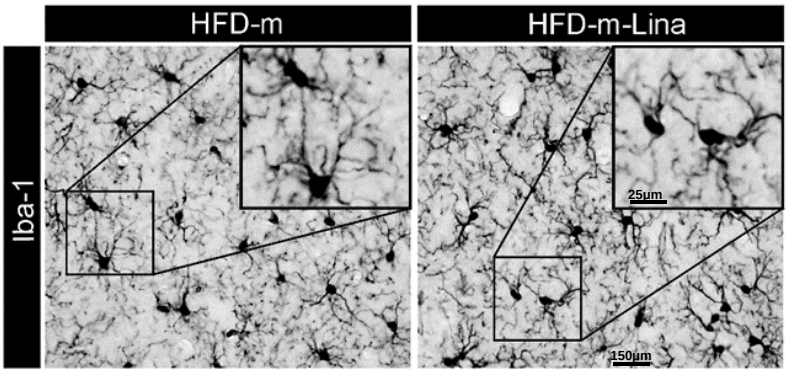
Figure 6.9 – Effect of linagliptin on GFAP-positive cells in the striatum of T2D in middle-aged mice. Representative microphotographs of GFAP+ staining (A), and density of GFAP+ cells (B). Data are mean ± SEM of the indicated number of mice. Statistical significance:

*P<0.05 *vs.* HFD-m mice, by unpaired t test. Arrows indicate the populations of GFAP+ glial cells.

6.4.8 - Linagliptin partially reduced neuroinflammation in middle-aged T2D mice

Similar to the results obtained for the GFAP staining, we demonstrated a moderate reduction in the neuroinflammatory environment, consisting in a linagliptin-associated lowering of Iba-1+ cell body volume, but not in the density of microglia in the striatum of diabetic mice (Fig. 6.10).

A.



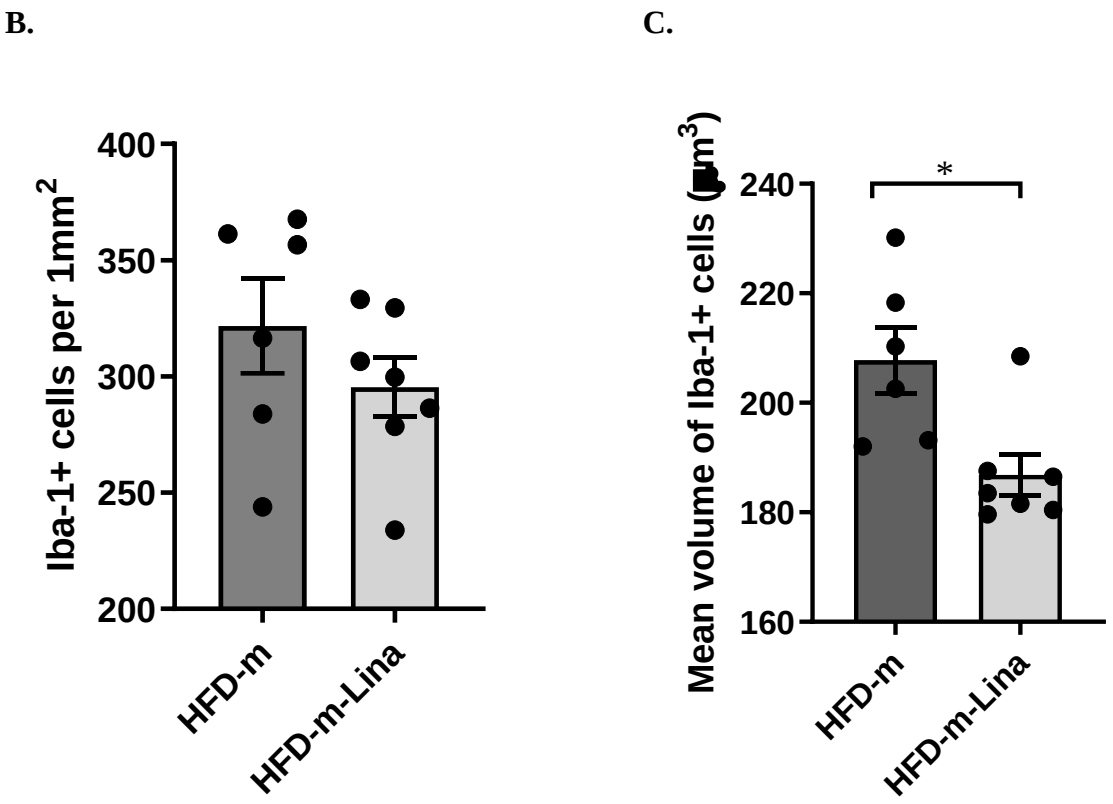


Figure 6.10 – Effect of linagliptin on Iba-1+ cells in the striatum of T2D in middle-aged mice. Representative microphotographs of Iba-1+ staining (A), and density (B) and volume of Iba-1+ cells (C). Data are mean ± SEM of the indicated number of mice. Statistical significance: **P*<0.05 *vs.* HFD-m mice, by unpaired t test.

Altogether, the results of the impact of linagliptin in middle-aged, HFD-fed mice, with a striatal reduction in astrocytes cells and microglial impact, suggest a normalization at some extent of the damaging effects of T2D-like pathology and aging in striatum.

6.5 - DISCUSSION

We demonstrated that aging induces structural alterations independent of T2D that include a decline of the nigrostriatal dopaminergic system. We showed that aging collapses the important support of striatal PV+ interneurons and increased the presence of astrocytes and microglia. Interestingly, we also showed that some of these aging-induced damaging effects may be prevented by a chronic (3 months) linagliptin

treatment. This may involve the linagliptin-induced inhibition of DPP-4, ultimately counteracting the glial alterations under such conditions.

As previously described, parvalbumin-expressing interneurons are important in the generation of network oscillations, thus mediating neural plasticity and affecting cognitive function (Hu *et al.*, 2014). Age-related and region-specific changes of PV+ interneurons are not well understood, justifying the undergoing studies to clarify the role of aging under such conditions. Although Ueno and coworkers did not find alterations in the number of PV+ interneurons in the whole cortex of aged mice, they showed a decrease in the expression levels of PV protein in such PV interneurons with aging (Ueno *et al.*, 2018). In turn, Dugan *et al*. demonstrated a clear reduction of PV+ interneurons in prefrontal cortex and hippocampus of aged mice (Dugan *et al.*, 2009). Moreover, two recent studies reported a consistent loss of hippocampal PV+ cells in AD mice. Cattaud and coworkers showed a significant drop in the total number of PV+ interneurons in the hippocampus during normal aging and an earlier decrease in the number of PV+ interneurons in AD Tg2576 mice (Cattaud *et al.*, 2018). Zallo *et al.* demonstrated that PV+ interneurons within the hippocampal CA1 region of aged 3xTg-AD mice were highly vulnerable (Zallo *et al.*, 2018). Interestingly, striatal PV+ interneurons appear to play a role in neuroplasticity and contribute to dystonia in Huntington disease (HD), since a large and rapid decrease in striatal PV+ cells was observed during HD progression (Reiner *et al.*, 2013).

The general compromise of cell integrity and function with aging is considered the main responsible for the development of long-term complications and age-related diseases. Accordingly, microglia and astrocytes lose the ability to maintain a healthy CNS environment upon aging, thereby promoting a mild (albeit chronic) inflammatory state characteristic of aging (Palmer and Ousman, 2018). Therefore, it is not surprising that accumulating evidence suggest that the development and progression of neurodegenerative diseases may be partially due to such neuroinflammatory environment and its subsequent damage, which may ultimately impair cognitive and motor function (Spittau, 2017; Ransohoff, 2016). Our results showing a dramatic increase in GFAP+ astrocytes, in microglial cells and in their activation, further support this heightened inflammatory state of aging. They also reinforce the hypothesis that inflammation is a risk factor for, *e.g.*, PD and AD.

In the second part of the study we showed the neuroprotective effects induced by the DPP-4i, linagliptin. Although some studies have associated the neuroprotection by DPP-4i with the rescue of mitochondrial function, insulin resistance, inflammation, and apoptosis, or even with the inhibition of other peptides within the brain, the specific underlying signaling pathways involved in such DPP-4i effects remain mostly unclear (Avogaro and Fadini, 2018; Sa-Nguanmoo *et al.*, 2017). This evidence suggests that DPP-4i-mediated neuroprotection may be due to their peripheral effects mainly involving the inhibition of the degradation of incretin hormones (such as GLP-1 and GIP), and the subsequent increase in insulin to-glucagon ratio and reduction of HbA1c (Deacon and Holst, 2013). In the present study we cannot exclude that the linagliptin-induced recovery of the dopaminergic system upon aging may involve a mechanism dependent of glycemia regulation and/or via GLP-1. This is in line with the notion that the rescue of motor function by anti-diabetic treatments may involve a wide range of central and peripheral mechanisms. The anti-aging effect of linagliptin in the dopaminergic system is further supported by the increased survival rate and an amelioration of cognitive impairment in a mouse model of premature aging (Hasegawa *et al.*, 2017).

Despite the lack of exacerbated neuronal/glial alterations in striatum induced by T2D in the present study, in a recently published paper we found that a 12-months HFD-induced T2D impairs the release of dopamine in striatum during aging (Lietzau *et al.*, 2020). Indeed, extracellular dopamine release was reduced in basal conditions and after an amphetamine challenge in middle-aged diabetic mice (HFD-m), being reversed by linagliptin administration (Lietzau *et al.*, 2020).

Overall, these results suggest that T2D impairs the sensorimotor function and facilitates the early pathophysiological hampering of the nigrostriatal dopaminergic system. However, further studies are needed to clarify these issues. On the other hand, the linagliptin-mediated neuroprotection suggests that glycemic regulation and insulin action may prevent motor disorders involving the nigrostriatal dopaminergic system. These novel observations may help to consolidate of the association between T2D, sensorimotor dysfunction and PD, ultimately improving the clinical significance of DPP-4i in neurodegeneration. To our knowledge this is the first study showing that obesity/T2D dramatically impairs the function of the nigrostriatal dopaminergic system in the middle-aged mouse.

Chapter 7

The positive impact of Liraglutide therapy in 3xTg-AD mice

Adapted from: Candeias E, Duarte AI, Alves IN, Mena D, Silva DF, Machado NJ, Campos EJ, Santos MS, Oliveira CR, Moreira PI (2020) Liraglutide Protects Against Brain Amyloid-beta1-42 Accumulation in Female Mice with Early Alzheimer's Disease-Like Pathology by Partially Rescuing Oxidative/Nitrosative Stress and Inflammation. *Int J Mol Sci,* 21. doi: 10.3390/ijms21051746.

Liraglutide Protects Against Brain Amyloid-$\beta_{1–42}$ Accumulation in Female Mice with Early Alzheimer Disease-Like Pathology by Partially Rescuing Oxidative/Nitrosative Stress and Inflammation

7.1 - ABSTRACT

Alzheimer disease (AD) is the most common form of dementia worldwide, being characterized by the deposition of senile plaques, neurofibrillary tangles (enriched in the amyloid beta (Aβ) peptide and hyperphosphorylated tau (p-tau), respectively) and memory loss. Aging, type 2 diabetes (T2D) and female sex (especially after menopause) are risk factors for AD, but their crosslinking mechanisms remain unclear. Most clinical trials targeting AD neuropathology failed and it remains incurable. However, evidence suggests that effective anti-T2D drugs, such as the glucagon-like peptide-1 (GLP-1) mimetic and neuroprotector liraglutide, can be also efficient against AD. Thus, we aimed to study the benefits of a peripheral liraglutide treatment in AD female mice. We used blood and brain cortical lysates from 10-month-old 3xTg-AD (triple transgenic mouse model of AD) female mice, treated for 28 days with liraglutide (0.2 mg/kg, once/day) to evaluate parameters affected in AD (*e.g.*, Aβ and p-tau, motor and cognitive function, glucose metabolism, inflammation and oxidative/nitrosative stress). Despite the limited signs of cognitive changes in mature female mice, liraglutide only reduced their cortical $A\beta_{1–42}$ levels. Liraglutide partially attenuated brain estradiol and GLP-1 and activated protein kinase A (PKA) levels, oxidative/nitrosative stress and inflammation in these AD female mice. Our results support the earlier use of liraglutide as a potential preventive/therapeutic agent against the accumulation of the first neuropathological features of AD in females.

Keywords: Alzheimer disease; brain protection; female sex; GLP-1 mimetics; liraglutide

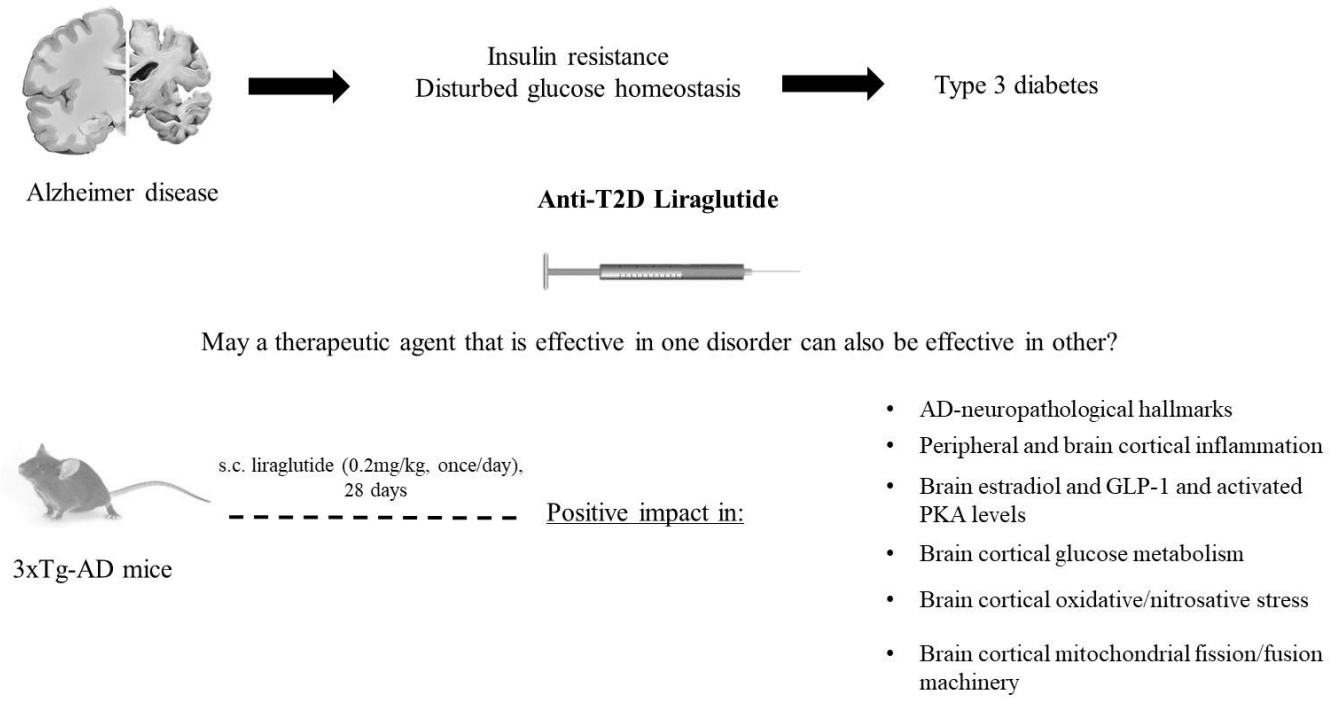


Figure 7.1 - Graphical abstract.

7.2 - INTRODUCTION

Alzheimer disease (AD) is the most common neurodegenerative disorder, neuropathologically characterized by the accumulation of senile plaques and neurofibrillary tangles (mainly composed of amyloid beta (Aβ) peptide and hyperphosphorylated tau protein (p-tau), respectively (Grundke-Iqbal *et al.*, 1986; Glenner and Wong, 1984). Its most common clinical symptom is the progressive loss of memory (Lopera *et al.*, 1997).

Two-thirds of AD patients are women, >60% of them at menopause (Brookmeyer *et al.*, 1998). This renders female sex the major risk factor for sporadic AD after aging (Farrer *et al.*, 1997), with its pathophysiological action starting years to decades before the onset of clinical symptoms, most likely at midlife—the so-called prodromal or preclinical phase (Sperling *et al.*, 2014). Indeed, studies showed that perimenopausal and menopausal women have a higher metabolic decline and Aβ levels, alongside a greater atrophy of grey and white matter relative to premenopausal women and age-matched men (Mosconi *et al.*, 2017a; Mosconi *et al.*, 2017b). Although the involved mechanisms remain debatable, the hormonal fluctuations affecting women from midlife until advanced ages may render them more vulnerable to brain changes and AD

(Mosconi *et al.*, 2017a; Mosconi *et al.*, 2017b; Fisher *et al.*, 2018; Mosconi *et al.*, 2018). In this respect, early changes in serum estrogen levels were correlated with cognitive impairment years later in aged women (Laughlin *et al.*, 2010), and with cortical and hippocampal senile plaque formation and memory deficits in AD female mice (Li *et al.*, 2013; Heys *et al.*, 2011; Colucci *et al.*, 2006; Sobow and Kloszewska, 2004; Aragno *et al.*, 2002; Ptok *et al.*, 2002). This, together with the estimates that 2/3 of AD caregivers are women render them at the epicenter of this epidemic (Mosconi *et al.*, 2017a; Mosconi *et al.*, 2017b).

AD is closely connected with diabetes (particularly type 2 diabetes; T2D) and obesity—both considered risk factors for AD. Although evidence suggests that AD patients may be more prone to develop co-morbid diabetes or obesity, this remains debatable (Camkurt *et al.*, 2018; Loera-Valencia *et al.*, 2019; Duarte *et al.*, 2018a). Nevertheless, the features shared by these pathologies (*e.g.*, impaired insulin signaling, and brain glucose transport and metabolism, mitochondrial anomalies, redox imbalance, inflammation and cognitive deficits (Duarte *et al.*, 2018a; De Felice and Ferreira, 2014)), alongside the failure of most AD clinical trials, led to the hypothesis that antidiabetic drugs may have a therapeutic potential against AD. Among them, glucagon-like peptide-1 (GLP-1) analogs are highly promising, with a minimal hypoglycemic risk. Similar to endogenous GLP-1, they tightly regulate postprandial blood glucose-dependent insulin secretion, with a subsequent fall in glycemia (Nadkarni *et al.*, 2014). GLP-1 is also ubiquitously expressed in the central nervous system (CNS), particularly in the hypothalamus, cortex, hippocampus, striatum, substantia nigra, brainstem and subventricular zone, where it may play a pivotal role (Hamilton *et al.*, 2011). Indeed, modulation of GLP-1 receptor protected against neurodegenerative events, neuronal death and cognitive decline (Li *et al.*, 2012; Gault and Holscher, 2008). Additionally, the GLP-1 mimetic liraglutide mitigated synaptic loss and neuropathology, and improved learning and memory in male AD mice (Chen *et al.*, 2017; McClean *et al.*, 2011). Liraglutide also rescued hyperhomocysteinemia-induced AD pathology and memory deficits in rats (Zhang *et al.*, 2019). Although the involved mechanisms remain unclear, liraglutide may recover brain insulin receptors (IR) and synapses after Aβ oligomer injection, ultimately improving memory function in mice and in non-human primates (Batista *et al.*, 2018). Liraglutide also hampered Aβ plaque formation (Han *et al.*, 2013), astrocyte and microglia-mediated inflammation

(Long-Smith *et al.*, 2013) and promoted neurogenesis and neuronal proliferation (Hunter and Holscher, 2012).

The lack of efficient AD-modifying therapies may result from studies performed in already symptomatic cohorts (with synaptic and neuronal deficits) and/or from the underestimation of sex differences in AD pathophysiology (Andrieu *et al.*, 2015). Moreover, most studies were performed in the hippocampus, despite the AD effects on wide areas of cerebral cortex (Masters *et al.*, 2015; Harris and Pierpoint, 2012) (including the frontal cortex) that underlie cognitive function and metabolic regulation (Stuss and Knight, 2013). Thus, there is an urgent need to uncover the role of female sex on brain cortical AD pathophysiology and progression, and to establish novel therapeutic strategies against the disease. These, by starting during the prodromal phase of AD, may efficiently prevent or delay its onset, or blunt its progression (Andrieu *et al.*, 2015). In this perspective, we aimed to evaluate the therapeutic benefits of a chronic (28-day) liraglutide treatment in mature female mice with AD-like pathology. Thus, we analyzed several brain parameters traditionally affected by AD, namely glucose metabolism, mitochondrial function/dynamics, inflammation, oxidative stress, neuropathological features and motor and cognitive behavior.

As far as we know, only one study evaluated the effects of an 8-week liraglutide treatment in the 3xTg-AD (triple transgenic mouse model of AD) mice, but in middle-aged (7–9 month-old) males (Chen *et al.*, 2017). This and our previous study in 11-month-old 3xTg-AD male mice (Carvalho *et al.*, 2013) led us to use brain cortices from mature (10-month-old) 3xTg-AD female mice displaying AD-like pathology, treated with liraglutide for a shorter time (4 weeks). Our results suggest that, despite the limited signs of cognitive impairment in these mature female mice, liraglutide treatment only mitigated the increased accumulation of brain cortical $A\beta_{1-42}$. The drug also partially normalized their brain estradiol, GLP-1 content and protein kinase A (PKA), partially reducing their plasma and brain inflammatory and oxidative stress markers, possibly due to the stimulation of glucose 6-phosphate dehydrogenase (G6PDH) (and its downstream antioxidant properties) and mitochondrial dynamics. As far as we know, this study constitutes a first approach to the use of GLP-1 mimetics (namely liraglutide) to mitigate some of the earlier AD-like pathological features in females. Further studies are needed to reinforce the need for a more tailormade, sex/gender-based medicine.

7.3 - MATERIAL AND METHODS

7.3.1 - Materials

Bovine serum albumin (BSA), phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), Tween 20, thiobarbituric acid (TBA) and mouse monoclonal β-actin (#A5441) antibody were obtained from Sigma-Aldrich (St. Louis, MO, USA). Polyvinylidene difluoride (PVDF) Immobilon-P membranes and rabbit polyclonal glucose transporter 1 (GLUT1, #CBL242) antibody were obtained from Millipore (Billerica, MA, USA). Mouse monoclonal GLUT4 antibody (#2213S) was obtained from Cell Signaling (Leiden, The Netherlands). Mouse monoclonal mitochondrial dynamin-like 120 kDa protein (OPA1) antibody (#612607) was obtained from BD Biosciences (Oeiras, Portugal). Rabbit polyclonal mitochondrial fission 1 protein (Fis1, #NB100-56646) antibody was obtained from Novus Biologicals (Abingdon, United Kingdom). Anti-mouse and anti-rabbit secondary antibodies (#RPN5781 and #RPN5783), and enhanced chemifluorescence (ECF) reagent were purchased from Amersham Biosciences (Little Chalfont, UK). Rat Insulin Enzyme Immunoassay kit (#A05105) was purchased from SPI-BIO, Bertin Pharma (Montigny le Bretonneux, France). Estradiol EIA kit (#582251) and 8-hydroxy-2-deoxy guanosine EIA (#589320) kit were purchased from Cayman Chemical (Ann Arbor, USA). QuantiChrom Glucose Assay kit (#DIGL-100) was purchased from BioAssay Systems (Hayward, CA, USA). Rat Amyloid Beta Peptide 1–42 ELISA kit (#LTI KMB3441) was purchased from EIAab Science Co. (Wuhan, China). Mouse β Amyloid 1–40 ELISA kit (#LTI KMB3481) and Tau [pS396] Human ELISA Kit (#LTI KHB7031) were purchased from Invitrogen (Camarillo, CA, USA). Trichloroacetic acid (TCA) was purchased from Calbiochem (Merck KGaA, Darmstadt, Germany). Rat GLP-1 ELISA Kit (#E-EL-R0059) was purchased from Elabscience (Wuhan, Hubei, China). Rat C-Reactive Protein (CRP) ELISA Kit (#88-7501-28), Rat interleukin (IL)-1β Platinum ELISA kit (#BMS630) and Rat IL-10 Platinum ELISA kit (#BMS629) were purchased from eBioscience (Vienna, Austria). Protein kinase A (PKA) kinase activity kit (#ADI-EKS-390A) was purchased from Enzo Life Sciences, Grupo Taper SA (Sintra, Portugal).

All other chemicals used were of the highest grade of purity commercially available.

7.3.2 - Animal Housing and Treatment

Following EU and Portuguese legislation (Directive 2010/63/EU; DL113/2013, August 7th) and ARRIVE guidelines (Kilkenny *et al.*, 2010), 10 month-old wild-type (WT) (control) and 3xTg-AD female mice (a genetic model for AD that develops an age-related progressive neuropathological phenotype) (Carvalho *et al.*, 2012) 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 (Project ORBEA_61_2013/24072013). Following the "3Rs" Reduction principle established by FELASA, in a first approach we used the brain cortical GLP-1 levels of saline-treated WT and 3xTg-AD female mice (Table 7.II) to estimate the number of animals required for this study. Briefly, by using the Wilcoxon-Mann-Whitney test applied to their independent means and standard deviations on the G-Power software (Faul *et al.*, 2007), an alpha error of 0.05 and a power of 80%, we estimated that a total of six mice should be used for the overall study. In line with this and aiming to increase the power of our hypothesis, we used a minimum of four mice per parameter.

Mice were maintained at our animal colony (Animal Research Center, University of Coimbra) in static microisolator cages (3–4 mice/cage) with a filter top and bedding and nesting materials, under controlled light (12h day/night cycle) and humidity (45–65%) and *ad libitum* standard hard pellets chow and sterilized and acidified water (pH 2.5–3). Signs of distress were carefully monitored. Mice were randomly divided into three experimental groups: in the first one, 14 3xTg-AD female mice were daily, subcutaneously (s.c.) injected with liraglutide (0.2mg/kg), for 28 days, whereas the remaining two groups (10 wild type and 12 3xTg-AD mice; mice with AD-like pathology were subjected to random assignments) received saline injection (0.9% sterile NaCl). Although not expected, a rapid decrease in body weight >15–20% was defined as a humane endpoint for the study.

7.3.3 - Body and Brain Weight

Body weight was monitored once/week throughout the study. Immediately before euthanasia, animals were also weighed. After euthanasia, brains were immediately removed and weighed. Results were expressed as body weight or brain weight (g).

7.3.4 - Collection of Peripheral Blood and Routine Biochemical Analyses

Mice were fasted for ~6h (starting early in the morning) and immediately after their euthanasia blood was immediately collected directly from the heart by transcardial punction to commercially-available blood collection tubes containing EDTA (Vacuette® K3E/EDTA3K; Greiner Bio One, Kremsmünster, Austria) to isolate plasma (as detailed below). One drop of blood was used to determine fasting or occasional blood glucose levels by the glucose oxidase reaction, using a glucometer (Glucometer-Elite, Bayer SA, Portugal) and compatible stripes. Results were expressed as mg glucose/dL blood.

Blood glycated hemoglobin (HbA1c) was measured with the Multi-Test HbA1c (A1C Now+, Bayer SA, Portugal) and results expressed as %. The remaining blood was centrifuged at 572× *g* for 10 min, at 4 ˚C, in a Sigma 2–16 PK centrifuge. The resulting plasma was used to determine fasting insulin levels through the Insulin Enzyme Immunoassay kit, according to the manufacturer's instructions.

Absorbance was read at 405 nm in a SpectraMax Plus 384 multiplate reader, when maximum binding (B_0) wells reached 0.2–0.8 arbitrary units (a.u.) Results were expressed as ng/mL plasma.

Plasma estradiol levels were measured by the Estradiol EIA kit, according to the manufacturer's instructions. Absorbance was read at 450 nm, in a SpectraMax Plus 384 multiplate reader. Results were expressed as pg/mL plasma.

7.3.5 - Isolation and Preparation of Brain Cortical Homogenates

After euthanasia, brains were immediately removed and cortices dissected and snap-frozen for further studies. Brain cortices were then homogenized at 0–4 ˚C in lysis buffer, containing (in mM): 25 HEPES, 2 $MgCl_2$, 1 EDTA, 1 EGTA, pH 7.4, supplemented with 2 mM DTT, 100 µM PMSF and commercial protease and phosphatase inhibitors cocktails. The crude homogenate was centrifuged at 17,968× *g* for 10 min, at 4 ˚C in a Sigma 2–16K centrifuge to remove the nuclei, and the resulting supernatant was collected. Pellet was further resuspended in supplemented buffered solution and centrifuged again at 17,968× *g* for 10 min, at 4 ˚C. The supernatant was added to the previously obtained one and protein content determined by the Bio-Rad Protein Assay, according to the manufacturer's instructions.

7.3.6 - Evaluation of AD Pathological Hallmarks

Brain cortical $A\beta_{1–42}$ levels were determined in 10 µL brain cortical homogenates by the Amyloid Beta Peptide 1–42 ELISA kit, according to the manufacturer's instructions. Absorbance was determined at 450 nm, in a SpectraMax Plus 384 multiplate reader. Results were expressed as pg/mg protein.

Brain cortical $A\beta_{1–40}$ levels were determined in 10 µL of brain cortical homogenates by the β-Amyloid 1–40 ELISA kit, according to the manufacturer's instructions. Absorbance was determined at 450 nm, in a SpectraMax Plus 384 multiplate reader. Results were expressed as pg/mg protein.

Brain cortical levels of p-tau protein at the serine 396 residue (Tau pSer396) were determined in 10 µL of brain cortical homogenates by the Tau [pS396] Human ELISA Kit, according to the manufacturer's instructions. Absorbance was read at 450 nm in a SpectraMax Plus 384 multiplate reader. Results were expressed as pg/mg protein.

7.3.7 - Behavioral Analyses

At the end of treatment, mice were transported in their home cages to the behavioral testing room and allowed to acclimate to the room for at least 2h prior to each test. Behavioral tests were performed in consecutive days, by experienced observers blind to the experimental conditions.

1. Open Field Behavior Test

Open field behavior testing allows the assessment of the locomotor and behavioral activity in rodents (Gould *et al.*, 2009). Motor activity was evaluated during night cycle in an open field squared arena with grey open-topped boxes (50 cm wide × 50 cm deep × 40 cm high), using the Stoelting ANY-MAZE video tracking system (Stoelting Co., Wood Dale, IL, USA), detecting position of the animal's head. Mice were placed individually in the corner of the open field arena and were recorded for a 30-min period. Data were collected every 5 min.

2. Y-maze Behavior Test

Short-term spatial memory was evaluated using the modified Y-maze test, based on the innate preference of animals to explore areas that have not been previously explored

(Soares *et al.*, 2013). Briefly, using a Y-shaped plexiglass apparatus consisting of three arms (18 cm long, 6 cm wide and 6 cm high) separated by equal angles, mice were subjected to a training session whereby they freely explored two arms (Start and Other) for 8 min, while the third one (Novel) was blocked (Akwa *et al.*, 2001; Dellu *et al.*, 1997; Dellu *et al.*, 1992). After a 120-min inter-trial interval, mice were subjected to the test session, after the removal of the wall that blocked the Novel arm and its opening for free exploration of the three arms for 8 min. Memory performance was given by the percentage of time spent in the novel arm over the time spent exploring all arms.

3. Morris Water Maze Test

Spatial memory was assessed by the Morris water maze (MWM) test, as described by Morris *et al*. (Morris *et al.*, 1982), with slight modifications (Soares *et al.*, 2013). Briefly, tests were performed in a circular swimming pool made of grey-painted fiberglass, 1.2 m inside diameter, 0.8 m high, which was filled to a depth of 0.6 m with water maintained a $23 \pm 2\ ^{\circ}C$. The target platform ($10 \times 10\ cm^2$) of transparent acrylic resin was submerged 1–1.5 cm beneath the water surface and it was cued by a 7-cm diameter white ball attached to the top of the platform and protruding above the water. Starting points were marked on the outside of the pool as north (N), south (S), east (E) and west (W). Four distant cues ($55 \times 55\ cm^2$) were placed 30 cm above the upper edge of the water tank and the position of each symbol marked the midpoint of the perimeter of a quadrant (circle = NE quadrant, square = SE quadrant, cross = SW quadrant and diamond = NW quadrant). A monitor and a video-recording system were installed in an adjacent room.

Mice were submitted to a cued version of the water maze (Prediger *et al.*, 2006), consisting of four training days and four consecutive trials per day, during which the animals were left in the tank facing the wall and were then allowed to swim freely to the submerged platform placed in the center of one of the four imaginary quadrants of the tank. The initial position in which the animal was left in the tank was one of the four vertices of the imaginary quadrants of the tank, by the following order: north, south, east and west. If the mouse did not find the platform during a period of 60 s, it was gently guided to it. After the animal had escaped to the platform, it remained on it for 10 s and was then removed from the tank for 20 s before being placed in the next random initial position. Test session (day five) consisted of a single trial, in which the platform was removed and each mouse was allowed to swim for 60 s in the maze. The

experiments were recorded and the scores for latency of escape from the starting point to the platform and swimming speed were later measured with the ANY-MAZE™ video tracking system.

7.3.8 - Evaluation of Inflammation Markers

Inflammation markers were evaluated in plasma and brain cortical homogenates, by using the CRP ELISA Kit, IL-10 Platinum ELISA kit and IL-1β Platinum ELISA kit, according to the manufacturer's instructions. Briefly, 7.5 µL of plasma and 5 µL of each brain cortical homogenate were used to determine CRP levels, whereas 10 µL of plasma and each brain cortical homogenate were used for IL-10 and IL-1β levels. Absorbance was read at 450 nm, in a SpectraMax Plus 384 multiplate reader. Results were expressed as ng/mL plasma and ng/mg protein for CRP, and as pg/mL plasma and pg/mg protein for IL-1β and IL-10.

7.3.9 - Evaluation of Brain Cortical Hormones' Levels

Brain cortical estradiol levels were measured in 10 µL of each sample (with the remaining volumes decreased to half) by using the Estradiol EIA kit, according to the manufacturer's instructions. Absorbance was determined by a SpectraMax Plus 384 multiplate reader, at 450 nm. Results were expressed as pg/mg protein.

Brain cortical GLP-1 levels were measured in 20µL of each sample (working dilution of 1:5) by the Rat GLP-1 ELISA Kit. Absorbance was determined at 450 nm, in a SpectraMax Plus 384 multiplate reader. Results were expressed as pg/mg protein.

7.3.10 - Assessment of Brain Cortical PKA Activity

Active PKA kinase was determined in 5 µL of each sample (working dilution of 1:6) by the PKA kinase activity kit. Absorbance was determined at 450 nm, in a SpectraMax Plus 384 multiplate reader. Results were expressed as ng/mg protein.

7.3.11 - Assessment of Brain Cortical Glucose Levels

Brain cortical glucose levels were determined by the QuantiChromTM Glucose Assay kit, according to the manufacturer's instructions, in 5 µL of each brain cortical homogenate. Absorbance was read at 630 nm, in a SpectraMax Plus 384 multiplate reader. Results were expressed as mg/mg protein.

7.3.12 - Determination of Brain Markers for Glycolysis and Pentose Phosphate Pathway

Glycolytic metabolism and pentose phosphate pathways (PPP) were given by the activity of the PPP enzyme G6PDH, and by the levels of pyruvate and lactate in mouse brain cortical lysates.

Pentose phosphate pathway was given by the activity of G6PDH, that catalyzes the formation of 6-phosphogluconolactone from glucose-6-phosphate (G6P), at the expense of $NADP^+$, according to a previously described method (Garcia-Nogales *et al.*, 1999). Briefly, 5 µL of each brain cortical lysate were incubated in a reaction buffer containing 50 mM Tris-HCl (pH 7.5) and supplemented with 50 µM $MgCl_2$ and 7.2 µM $NADP^+$. Absorbance was read at 340nm, at 37 ˚C, during 2 min, with readings of 20 s intervals, in a SpectraMax Plus 384 microplate reader. Then, the reaction was initiated by the addition of 0.5 mM G6P, and the absorbance continuously read for 150 s, with 20 s intervals. G6PDH activity was calculated using a $\varepsilon 340\ nm = 6220\ M^{-1}cm^{-1}$. Results were expressed as µM/s/mg protein.

Pyruvate levels were determined by the Pyruvate Colorimetric/Fluorometric assay kit, according to the manufacturer's instructions, in 5 µL of brain cortical lysate (working dilution 1:10). Absorbance was read at 570 nm, in a SpectraMax Plus 384 microplate reader. Results were expressed as nmol/mg protein.

Lactate levels were determined by the Lactate Colorimetric/Fluorometric assay kit, according to the manufacturer's instructions, in 5 µL of each brain cortical homogenate (working dilution 1:10). Absorbance was read at 570 nm, in a SpectraMax Plus 384 microplate reader. Results were expressed as nmol/mg protein.

7.3.13 - Evaluation of Oxidative/Nitrosative Stress Markers

Carbonyl groups were determined according to Fagan *et al*. (Fagan *et al.*, 1999), with slight modifications. Briefly, 5 µL of each brain cortical homogenate were dissolved in 71 µL TCA 20%, and centrifuged at 9167× *g*, for 3 min, in a Sigma 2–16K centrifuge. The pellet obtained was incubated for 1h, at room temperature, in 35 µL DNPH 10 mM (freshly prepared in 2M HCl) protected from light and with vortex agitation every 10min. Then, 35 µL TCA 20% were added and the mixture was centrifuged at 11,092× *g*, for 3 min. The resulting pellet was mixed with 71 µL ethanol:ethyl acetate (1:1, *v*/*v*), and centrifuged again at 9167× *g*, for 3 min. Then, the pellet was incubated in 64.3 µL guanidine 6M (prepared in PBS, pH 6.5), for 15 min, at 37 ˚C, and centrifuged at 9167× *g* for 3 min. For all samples, a blank was prepared, which was incubated with HCl 2M instead of 2,4-dinitrophenylhydrazine (DNPH). Carbonyl content was calculated from the maximum absorbance, at 360 nm, measured in a SpectraMax Plus 384 multiplate reader, and an $\varepsilon_{360nm} = 22 \times 10^3\ M^{-1}cm^{-1}$. The results were expressed as µmol/mg protein.

Levels of the DNA oxidation marker 8-hydroxy-2-deoxy guanosine (8-OHdG) were determined in 10 µL of brain cortical homogenates by the 8-OHdG EIA kit (Cayman Chemical Co.), according to the manufacturer's instructions. Absorbance was read at 405 nm, in a SpectraMax Plus 384 multiplate reader. Results were expressed as pg/mg protein.

Nitrite levels were indirectly given by the $NO^{\bullet}$ production upon the reaction with Griess reagent, according to Green *et al.* (Green *et al.*, 1981). Briefly, 100 µg of each brain cortical homogenate were diluted in 100 µL phosphate buffer and incubated, for 10 min, in 100 µL Griess reagent (containing 1% sulfanilamide in 2.5% phosphoric acid, plus 0.1% n-(1-naphthyl) ethylenediamine dihydrochloride), protected from light. Absorbance was read at 550nm, in a SpectraMax Plus 384 multiplate reader. Nitrite content was calculated using a standard curve of sodium nitrite. Results were expressed as pmol/mg protein.

7.3.14 - Western Blot Analyses

Samples containing denatured brain cortical homogenates (50 µg per lane) were subjected to sodium dodecyl sulfate (SDS)/polyacrylamide gel electrophoresis (SDS/PAGE) (8–15%) and transferred onto PVDF membranes. Then, membranes were

blocked for 1h at room temperature in Tris-buffered saline (TBS, pH 7.4) plus 1% or 5% BSA and 0.05% Tween 20. Membranes were then incubated overnight at 4ºC with rabbit GLUT1 (1:1000), mouse GLUT4 (1:1000), rabbit Fis1 (1:750) and mouse OPA1 (1:1000) primary antibodies. Membranes were then incubated with the respective anti-rabbit or -mouse secondary IgG antibodies (1:10,000), for 2h, at room temperature, and developed using ECF. Immunoreactive bands were visualized by the VersaDoc Imaging System (Bio-Rad, Hercules, CA, USA). Fluorescence signal was analyzed using the QuantityOne software and the results given as INT/mm^2. Of note, membranes were then reprobed with the corresponding mouse β-actin (1:5000) primary antibody. Results were presented as the ratio between total protein vs. β-actin.

7.3.15 - Statistical Analysis

Authors performed the statistical analysis using SPSS version 24.0 (IBM Corp., Armonk, NY, USA). The extreme outliers were discarded, based on the 3× IQR criterion. The Shapiro-Wilk test was used to assess the normality of data ($p > 0.05$), since the number of mice/group were considered small (*i.e.*, $n < 50$). The normally distributed data were evaluated concerning the homogeneity of variance, using the Levene's test ($p > 0.05$). For data with a Gaussian distribution, a parametric one-way analysis of variance (ANOVA) was performed to determine whether there were significant overall differences ($p < 0.05$) between the mean of more than two groups. To determine which groups differed from the rest ($p < 0.05$), the Fisher's Least Significant Difference (LSD), Bonferroni or the Games-Howell *post-hoc* tests were used. For data with a non-Gaussian distribution, a non-parametric Mann-Whitney test was used ($p < 0.05$). In this study, the groups analyzed were the brain cortical homogenates, blood or plasma from mature female WT mice, 3xTg-AD and 3xTg-AD + Liraglutide mice. Statistical significance was defined as $p < 0.05$.

Graphic artwork was obtained using the GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA, USA). Data were presented as mean $\pm$ SE of the indicated number of mice/group, run in duplicate.

7.4 - RESULTS

7.4.1 - Effect of Liraglutide Treatment on Brain and Peripheral Features in Female Mice

The key neuropathological hallmarks of AD are the deposition of Aβ and hyperphosphorylated tau that occur early in disease pathology in brain areas such as the hippocampus and cortex, long before its clinical diagnosis that relies mostly on memory loss and, to a lower extent, in a few biomarkers (Camkurt *et al.*, 2018; Serrano-Pozo *et al.*, 2011). However, the precise crosslinking mechanisms that occur across this timeframe remain debatable.

Similar to our previous study in 11-month-old 3xTg-AD male mice (Carvalho *et al.*, 2013), here we observed a significant increase in brain $A\beta_{1–42}$, $A\beta_{1–40}$ and p-tau (Ser396) levels in 3xTg-AD female mice compared to WT ones. Liraglutide treatment only reduced brain $A\beta_{1–42}$ levels (for $A\beta_{1–42}$: $F(2,14) = 15.206$; $p < 0.0001$; for $A\beta_{1–40}$: $F(2,14) = 4.597$; $p = 0.029$; for p-tau (Ser396): $F(2,11) = 10.178$; $p = 0.003$; Figure 7.2A–C). Despite this and our previous observations in mature 3xTg-AD male mice (Carvalho *et al.*, 2013), our mature 3xTgAD female mice only showed partial deficits in motor and cognitive performance compared to WT ones (Figure 7.3), as given by the slightly lower distance travelled in total ($F(2,18) = 0.609$; $p = 0.554$) and in the center of the open field arena ($F(2,17) = 2.141$; $p = 0.148$), and also by the time spent in its center ($Z = −0.387$, $p = 0.755$ for 3xTg-AD vs. WT mice; $Z = −0.579$, $p = 0.613$ for 3xTg-AD + Lira vs. WT mice; $Z = −0.429$, $p = 0.731$ for 3xTg-AD + Lira vs. 3xTg-AD mice), suggesting a thigmotaxic behavior that may be due to increased anxiety/fearfulness (Figure 7.3A–C). These were mirrored by their lower number of entries into the novel arm of the Y-maze ($F(2,20) = 8.454$; $p = 0.002$), despite no significant changes in the time spent in its start arm: $F(2,21) = 0.259$; $p = 0.774$) (Figure 7.3D, E), and the slightly reduced number of crossings of the Morris water maze ($Z = −1.787$, $p = 0.081$ for 3xTg-AD vs. WT mice; $Z = −0.059$, $p = 0.955$ for 3xTg-AD + Lira vs. WT mice; $Z = −1.619$, $p = 0.138$ for 3xTg-AD + Lira vs. 3xTg-AD mice; for escape latency: $Z = −0.698$, $p = 0.536$ for 3xTg-AD vs. WT mice; $Z = −0.901$, $p = 0.408$ for 3xTg-AD + Lira vs. WT mice; $Z = −0.457$, $p = 0.710$ for 3xTg-AD + Lira vs. 3xTg-AD mice) (Figure 7.3F–H), suggesting that the impairment in short-term spatial memory was not accompanied by significant changes in long-term spatial

memory. Liraglutide administration only exerted limited benefits in these motor and cognitive deficits in mature 3xTg-AD female mice.

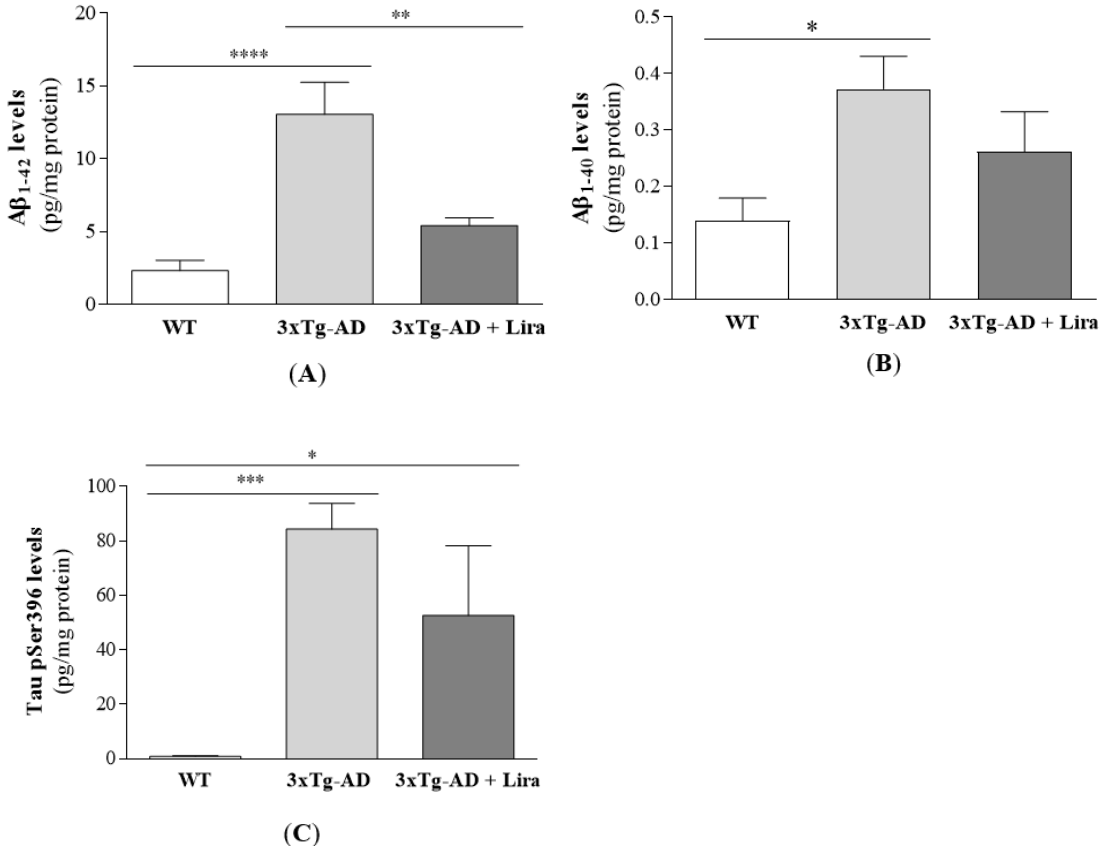
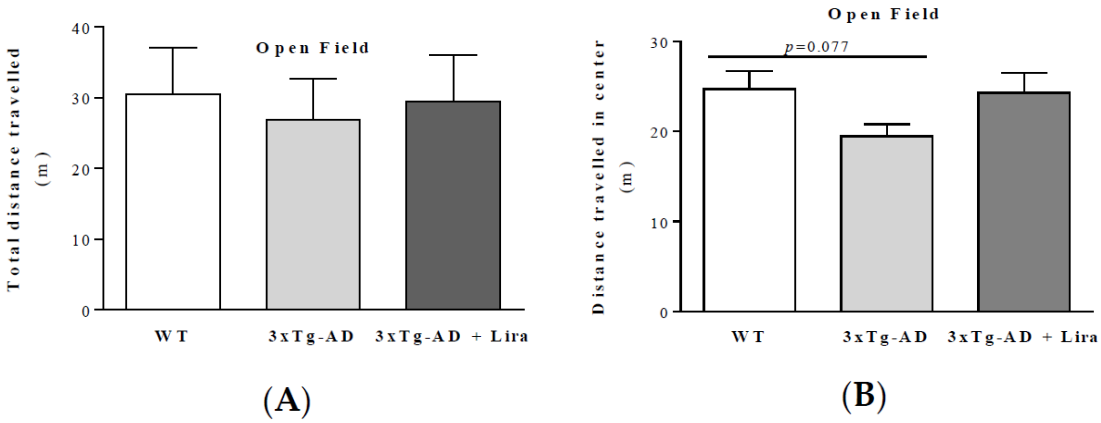
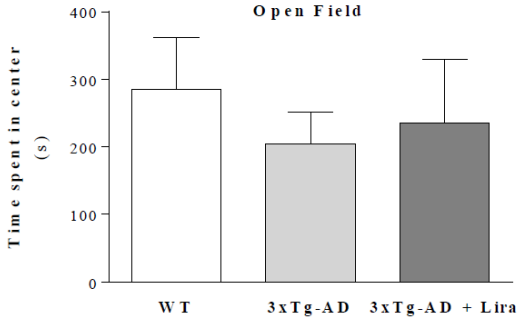
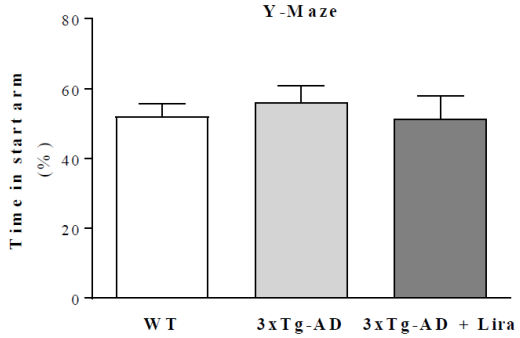


Figure 7.2 - Effect of liraglutide on brain cortical AD-like hallmarks in 3xTg-AD female mice. Brain cortical $A\beta_{1–42}$ (**A**), $A\beta_{1–40}$ (**B**) and Tau pSer396 levels (**C**) were determined. Data are the mean ± SE from 4–6 mice/group. Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ or **** $p < 0.0001$, by the one-way ANOVA with the Bonferroni and Fisher LSD post-hoc tests for multiple comparisons.

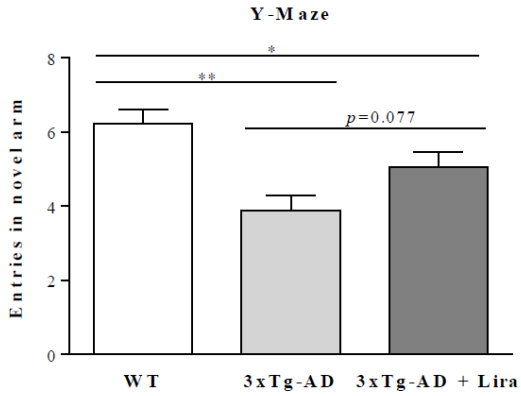



Open Field
Time spent in center (s)
400
300
200
100
0
WT
3xTg-AD
3xTg-AD + Lira

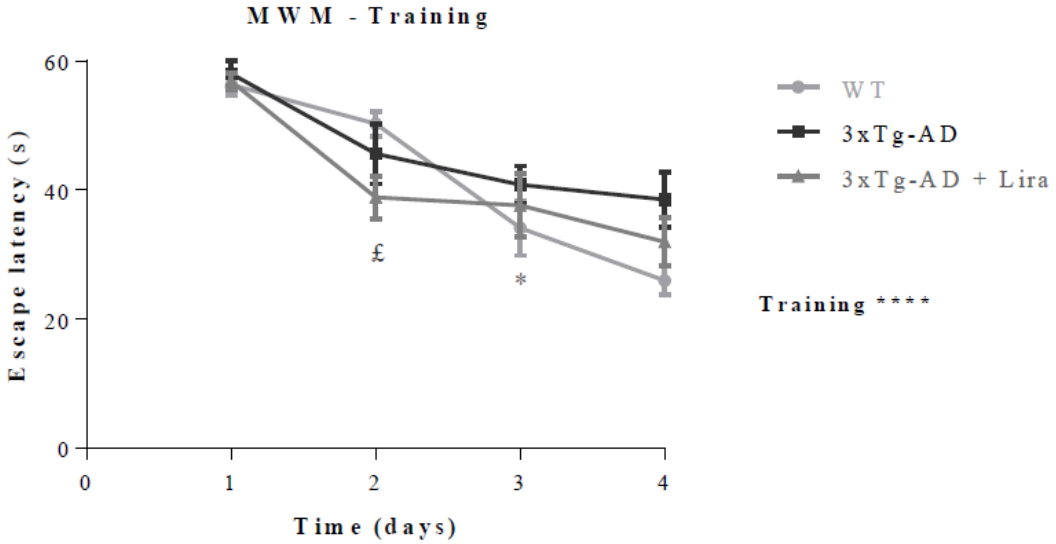
(C)


Y-Maze
Time in start arm (%)
80
60
40
20
0
WT
3xTg-AD
3xTg-AD + Lira

(D)


Y-Maze
Entries in novel arm
8
6
4
2
0
*
**
p=0.077
WT
3xTg-AD
3xTg-AD + Lira

(E)


MWM - Training
Escape latency (s)
60
40
20
0
£
*
0
1
2
3
4
Time (days)
WT
3xTg-AD
3xTg-AD + Lira
Training ****

(F)

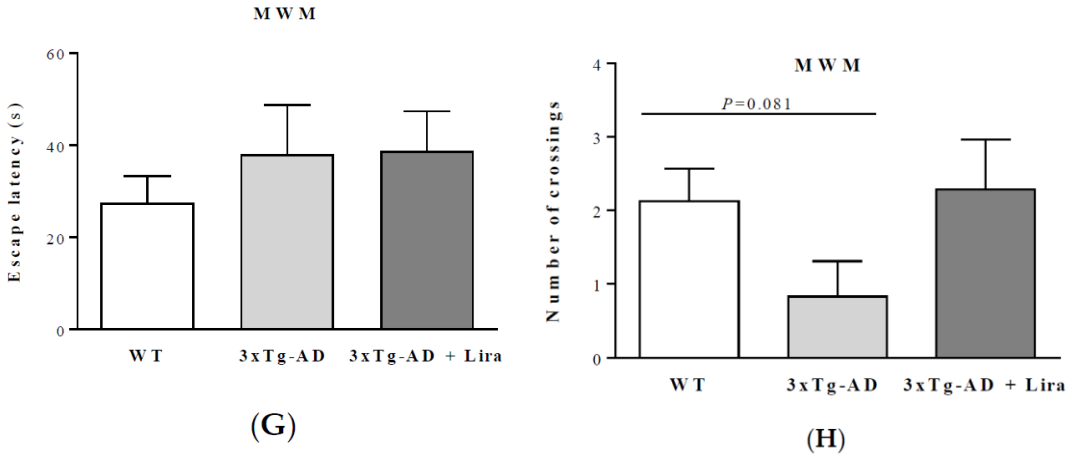


Figure 7.3 - Effect of liraglutide on behavioral performance in female mice with early AD-like pathology. Total distance travelled (**A**), and distance travelled (**B**) and time spent in the center (**C**) of the open field area during the open field test; time spent in start arm during training (**D**) and number of entries into the novel arm during testing session (**E**) in the Y-maze test; escape latency across trainings days (**F**) and testing session (**G**), and the number of crossings during testing session (**H**) of the Morris Water Maze test were assessed. Data are the mean ± SE from 6–10 mice/group. Statistical significance: * $p < 0.05$ or ** $p < 0.01$, by the one-way ANOVA with the Fisher LSD post-hoc test for multiple comparisons (for a Gaussian distribution: A,B,D,E), or by the non-parametric Mann-Whitney test (for a non-Gaussian distribution: C,G,H). Regarding Figure 2F, statistical significance: * $p < 0.05$ in WT day 3 vs. WT day 2, $^{£}p < 0.05$ in 3xTg-AD + Lira day 2 vs. 3xTg-AD + Lira day 1, **** $p < 0.0001$ by two-way ANOVA, with the Tukey post-hoc test for multiple comparisons.

These results suggest that our mature 3xTg-AD female mice model an early symptomatic stage of the disease, displaying early AD-like pathology with still limited signs of cognitive deficits.

Peripheral and brain inflammation constitutes another prominent feature of AD (De Luigi *et al.*, 2002; Yang *et al.*, 2018). In line with this, we observed a massive increase in the pro-inflammatory CRP and IL-1β markers in plasma from the 3xTg-AD female mice, whereas the anti-inflammatory IL-10 was only slightly decreased (by 34%) compared to WT female mice ($F(2,16) = 2.974$; $p = 0.08$ for plasma CRP levels; for plasma IL-10 levels: $Z = -0.857$, $p = 0.445$ for 3xTg-AD vs. WT mice; for plasma IL-1β levels: $Z = -2.882$, $p = 0.002$ for 3xTg-AD vs. WT mice; Table 7.I). Liraglutide treatment tended to normalize the plasma inflammatory markers (for plasma IL-10

levels: $Z = -0.319$, $p = 0.805$ for 3xTg-AD + Lira vs. WT mice; $Z = -1.286$, $p = 0.234$ for 3xTg-AD + Lira vs. 3xTg-AD mice; for plasma IL-1β levels: $Z = -2.00$, $p = 0.051$ for 3xTg-AD + Lira vs. WT mice; $Z = -1.143$, $p = 0.295$ for 3xTg-AD + Lira vs. 3xTg-AD mice; Table 7.I). Similar to the well-described neuroinflammation markers in AD patients and animal models (Moussa *et al.*, 2017; Nazem *et al.*, 2015), the brains from 3xTg-AD female mice showed a significant increase in the pro-inflammatory CRP ($F(2,11) = 9.337$; $p = 0.004$) and in the anti-inflammatory cytokine IL-10 levels ($F(2,14) = 2.447$; $p = 0.123$) compared to WT female mice (Figure 7.4). Liraglutide treatment decreased their brain CRP and IL-10 levels (although the later was not statistically significant) (Figure 7.4). Unexpectedly, no significant alterations occurred in IL-1β levels in the brains from 3xTg-AD female mice (data not shown). These results further reinforce the notion that our 3xTg-AD female mice model an asymptomatic stage of the disease, displaying early AD-like neuropathology without substantial signs of cognitive deficits. This is further supported by the lack of significant alterations in brain weight ($F(2,22) = 0.742$; $p = 0.868$; Table 7.I) or in pre- and postsynaptic markers between experimental groups (data not shown).

Table 7.I - Effect of liraglutide administration on peripheral features of female mice with early AD-like pathology.

	WT	**3xTg-AD**	**3xTg-AD + Lira**
Body weight (g)	29.1 ± 1.2 (n=10) (95% CI: 26.3-31.8)	23.3 ± 0.6**** (n=12) (95% CI: 22.1-24.6)	23.3 ± 0.4 (n=14) (95% CI: 22.6-24.1)
Brain weight (g)	0.5 ± 0.01 (n=7) (95% CI: 0.45–0.51)	0.4 ± 0.03 (n=8) (95% CI: 0.36–0.52)	0.5 ± 0.03 (n=10) (95% CI: 0.42–0.54)
HbA_{1c} (%)	4.3 ± 0.2 (n=10) (95% CI: 3.74–4.84)	4.4 ± 0.1 (n=11) (95% CI: 4.17–4.65)	4.4 ± 0.1 (n=12) (95% CI: 4.13–4.57)

Occasional glycemia (mg glucose/dL blood)	132.8 ± 3.3 (n=9) (95% CI: 125.2–140.3)	121.2 ± 7.3 (n=12) (95% CI: 105.2–137.2)	128.1 ± 10.5 (n=14) (95% CI: 105.6–150.7)
Fasting glycemia (mg glucose/dL blood)	126.4 ± 4.7 (n=9) (95% CI: 115.6–137.7)	110.3 ± 8.2 (n=12) (95% CI: 92.4–128.3)	127.6 ± 6.5 *P*=0.073 (n=14) (95% CI: 113.7–141.6)
Fasting insulin levels (ng/mL plasma)	1.5 ± 0.8 (n=8) (95% CI: 0.07–6.97)	2.5 ± 0.8 (n=11) (95% CI: 0.72–4.23)	1.4 ± 0.4 (n=11) (95% CI: 0.56–2.13)
HOMA-IR	11 ± 5.9 (n=8) (95% CI: 0.7–59.8)	15.2 ± 5.0 (n=11) (95% CI: 4.05–26.37)	11.3 ± 3.1 (n=11) (95% CI: 4.37–18.26)
HOMA-β	456.0 ± 191.0 (n=10) (95% CI: -76.25–511.26)	262.1 ± 93.01 (n=9) (95% CI: 47.60–476.5)	170.9 ± 39.2 (n=11) (95% CI: 72–268)
Estradiol levels (pg/mL plasma)	184.1 ± 15.1 (n=7) (95% CI: 147.2–220.9)	230.8 ± 24.3 *P*=0.07 (n=6) (95% CI: 168.3–293.3)	244.9 ± 9.5 *P*=0.023 (n=6) (95% CI: 220.3–269.4)
C-Reactive Protein levels (ng/mL plasma)	31.9 ± 6.1 (n=6) (95% CI: 16.25–47.51)	74.3 ± 17.6* (n=6) (95% CI: 29.10–119.4)	60.8 ± 10.7 (n=7) (95% CI: 34.53–86.98)
IL-10 levels (pg/mL plasma)	551.5 ± 134.6 (n=7)	364.6 ± 81.6 (n=6)	494.3 ± 54.5 (n=7)

	(95% CI: 222.1–880.9)	(95% CI: 154.8–574.3)	(95% CI: 361.1–627.6)
IL-1β levels (pg/mL plasma)	43.2 ± 12.3 (n=6) (95% CI: 11.66–74.66)	821.6 ± 400.7* (n=6) (95% CI: -208.3–1852)	355.4 ± 159.3 (n=7) (95% CI: -34.38–745.3)

Data are mean ± SE of the indicated number of mice/group. Statistical significance: * p < 0.05, ** p < 0.01 or **** p < 0.0001 vs. WT female mice, by the one-way ANOVA with the Fisher LSD post-hoc test for multiple comparisons (for a Gaussian distribution), or by the non-parametric Mann-Whitney test (for a non-Gaussian distribution: occasional glycemia, fasting insulin levels, HOMA-IR, HOMA-β, plasma IL-10 and IL-1β levels). HbA1c: glycated hemoglobin A1c, HOMA-IR: homeostatic model assessment for insulin resistance, HOMA-β: homeostatic model assessment for β-cell function.

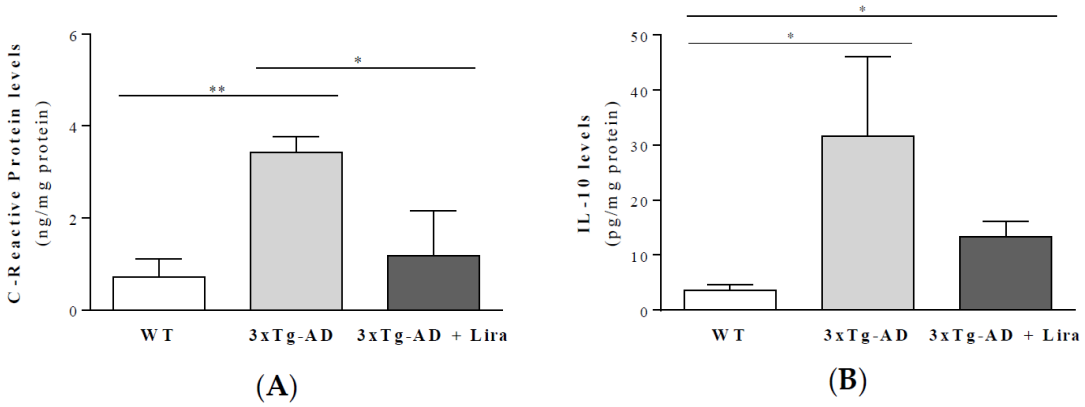


Figure 7.4 - Effect of liraglutide on brain cortical inflammation markers in female mice with early AD-like pathology. Brain cortical C-Reactive Protein (**A**) and IL-10 (**B**) were determined. Data are the mean ± SE from 3–6 mice/group. Statistical significance: * $p < 0.05$ or ** $p < 0.01$, by the one-way ANOVA with the Fisher LSD or Games-Howell post-hoc tests for multiple comparisons.

Other feature of AD is body weight loss (Gillette-Guyonnet *et al.*, 2000), whereas peripheral metabolic anomalies remain controversial (Raji *et al.*, 2009). Accordingly, our female mice with early AD-like pathology showed a 20% reduction in body weight

that, nonetheless, was not recovered by liraglutide treatment ($F(2,33) = 19.7$; $p < 0.0001$; Table 7.I). Conversely, plasma estradiol levels were slightly increased (between 25–33%) in female mice with early AD-like pathology (treated or not with liraglutide) compared to WT mice ($F(2,16) = 3.568$, $p = 0.052$; Table 7.I). No significant alterations occurred in the peripheral glucose homeostasis markers occasional ($Z = -0.139$, $p = 0.169$ for 3xTg-AD vs. WT mice; $Z = -0.129$, $p = 0.201$ for 3xTg-AD + Lira vs. WT mice; $Z = -0.129$, $p = 0.899$ for 3xTg-AD + Lira vs. 3xTg-AD mice) and fasting glycemia ($F(2,32) = 1.914$, $p = 0.153$), HbA_{1c} ($F(2,30) = 0.142$, $p = 0.868$), plasma insulin ($Z = -0.352$, $p = 0.756$ for 3xTg-AD vs. WT mice; $Z = -0.07$, $p = 0.973$ for 3xTg-AD + Lira vs. WT mice; $Z = -0.558$, $p = 0.606$ for 3xTg-AD + Lira vs. 3xTg-AD mice), HOMA-IR ($Z = -0.494$, $p = 0.654$ for 3xTg-AD vs. WT mice; $Z = -0.635$, $p = 0.557$ for 3xTg-AD + Lira vs. WT mice; $Z = -0.230$, $p = 0.847$ for 3xTg-AD + Lira vs. 3xTg-AD mice) or HOMA-β ($Z = -1.251$, $p = 0.236$ for 3xTg-AD vs. WT mice; $Z = -0.622$, $p = 0.573$ for 3xTg-AD + Lira vs. WT mice; $Z = -0.572$, $p = 0.604$ for 3xTg-AD + Lira vs. 3xTg-AD mice) between experimental groups (Table 7.I).

7.4.2 - Liraglutide Partially Normalizes Brain Levels of Estradiol and GLP-1-Related Signaling in Female Mice with Early AD-Like Pathology

AD pathology has been associated with impaired levels and/or activity of hormones and signaling pathways (Mosconi *et al.*, 2018; Duarte *et al.*, 2018a; Sepulveda *et al.*, 2019). Thus, we aimed to analyze the role of peripheral liraglutide treatment on brain estradiol and GLP-1 levels and downstream signaling in female mice with early AD-like pathology.

Similar to the periphery, levels of brain estradiol and GLP-1 were increased in female mice with early AD-like pathology compared to WT ones (for brain GLP-1 levels: $F(2,13) = 2.686$; $p = 0.106$; for brain estradiol levels: $Z = -2.191$, $p = 0.030$ for 3xTg-AD vs. WT mice; Table 7.II). Liraglutide treatment tended to normalize both estradiol and GLP-1 levels (for brain estradiol levels: $Z = -1.358$, $p = 0.222$ for 3xTg-AD + Lira vs. WT mice; $Z = -0.548$, $p = 0.662$ for 3xTg-AD + Lira vs. 3xTg-AD mice; Table 7.II). Despite no significant alterations in brain insulin levels nor in IR, GLP-1R or activated Akt between cohorts (data not shown), female mice with early AD-like pathology had a massive decrease in brain active PKA kinase that tended to recover

with liraglutide ($Z = -2.562$, $p = 0.009$ for 3xTg-AD vs. WT mice; $Z = -0.548$, $p = 0.662$ for 3xTg-AD + Lira vs. WT mice; $Z = -0.913$, $p = 0.429$ for 3xTg-AD + Lira vs. 3xTg-AD mice; Table 7.II). These results suggest an impairment in brain GLP-1R-mediated signaling in 3xTg-AD female mice that tended to be normalized by liraglutide administration (Table 7.II).

Table 7.II - Effect of liraglutide administration on brain cortical hormones' levels and signaling in female mice with early AD-like pathology.

	WT	**3xTg-AD**	**3xTg-AD + Lira**
Estradiol levels (pg/mL/mg protein)	5.62 ± 1.19 (n=5) (95% CI: 2.31–8.93)	15.2 ± 2.7* (n=6) (95% CI: 8.27–22.11)	12.2 ± 3.3 (n=5) (95% CI: 3.1–21.24)
GLP-1 levels (pg/mL/mg protein)	5.9 ± 2.5 (n=5) (95% CI: -1.14–12.94)	21.1 ± 6.5* (n=6) (95% CI: 4.52–37.74)	15.0 ± 2.8 (n=5) (95% CI: 7.29–22.76)
Active PKA kinase (ng/assay/mg protein)	0.01 ± 0.004 (n=6) (95% CI: -0.0005–0.02)	0.001 ± 0.0004** (n=6) (95% CI: 0.0001–0.002)	0.009 ± 0.005 (n=5) (95% CI: -0.0048–0.022)

Data are mean ± SE of the indicated number of mice/group. Statistical significance: * $p < 0.05$, ** $p < 0.01$ vs. WT mice, by the one-way ANOVA with the Fisher LSD or Games-Howell post-hoc tests for multiple comparisons (for a Gaussian distribution), or with the non-parametric Mann-Whitney test (for a non-Gaussian distribution: brain estradiol levels and active PKA kinase).

7.4.3 - Liraglutide Promotes Brain Glucose Metabolism via the Oxidative Branch of the Pentose Phosphate Pathway in Female Mice with Early AD-Like Pathology

Another feature of AD is the impairment in brain glucose transport and metabolism (Szablewski, 2017; Mosconi, 2013). Therefore, we next evaluated the effect of

liraglutide administration on brain cortical markers for glucose transport and downstream metabolism.

Despite no significant alterations in GLUT4 and GLUT8 expression between experimental groups, brains from female mice with early AD-like pathology had higher glucose levels ($F(2,14)=2.433$, $p=0.046$ and slightly increased GLUT1 expression than WT mice (Figure 7.5A, B). Liraglutide treatment did not significantly affect brain GLUT1 and GLUT4 (an insulin-sensitive glucose transporter; $F(2,13) = 4.491$, $p = 0.033$) or glucose content in early AD-like female mice compared to 3xTg-AD female mice (Figure 7.5A–C).

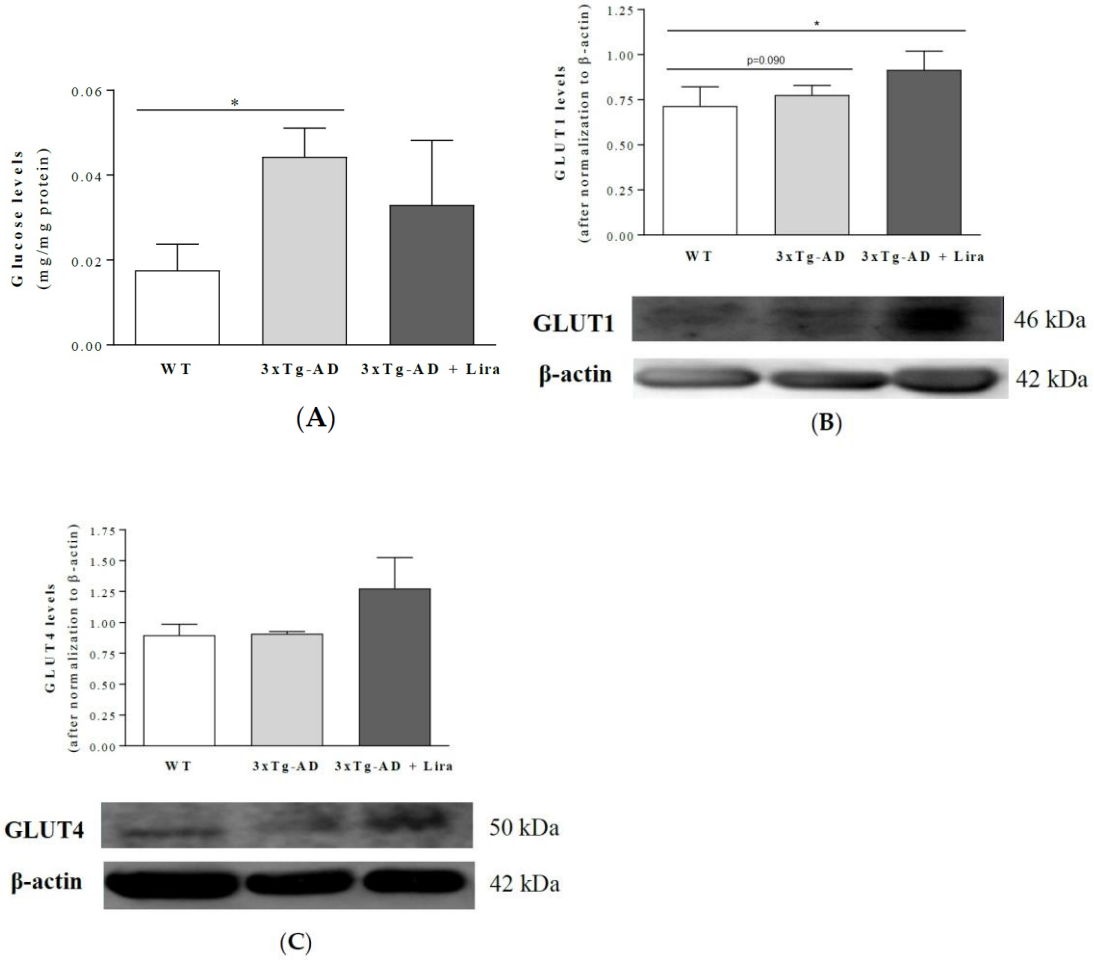


Figure 7.5 - Effect of liraglutide on brain cortical glucose levels and transporters in mature female mice with early AD-like pathology. Brain cortical glucose (**A**), and GLUT1 (**B**) and GLUT4 protein levels (**C**) were evaluated and normalized to β-actin levels, and representative Western blotting images displayed. Data are the mean ± SE from 5–6

mice/group. Statistical significance: * $p < 0.05$, by the one-way ANOVA with the Fisher LSD or Games-Howell post-hoc tests for multiple comparisons.

Moreover, liraglutide abrogated the decrement in the activity of G6PDH (the limiting enzyme from the oxidative branch of the pentose phosphate pathway) in brains from female mice with early AD-like pathology ($Z = -2.309$, $p = 0.029$ for 3xTg-AD vs. WT mice; $Z = -2.309$, $p = 0.029$ for 3xTg-AD+ Lira vs. WT mice; $Z = -2.309$, $p = 0.029$ for 3xTg-AD + Lira vs. 3xTg-AD mice; Figure 7.6A). Regarding glycolysis markers, liraglutide decreased brain pyruvate levels ($F(2,15) = 5.210$, $p = 0.019$) without significant changes in those of lactate in female mice with early AD-like pathology compared to the saline-treated ones (for lactate levels: $Z = 0$, $p = 1$ for 3xTg-AD vs. WT mice; $Z = -0.838$, $p = 0.421$ for 3xTg-AD + Lira vs. WT mice; $Z = -0.548$, $p = 0.662$ for 3xTg-AD + Lira vs. 3xTg-AD mice; Figure 7.6B, C).

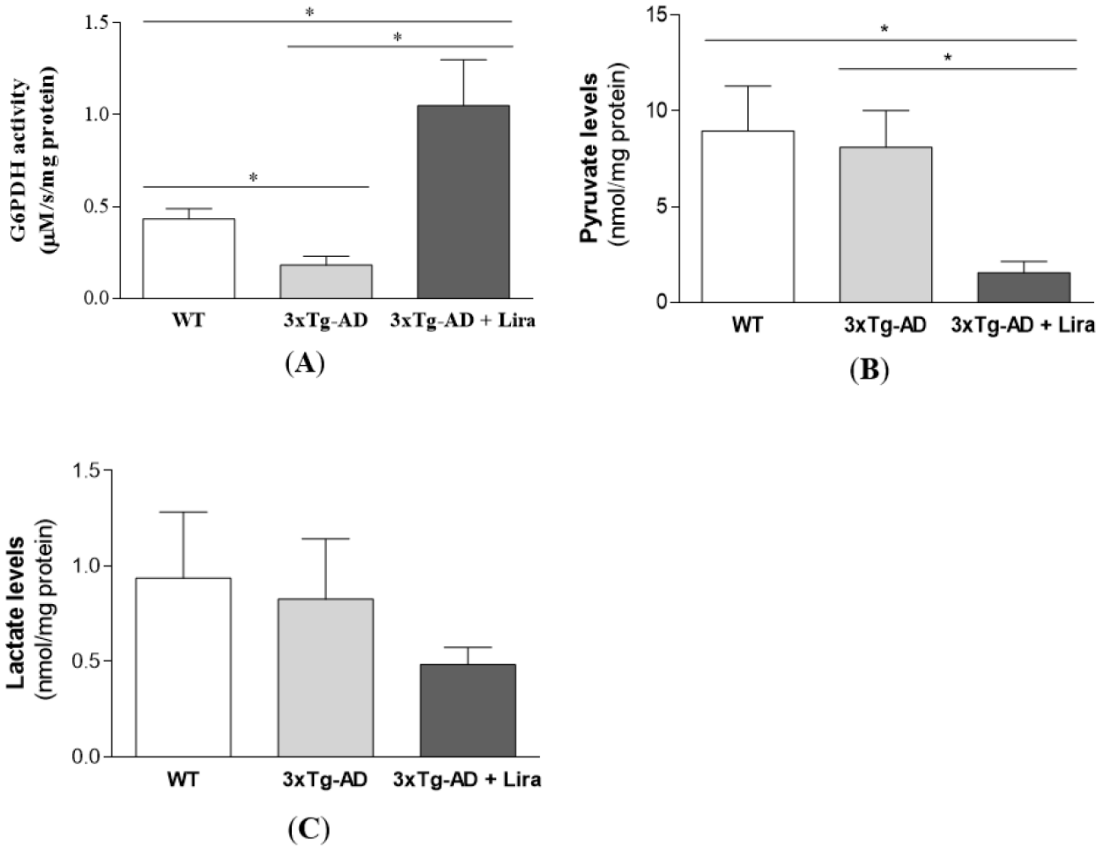


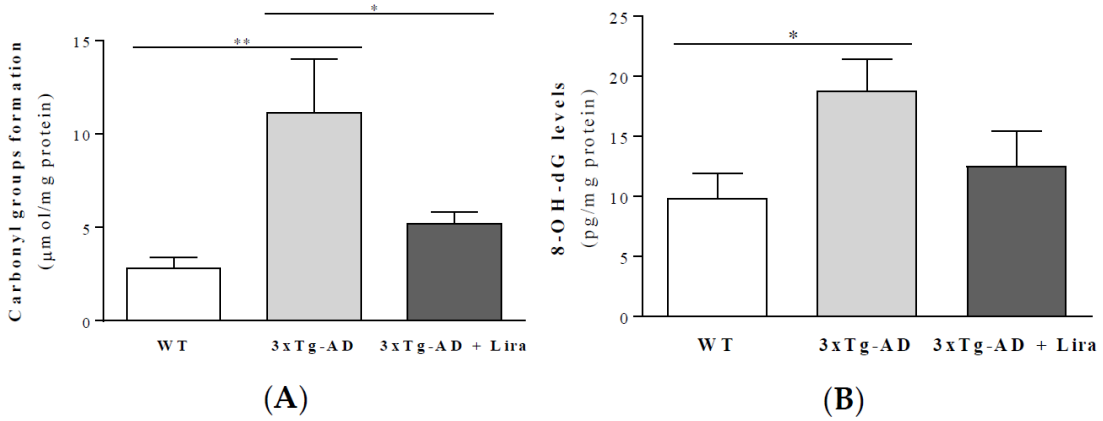
Figure 7.6 - Effect of liraglutide on brain cortical glucose metabolism in female mice with early AD-like pathology. Brain cortical G6PDH activity (**A**), and pyruvate (**B**) and lactate levels (**C**) were determined. Data are the mean ± SE from 4–6 mice/group. Statistical

significance: * $p < 0.05$, by the one-way ANOVA with the Fisher LSD post-hoc test for multiple comparisons (for a Gaussian distribution), or with the non-parametric Mann-Whitney test (for a non-Gaussian distribution: GAPDH activity and lactate levels).

These results suggest that liraglutide-mediated stimulation of G6PDH may be beneficial against brain oxidative stress in female mice with early AD-like pathology.

7.4.4 - Liraglutide Partially Rescues Brain Oxidative/Nitrosative Stress Markers in Female Mice with Earl AD-Like Pathology

From the above and since increased oxidative and nitrosative stress was demonstrated in both human and rodent AD brains (including the 3xTg-AD mice) (Resende *et al.*, 2008; Nunomura *et al.*, 2001), we next evaluated the effect of liraglutide on brain oxidative/nitrosative stress markers. Accordingly, brains from female mice with early AD-like pathology showed a slight increase in TBARS (by ~1.4-fold; $F(2,13) = 2.819$, $p = 0.096$; Supplementary Figure 7.1A) and nitrite levels (by ~1.4-fold; $F(2,15) = 4.30$, $p = 0.033$), and significantly higher carbonyl groups (by ~4-fold; $F(2,14) = 5.755$, $p = 0.015$) and 8-OHdG levels (by ~1.9-fold; $F(2,15) = 3.559$, $p = 0.054$) compared to WT mice (Figure 7.7A–C). Liraglutide tended to normalize the 8-OHdG content (Figure 7.7B), while those of TBARS, carbonyl groups and nitrites were significantly reversed by the drug in female mice with early AD-like pathology (Figure 7.7A, C; Supplementary Figure 7.1A).



(**A**) (**B**)

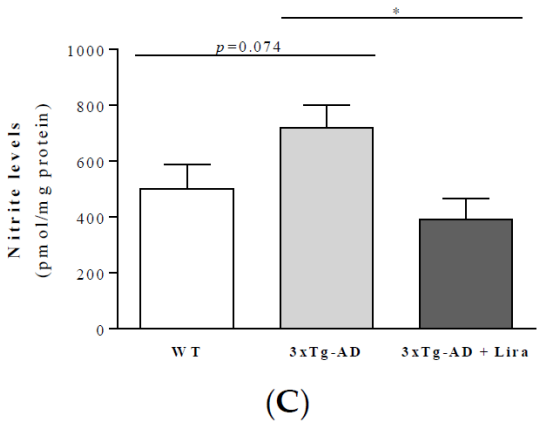
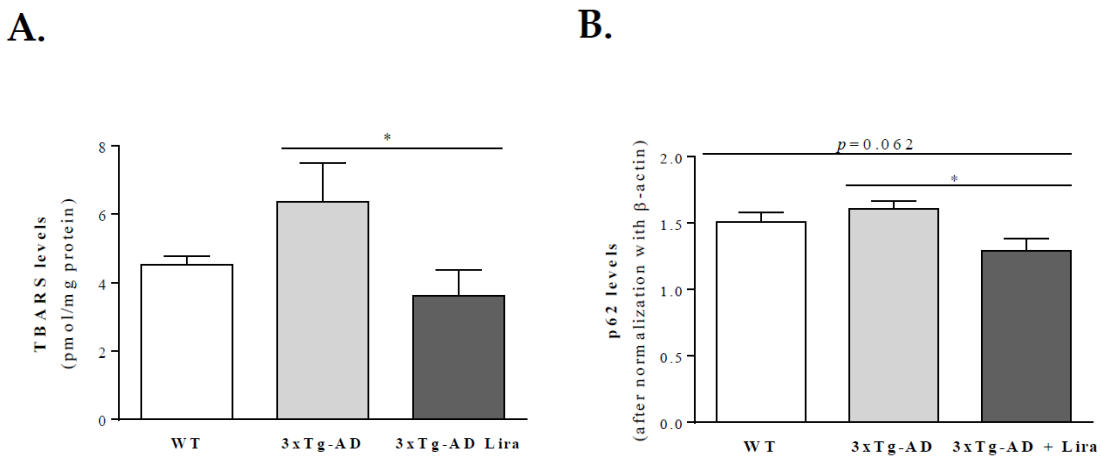


Figure 7.7 - Effect of liraglutide on brain cortical oxidative and nitrosative stress markers in female mice with early AD-like pathology. Brain cortical carbonyl groups formation (**A**), 8-OHdG (**B**) and nitrites levels (**C**) were determined. Data are the mean ± SE from 5–7 mice/group. Statistical significance: * $p < 0.05$ or ** $p < 0.01$, by the one-way ANOVA with the Fisher LSD post-hoc test for multiple comparisons.

Recent evidence suggests that, besides its pivotal role in lysosomal-mediated autophagy, p62 may also be involved in oxidative defense, nutrient sensing and inflammation mechanisms (Sanchez-Martin *et al.*, 2019). Despite no significant alterations in brain p62 levels in female mice with early AD-like pathology, liraglutide treatment reduced its levels by 20% in these animals ($F(2,15) = 4.424$, $p = 0.031$; Supplementary Figure 7.1B).



Supplementary Figure 7.1 - Effect of liraglutide on brain cortical lipid oxidation and p62 markers in female mice with early AD-like pathology. Brain cortical TBARS (A) and p62

protein levels (B, after reprobing from membranes labeled to OPA1) were determined. Data are the mean ± SE from 5-6 mice/group. Statistical significance: *p<0.05, by the one-way ANOVA with the Fisher LSD post-hoc test for multiple comparisons.

These results suggest that peripheral treatment with liraglutide partially rescued brain oxidative stress markers in female mice with early AD-like pathology.

7.4.5 - Liraglutide Partially Attenuates the Altered Mitochondrial Fission/Fusion Proteins in Female Mice with Early AD-Like Pathology

Alongside the above-mentioned pathophysiological changes in AD, we previously showed alterations in brain mitochondrial dynamics (Santos *et al.*, 2010). Therefore, we aimed to study the role of liraglutide on brain markers for mitochondrial fission and fusion. We observed that liraglutide reversed the 2.6-fold increase in Fis1 levels in brains from female mice with early AD-like pathology ($F(2,15) = 5.358$, $p = 0.018$; Figure 7.8A), while the 1.8-fold lower OPA1 levels were only partially reversed upon liraglutide administration (by 1.6-fold) in female mice with early AD-like pathology ($F(2,15) = 3.636$, $p = 0.052$; Figure 7.8B).

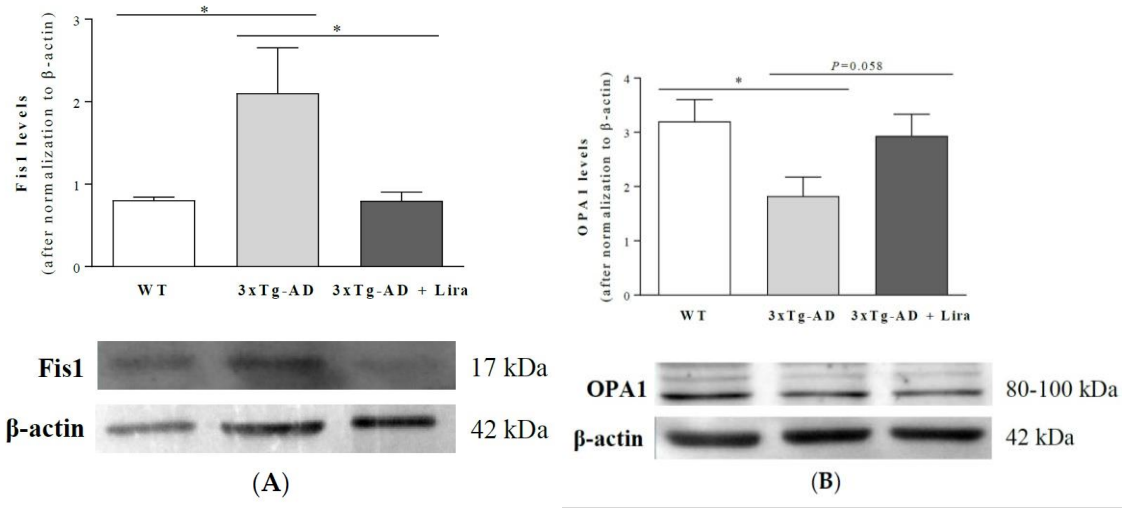


Figure 7.8 - Effect of liraglutide on brain cortical mitochondrial fission/fusion markers in female mice with early AD-like pathology. Brain cortical Fis1 (**A**) and OPA1 protein levels (**B**) were determined and normalized to β-actin levels, and representative Western blotting

images displayed. Data are the mean ± SE from 6 mice/group. Statistical significance: * $p < 0.05$, by the one-way ANOVA with the Fisher LSD post-hoc test for multiple comparisons.

These results suggest that peripheral treatment with liraglutide partially attenuated the dysfunctional brain mitochondrial fission/fusion machinery in female mice with early AD-like pathology.

7.5 - DISCUSSION

To the best of our knowledge, this study constitutes a first support to the use of GLP-1 mimetics (namely liraglutide) to mitigate some of the earlier AD-like pathological features in mature females. Contrary to our previous study in 11-month-old 3xTg-AD male mice that showed increased brain cortical and hippocampal Aβ levels and thigmotaxis, reduced exploratory activity, and deficits in learning and memory (Carvalho *et al.*, 2013), in the present study the massive rise in brain cortical Aβ and p-tau content in 11-month-old 3xTg-AD female mice (in line with the *Amyloid Cascade Hypothesis*—the basis for this mouse model) was accompanied by less pronounced signs of cognitive alterations. Liraglutide treatment only attenuated their increased brain $A\beta_{1-42}$ levels. This was accompanied by a slight reduction in their plasma and brain inflammatory markers upon liraglutide administration, which also tended to normalize estradiol and GLP-1 content, and PKA-mediated downstream signaling in female mice with early AD-like pathology. Interestingly, liraglutide partially mitigated their brain oxidative stress markers, possibly via the stimulation of G6PDH (and its downstream antioxidant properties) and by altering mitochondrial dynamics, ultimately rescuing the AD-like neuropathology in mature female mice.

Liraglutide administration attenuated memory deficits, Aβ plaques and oligomers, synaptic and tau pathology in APP/PS1 mice (McClean *et al.*, 2011) and in non-human primates infused with Aβ oligomers into the lateral cerebral ventricle (Batista *et al.*, 2018). The drug also mitigated the cognitive deficits and cerebral p-tau in diabetic rodents (Xiong *et al.*, 2013; Yang *et al.*, 2013). However, others failed to observe a significant effect of chronic liraglutide treatment on cerebral Aβ plaque formation in two transgenic APP/PS1 mouse models with low and high grade of amyloidosis (Hansen *et al.*, 2016). This suggested that distinct animal models for AD may display

distinct sensitivities to liraglutide treatment (Hansen *et al.*, 2016) . Indeed, a recent study demonstrated that a 2-week administration of liraglutide decreased memory deficits, p-tau and Aβ overproduction, and increased dendritic spines' density and synaptic proteins upon hyperhomocysteinemia (Zhang *et al.*, 2019). In this respect, liraglutide injection for 4 weeks only mitigated the brain $A\beta_{1–42}$ levels, without significantly affecting the $A\beta_{1–40}$ or p-tau(Ser396) (a known intermediary phosphorylated residue in AD pathology (Augustinack *et al.*, 2002; Hoffmann *et al.*, 1997)) in 3xTg-AD female mice with early AD-like pathology, which also presented less pronounced signs of motor, cognitive or synaptic defects (data not shown) (contrary to the previous observations of impaired motor activity and learning/memory in 3xTg-AD male mice (Chen *et al.*, 2017; Carvalho *et al.*, 2012)). This corroborates the slight delay in the onset of AD-like pathology in 3xTg-AD female mice described by Belfiore *et al.* (Belfiore *et al.*, 2019), together with the notion of a sexual dimorphism in the susceptibility to AD neuropathology, cognitive dysfunction and changes in brain energy metabolism under neuropathological conditions (Duarte *et al.*, 2018a; Yang *et al.*, 2018; Candeias *et al.*, 2017; Valencak *et al.*, 2017) (including the persistently lower metabolic brain age in women across their life span compared to men (Goyal *et al.*, 2019)). Since Yan *et al.* (Yan *et al.*, 2019) observed that peripheral 17β-estradiol treatment activates the estrogen receptor α and the downstream PI3K/Akt/Foxo1 signaling, recovering insulin sensitivity and glucose metabolism, one cannot exclude a role for the increased brain estradiol levels in this delay in AD-like neuropathology in 3xTg-AD female mice (as further discussed by Yang *et al.* (Yang *et al.*, 2018)). Accordingly, Yang *et al.* (Yang *et al.*, 2018) found that chronic 17β-estradiol administration to ovariectomized 3xTg-AD female mice recovered their spatial learning and memory, partially due to the recovery of PKA-CREB and downregulation of the p38-MAPK signaling. Hippocampal 17β-estradiol induced the release of glutamate from astrocytes, stimulating neuronal glutamate receptors, thereby modulating dendritic spine density and growth, and synapse formation and plasticity in developing and adult central nervous system (Haraguchi *et al.*, 2012; Dave *et al.*, 2010). Besides estradiol, the increased brain levels of GLP-1 in female mice with AD-like pathology may constitute an adaptive mechanism to delay the negative effects of less active PKA (its activation by hormones or neurotransmitters in multiple brain regions was shown to regulate feeding, energy expenditure and glucose homeostasis (Yang, 2018; Gejl *et al.*, 2013; Gejl *et al.*, 2012)). In line with this and with previous studies in

AD patients and rodent models (including mature 3xTg-AD male mice) (Carvalho *et al.*, 2013; Cova *et al.*, 2016), our female mice with early AD-like pathology had lower body weight that, contrary to other animal models (Duarte *et al.*, 2018b; Hansen *et al.*, 2015), was not recovered by liraglutide treatment.

The delay in AD-like neuropathology in our female 3xTg-AD mice is further supported by their apparently unaltered peripheral glucose metabolism and insulin sensitivity, in contrast with previously studied mature 3xTg-AD male mice (Carvalho *et al.*, 2012). Although it is well-known that metabolic disorders (such as insulin resistance, T2D and/or obesity) increase the risk for AD (Serrano-Pozo *et al.*, 2011; Carvalho *et al.*, 2012; Rollins *et al.*, 2019; Cardoso *et al.*, 2017a; Ott *et al.*, 1999; Leibson *et al.*, 1997), the opposite (*i.e.*, AD-induced peripheral glucose dysmetabolism and insulin insensitivity) remains a matter of debate (Morris *et al.*, 2018; Kilander *et al.*, 1993). This does not invalidate the repurpose of anti-type 2 diabetes drugs to prevent or delay AD progression. Indeed, increasing evidence demonstrates the beneficial effects of, *e.g.*, GLP-1 mimetics (including liraglutide) against AD (Camkurt *et al.*, 2018; Loera-Valencia *et al.*, 2019; Duarte *et al.*, 2018a). Among them, we emphasize the liraglutide-induced recovery of brain glucose metabolism (whose changes may start before the onset of brain atrophy and neurodegeneration) (Femminella *et al.*, 2019; Patching, 2017; Liu *et al.*, 2011; Nordberg *et al.*, 2010; Liu *et al.*, 2009; Liu *et al.*, 2008; Mosconi *et al.*, 2008). Although the precise nature of such metabolic improvement remains unknown, evidence suggests a role for the recovered neurovascular unit (involving a NF-κB-induced balance between the vasoconstrictor endothelin-1 and the vasodilator endothelial nitric oxide synthase (eNOS)) (Wicinski *et al.*, 2018b; Wicinski *et al.*, 2018a) and the normalization of (cerebral) blood flow on the increment of GLUTs levels and/or function (their loss, particularly of those at the blood-brain barrier, like GLUT1 and, to a lesser extent, GLUT4, constitutes an early event in AD pathology) (Gejl *et al.*, 2017; Winkler *et al.*, 2015). In addition, liraglutide-induced slowdown in brain glucose clearance may aid in the brain recovery of glucose uptake and/or metabolism (as our observations appear to partially confirm), ultimately, in improved cognitive performance (Gejl *et al.*, 2017; Liu *et al.*, 2008; Garcia-Caceres *et al.*, 2016; Hernandez-Garzon *et al.*, 2016; Jais *et al.*, 2016; Xiao-Yun *et al.*, 2011; Madadi *et al.*, 2008). However, others described that the tendentious increase in brain glucose metabolism induced by liraglutide upon AD was not accompanied by a rescue in cognitive function (Loera-Valencia *et al.*, 2019; Gejl *et al.*, 2016). Hopefully, this

apparent discrepancy will be clarified by a phase IIb trial involving the treatment of AD individuals with very mild dementia with liraglutide for 12-month (the ELAD trial) (Femminella *et al.*, 2019).

Oxidative/nitrosative stress and inflammation have been also widely demonstrated at the periphery (Lai *et al.*, 2017; Ramamoorthy *et al.*, 2012; Swardfager *et al.*, 2010; Moreira *et al.*, 2007a) and in brains (Nunomura *et al.*, 2001; Yao *et al.*, 2009; Cenini *et al.*, 2008; Calabrese *et al.*, 2006) of human subjects and rodent models of AD (Yang *et al.*, 2018; Resende *et al.*, 2008; Baker *et al.*, 2018; Choi *et al.*, 2018). Several authors suggested that impaired redox status, Aβ deposition, neurofibrillary tangles and neuronal damage (Placido *et al.*, 2015; O'Connor *et al.*, 2008) play a key role in AD pathogenesis, most likely by activating microglia and inflammation-mediated neurotoxicity (Herrup, 2010; Perry *et al.*, 2010). Accordingly, our female mice with early AD-like pathology had increased oxidative stress and serum and brain CRP and IL-1β levels. Indeed, high IL-1β levels occurred in AD patients and in mild cognitive impaired subjects (Forlenza *et al.*, 2009; Shaftel *et al.*, 2008), and activated microglia and astrocytes were recently correlated with the levels of hippocampal Aβ and p-tau, and the severity of AD pathology in 3xTg-AD mice (Yang *et al.*, 2018). This hippocampal Tau hyperphosphorylation may arise from an upregulation of the p-38-MAPK cascade in AD, while the downregulation of cAMP-PKA-CREB signaling (as partially observed in Table 7.II) may impair synaptic plasticity and memory formation (Yang *et al.*, 2018). Importantly, the role of the anti-inflammatory cytokine IL-10 in AD brain remains controversial, since recent studies in APP mice suggested that it may inhibit microglial Aβ clearance, promoting Aβ plaque generation and cognitive impairment (rather than delaying AD progression) (Chakrabarty *et al.*, 2015). Furthermore, brain immunity was improved in IL-10-deficient APP mice that also showed lower cerebral amyloidosis (Guillot-Sestier *et al.*, 2015). Hence, the increased brain IL-10 content in female mice with early AD-like pathology appears to precede their typical behavioral deficits, possibly exacerbating the brain damage elicited by IL-1β, CRP and oxidative/nitrosative stress and allowing AD progression. In line with previous studies (McClean *et al.*, 2011; He *et al.*, 2018), liraglutide partially mitigated brain oxidative stress and inflammation markers in female mice with early AD-like pathology.

Similar to liraglutide's anti-inflammatory mechanisms, those underlying its anti-oxidative stress properties remain poorly understood. These may involve the activation

of Akt and eNOS, with the subsequent stimulation of antioxidant defenses (*e.g.*, glutathione, catalase, superoxide dismutase) and reduction of reactive oxygen species (ROS) formation, as observed in ischemic stroke (Wicinski *et al.*, 2019; Shiraki *et al.*, 2012). Despite no significant alterations in active Akt in our conditions, one cannot exclude the involvement of the parallel MAPK/ERK signaling cascade (Wicinski *et al.*, 2019), known to mediate its antioxidant, anti-inflammatory, anti-apoptotic and pro-cognition roles (Han *et al.*, 2016; Zhu *et al.*, 2016; Zhou *et al.*, 2015a; Briyal *et al.*, 2014; Hamamoto *et al.*, 2013; Sato *et al.*, 2013; Talbot *et al.*, 2012; Zhang *et al.*, 2015b), as well as its benefits in AD symptoms and features (Femminella *et al.*, 2019). Liraglutide-mediated NF-κB inhibition and Sirt1 may also recover mitochondrial membrane integrity and complex I activity, improving mitochondrial function (as reported in epilepsy, ischemia or toxin exposure) (Wicinski *et al.*, 2019; Camkurt *et al.*, 2018; Jalewa *et al.*, 2016; Wang *et al.*, 2018; Ji *et al.*, 2016; Li *et al.*, 2016b; Zhang *et al.*, 2016b; Sharma *et al.*, 2014; Velmurugan *et al.*, 2012; Lozano *et al.*, 2009), and further protecting against oxidative stress (Wicinski *et al.*, 2019; Briyal *et al.*, 2014; He *et al.*, 2020; Tong *et al.*, 2016), which may also rely on the inhibition of myeloperoxidase (via Nrf2/heme oxygenase-1 downregulation of NADPH oxidase or PKCα membrane translocation, as reported in diabetic and stroke brain) (Deng *et al.*, 2018). Importantly, the lower G6PDH activity (a pivotal enzyme from the oxidative branch of the pentose phosphate pathway also involved in the regulation of nicotinamide adenine dinucleotide phosphate (NADPH) and of the key antioxidant reduced glutathione, GSH) observed in brains from female mice with early AD-like pathology further support an increased oxidative stress, in agreement with the G6PDH inhibition in *postmortem* hippocampal regions (Bigl *et al.*, 1999) and prefrontal cortex synaptosomes (Ansari and Scheff, 2010) from AD human subjects. The liraglutide-mediated increase in G6PDH activity and decreased pyruvate levels in mature female mice with early AD-like pathology suggest that its antioxidant effects may involve the stimulation of the oxidative branch of the pentose phosphate pathway (rather than glycolysis) and/or a decrement in p62 levels. Since the liraglutide-induced changes in this stress-inducible protein were not accompanied by alterations in other autophagy markers (p62 is mostly known as a cargo receptor for the lysosomal-mediated autophagy degradation of detrimental and unnecessary components), we hypothesize that p62 may alternatively account for liraglutide's anti-oxidative stress or anti-inflammatory properties. Indeed, p62 was recently associated with Nrf2, mTOR

Complex 1 (mTORC1) and NF-κB signaling pathways and their role in oxidative stress, nutrient sensing and inflammation (Sanchez-Martin *et al.*, 2019). Besides the liraglutide's anti-inflammatory mechanisms discussed above, NF-κB inhibition was also found to reduce TNFα, IL-1β and IL-6 levels, and activated microglia and astrocytes (Gault and Holscher, 2008; Wang *et al.*, 2018; Dai *et al.*, 2013; McClean and Holscher, 2014; Parthsarathy and Holscher, 2013b; Barreto-Vianna *et al.*, 2017), while the downregulation of JNK and phosphorylated p38, and the consequent inhibition of caspases-8 and -3, may account for its anti-apoptotic actions (Wicinski *et al.*, 2019; Zhu *et al.*, 2016; Gao *et al.*, 2015; Wu *et al.*, 2015).

The increased Fis1 and decreased OPA1 levels in female mice with early AD-like pathology suggest a dysregulation in brain mitochondrial fission/fusion machinery, namely the promotion of fission and the impairment of fusion processes (Tian *et al.*, 2014; Alavi and Fuhrmann, 2013; Palmer *et al.*, 2013), respectively. OPA1 at the mitochondrial inner membrane is also involved, *e.g.*, in the maintenance of mitochondrial respiratory chain and membrane potential (Olichon *et al.*, 2003), cristae organization, mitochondrial DNA and apoptosis regulation (Elachouri *et al.*, 2011; Amati-Bonneau *et al.*, 2008; Hudson *et al.*, 2008), whereas Fis1 can also regulate the size and distribution of mitochondria in response to the local demand for ATP or calcium (Lees *et al.*, 2012). Hence, changes in brain OPA1 and Fis1 levels in female mice with early AD-like pathology may elicit alternative damaging mechanisms that were partially reversed by liraglutide.

Although not studied herein, the anti-amyloidogenic/tauogenic effects of liraglutide may also rely on the PI3K/MAPK/cAMP/PKA-mediated activation of brain insulin degrading enzyme (IDE) and/or the upregulation of Aβ transporters to promote Aβ trafficking and proteolytic degradation (Li *et al.*, 2018a; Li *et al.*, 2018b; Costa *et al.*, 2008a; Costa *et al.*, 2008b; Carro and Torres-Aleman, 2006; Carro *et al.*, 2006); on the inactive caspase-3-mediated blunt of neurofibrillary tangle formation (Wicinski *et al.*, 2019; Padurariu *et al.*, 2012; Rissman *et al.*, 2004; Ayala-Grosso *et al.*, 2002); on the regulation of brain neurotransmission (*e.g.*, GABAergic and glutamatergic) (Koshal and Kumar, 2016b; Koshal and Kumar, 2016a; McClean *et al.*, 2010; Babateen *et al.*, 2017; Gupta *et al.*, 2017; Gilman *et al.*, 2003), thus promoting synaptic plasticity; on the improvement of axonal sprouting and neurite outgrowth (He *et al.*, 2020; Li *et al.*, 2015b; Ma *et al.*, 2017b; Meier, 2012); and/or on increased neurogenesis (De Felice and Ferreira, 2014; Han *et al.*, 2013; McClean and Holscher, 2014; Wicinski *et al.*,

2017; Parthsarathy and Holscher, 2013a; Salcedo *et al.*, 2012), ultimately contributing to (AD) brain repair and cognitive function (He *et al.*, 2020; Dong *et al.*, 2017; Briyal *et al.*, 2012; Schaar *et al.*, 2010). Finally, in spite of the apparent lack of changes in the present study, we cannot underestimate the indirect peripheral effects of liraglutide in restoring insulin action and glucose homeostasis, as well as in blood pressure, body weight and lipid profiles (Gault and Holscher, 2008; Han *et al.*, 2013; McClean *et al.*, 2010; Gentilella *et al.*, 2019; Lin *et al.*, 2015; Salehi *et al.*, 2010; Onoviran *et al.*, 2019; Harkavyi *et al.*, 2008; Armstrong *et al.*, 2016; Blackman *et al.*, 2016; Simo *et al.*, 2015; Sun *et al.*, 2015).

Altogether, our results constitute a first approach to disentangle the complex puzzle underlying the use of the GLP-1 mimetic liraglutide as a potential preventive/therapeutic agent against some of the earlier AD-like pathological signs in female mice. Although further studies are needed (particularly in rodent models displaying risk factors for sporadic AD, including aging or diabetes), the different patterns in AD-related pathology between males and females and their response to medicines also reinforce the need for a more tailormade, sex/gender-based medicine.

Chapter 8

General Conclusion

8.1 - GENERAL CONCLUSION

The results presented in this Thesis uncover different pathological processes of T2D-associated neurodegeneration and neurodegenerative diseases. Altogether, with this work we were able to clarify: 1) the distinct susceptibility of middle-aged T2D females and males to develop AD-like pathology; 2) the signaling pathways, such as GLP-1/IGF-1 and autophagy, which mediate the protection of the anti-diabetic Ex-4 in T2D brain; 3) the positive impact of Ex-4 therapy on glucose transport and on the energetic status in the T2D brain; 4) the neuroprotective effects of the anti-diabetic linagliptin in the striatal pathway during aging and/or HFD-induced T2D; 5) the protection of the anti-diabetic liraglutide in females with early AD-like pathology.

By studying the brain cortices of middle-aged male and female T2D GK rats we cemented the evidence of the progression of T2D pathophysiology as a risk factor of AD, as well as some common signaling cascades between the two conditions. Moreover, the observed differential sex steroid hormones profiles/action in CNS suggests that in middle-aged females the brain steroid hormonal changes may precede those at the periphery. Nevertheless, brain cortices from female cohorts may also develop compensatory mechanisms through the maintenance of ER, IGF-1R and IR function, and of the downstream Akt- and ERK1/2-mediated signaling. This compensation may delay the deleterious brain changes associated with T2D, which include oxidative damage to lipids and DNA, amyloidogenic processing of amyloid precursor protein and increased tau protein phosphorylation, ultimately protecting the middle-aged perimenopausal T2D females (Candeias *et al.*, 2017) (Chapter 3).

By using the less protected middle-aged male GK rats (whose brain cortical alterations were more exacerbated and, as such, the beneficial impact of therapies could be more evident), we then explored the effects of a chronic, continuous and subcutaneous administration of Ex-4 to demonstrate its promising therapeutic potential against the chronic complications of T2D affecting the brain. The typical peripheral hallmarks of T2D were successfully rescued by Ex-4 therapy in middle-aged male GK rats, including the normalization in fasted and occasional glucose levels, HOMA-IR, HOMA-β, HbA1c levels, ipGTT and heart rate. This suggests that Ex-4 initiated a peripheral protection against T2D, most likely via an insulinotropic response and consequent attenuation of insulin resistance and of the abnormal glucose regulation. Additionally, we reported that peripheral Ex-4 counteracted the alterations in T2D brain

cortical GLP-1 and IGF-1 levels, with the subsequent stimulation of their downstream signaling cascades (namely the activity of PKA, the levels of the p110 (catalytic) subunit of the PI3K protein, the GSK-3β activation by phosphorylation at Tyr216 and the modulation of intracellular stress signaling through JNK activation and AMPK levels). As a consequence, we demonstrated that peripherally-administered Ex-4 stimulated brain cortical autophagy and inhibited cell death mechanisms in T2D rats (Candeias *et al.*, 2018) (Chapter 4).

In parallel, we demonstrated that the effect of peripheral Ex-4 therapy in middle-aged male GK rats further protected against their central metabolic dysfunction. This may involve the stimulation of several GLUT, SGLT and MCT isoforms, thus promoting brain glucose uptake and metabolism. More specifically, peripheral treatment with Ex-4 partially rescued the glycolytic metabolism, activated the pentose phosphate pathway, the TCA cycle and the formation of amino acid precursors that fuel this cycle and constitute pivotal neurotransmitters within middle-aged T2D male rat brains. Such benefits of subcutaneous Ex-4 were further accompanied by the improvement of brain mitochondrial respiratory chain activity and energy production, and by the activation of mitochondrial fusion mechanisms in middle-aged male GK rat brains. We also demonstrated that Ex-4 modulated their purines metabolism (by boosting the levels of adenosine and inosine), increased in the activity of Na^+/K^+ ATPase and rescued their shift to ketone bodies' metabolism (Manuscript in preparation) (Chapter 5).

By using young and middle-aged male C57BL/6 mice, as well as a middle-aged male mice with diet-induced obese T2D, we next demonstrated that: 1) the structural alterations in the nigrostriatal dopaminergic system (namely the loss of PV+ interneurons and the increase of GFAP+ and Iba-1+ cells in striatum) were dependent of aging; 2) equal effects were observed in T2D induced by chronic HFD diet in 14-months-old mice compared to the middle-aged C57BL/6 mice, but further impairments in the release of dopamine in T2D animals suggest that T2D is a key negative regulator of the sensorimotor function and a potential facilitator of an early pathophysiological impairment of the nigrostriatal dopaminergic system; 3) the chronic treatment with the DPP-4i linagliptin mitigated the glial alterations and the reduced basal and amphetamine-stimulated striatal extracellular dopamine in T2D mice (Lietzau *et al.*, 2020) (Chapter 6).

Finally, during the analysis of the effect of peripheral administration of the GLP-1RA liraglutide in a mature female model of the 3xTg-AD with early neuropathological features of AD, we first demonstrated that the saline-treated female mice presented less pronounced signs of AD (namely motor, cognitive and synaptic defects) than age-matched male cohorts used in previous studies from our and other's laboratories. This reinforces the existence of a sexual dimorphism in the susceptibility to AD neuropathology. Additionally, the chronic subcutaneous administration of liraglutide, partially prevented the earlier AD-like pathological features in mature female mice, namely their increased plasma IL-10 and IL-1β levels and brain cortical CRP and IL-10 levels. These were accompanied by a normalization in brain cortical: key neuropathological hallmark of AD ($A\beta_{1-42}$ levels); in GLP-1 levels and downstream signaling; in estradiol levels; in glucose metabolism (namely the activity of G6PDH and pyruvate levels); in the markers for oxidative/nitrosative stress (such as 8-OHdG content, TBARS, carbonyl groups and nitrites levels) and for mitochondrial dynamics (Fis1 and OPA1 levels) (Duarte *et al.*, 2020) (Chapter 7).

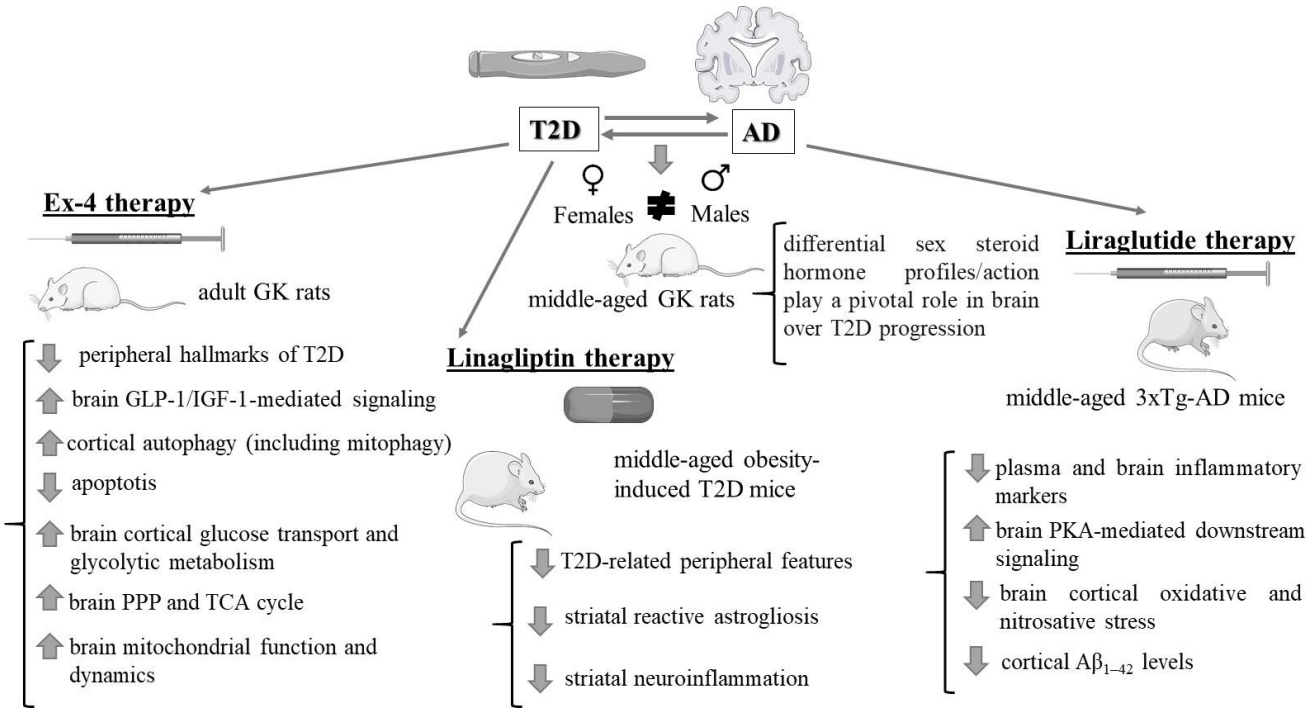


Figure 8.1 - Overview of the general conclusions of this thesis. Our studies revealed the different brain cortical susceptibility of middle-aged males and females in the progression of

type 2 diabetes (T2D) and the risk to develop Alzheimer disease (AD)-like pathology. The peripheral administration of the GLP-1R agonist exendin-4 (Ex-4) to the more vulnerable male Goto-Kakizaki (GK) T2D rat at midlife rescued the peripheral hallmarks of T2D, stimulated their brain cortical GLP-1/IGF-1-mediated signaling and autophagic mechanisms (including mitophagy), protecting against apoptosis. Ex-4 also rescued their brain cortical glucose transport and glycolysis, stimulated the pentose phosphate pathway (PPP) and tricarboxylic acid (TCA) cycle, improving mitochondrial function and dynamics.

Orally-administered DPP-4i linagliptin to a middle-aged male mouse with diet-induced obese T2D attenuated their T2D peripheral features, and reduced striatal reactive astrogliosis and neuroinflammation.

The chronic, peripheral administration of liraglutide to mature female 3xTg-AD mice reduced their plasma and brain inflammatory markers, activated brain PKA-mediated downstream signaling, mitigated oxidative/nitrosative stress and $A\beta_{1-42}$ levels.

GLP-1R: Glucagon-like peptide-1 receptor agonist; IGF-1: Insulin-like growth factor-1; DPP-4i: Dipeptidyl peptidase-4 inhibitor; PKA: Protein kinase A.

To sum up, our studies reinforce the association between T2D progression and its impact on cognition/memory, sensorimotor functions and the role as a risk factor for the development of neurodegenerative diseases, such as AD and PD. We also demonstrate the therapeutic potential of clinically used anti-T2D therapies, such as GLP-1 mimetics (Ex-4 and liraglutide) and DPP-4i (linagliptin) against peripheral T2D features and the CNS impairments associated with T2D, AD, PD and/or aging. Our studies also reinforce the urgent need of future investigations and clinical trials to establish a sex-specific time window for successful preventive measures in T2D, AD and aging-related neuropathology.

Chapter 9

References

9.1 - REFERENCES

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“Nobody is gonna hit as hard as life, but it ain’t how hard you can hit. It’s how hard you can get hit and keep moving forward. It’s how much you can take, and keep moving forward. That’s how winning is done.”

-Rocky Balboa

“Somewhere, something incredible is waiting to be known.”

-Carl Sagan