UNIVERSIDADE D
COIMBRA

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Effect of Dual Therapy with Liraglutide and Ghrelin on Brain Metabolism and Intracellular Stress in the Huntington´s Disease R6/2 Mouse Model

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

June 2019

Department of Life Sciences

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

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

Acknowledgements

I would like to thank the CNC - Center for Neuroscience and Cell Biology for providing me the technical facilities to perform the work presented in this Dissertation, and to the University of Coimbra for giving me the opportunity to continue my academic education at the Master in Cellular and Molecular Biology.

The realization of this work would not have been possible without the kind support and participation of several people that contributed in the most diverse forms.

I would like to express my gratitude to Doctor Ana I. Duarte for guiding this work and Thesis. I would also like to thank all her patience, availability, help and knowledge provided during this work.

I would like to thank Professor António Moreno for all the help and knowledge transmitted, especially in the measurement of mitochondrial respiratory chain enzymatic activities.

I would also like to acknowledge Professor Paula I. Moreira for the opportunity to perform the work that culminated in this Dissertation at her laboratory.

I would also like to thank all the members of the group *Metabolism, Mitochondria and Hormones in Brain Disorders* for all the support given during this year.

I would also like to acknowledge all technical staff from the CNC for frequently make my life easier during the experimental part of this work.

A special thanks also to the research and technical team from the *Brain Disease Biomarker Unit*, Lund Medical School, Lund University (Lund, Sweden), especially to Professor Maria Björqkvist and Dr. Marie Sjögren for all the scientific and technical support, at the distance of an e-mail or sms.

I leave a special thank to my colleague Maria Inês Alves for all the help and support. Without her, this work would not have been the same. She was always available to help me when I needed it, making the experience of co-working rewarding.

I also leave a very special thanks to my parents, Adélia and Pedro, and to my brother Hugo. In particular, I want to acknowledge my mother for all the support, dedication and effort.

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

Resumo

A doença de Huntington é uma doença neurodegenerativa rara, autossómica dominante, caracterizada pela expansão da repetição citosina-adenina-guanina no exão 1 do gene da huntingtina, resultando na formação da proteína huntingtina mutante. A doença de Huntington permanece incurável e sem prevenção, devido à ausência de medicamentos e biomarcadores cronicamente eficazes. Contudo, a existência de vias moleculares comuns entre a doença de Huntington e a diabetes tipo 2 (nomeadamente as alterações periféricas e cerebrais do metabolismo da glucose) sugere que, terapias eficientes no tratamento da diabetes tipo 2 podem sê-lo também no tratamento da doença de Huntington.

Desta forma, e na sequência de um estudo recente realizado no nosso laboratório, colocámos a hipótese de a co-administração periférica, crónica, de liraglutida (fármaco utilizado no tratamento da diabetes tipo 2 com efeitos neuroprotetores) e a grelina (hormona orexigénica que estimula o apetite e afeta o metabolismo periférico) recupera a função metabólica cerebral e protege contra o stress intracelular na doença de Huntington. Assim, no presente estudo pretendemos determinar os mecanismos moleculares subjacentes à neuroproteção mediada pela administração de liraglutida e grelina, sozinhas ou em conjunto, contra a disfunção metabólica cerebral e o stress intracelular associados à doença de Huntington, num modelo animal da patologia (o murganho R6/2).

Murganhos R6/2 com 9 semanas de idade, numa fase precoce da doença de Huntington, foram injetados por via subcutânea com liraglutida e/ou grelina, diariamente, durante 2 semanas. Seguidamente, avaliou-se o efeito da liraglutida e grelina em conjunto ou em separado nas vias metabólicas associadas à glucose e mitocôndria no córtex cerebral destes animais, mediante a utilização de técnicas de colorimetria e fluorimetria. Finalmente, analisou-se o efeito liraglutida e/ou grelina em marcadores de stress oxidativo/nitrosativo, através da utilização de técnicas colorimétricas e de ELISA.

A administração subcutânea da grelina *per se* ou em combinação com a liraglutida promoveu o metabolismo da glucose por via das pentoses fosfato e do ciclo do ácido tricarboxílico, bem como o aumento da formação de ATP através do sistema fosfocreatina/creatina cinase no córtex cerebral de murganhos R6/2 numa fase precoce da doença de Huntington. Além disso, a administração de grelina *per se* ou em conjunto com a liraglutida desencadeou mecanismos protetores contra o stress intracelular, traduzidos

por uma ligeira redução da lesão oxidativa no DNA no córtex cerebral de murganhos R6/2. Finalmente, a liraglutida *per se* ou em conjunto com a grelina recuperou a atividade do complexo IV da cadeia respiratória mitocondrial e a produção de corpos cetónicos.

Em conclusão, estes resultados mostram que a co-administração de liraglutida e grelina tem efeitos cerebrais benéficos numa fase precoce da doença de Huntington, nomeadamente através da recuperação do metabolismo cortical da glucose e da estimulação da cetogénese e do sistema da fosfocreatina/creatina cinase, protegendo contra a lesão oxidativa do DNA e, em última instância, contra os efeitos adversos da doença de Huntington no cérebro. Assim, podemos especular que a co-administração de grelina e liraglutida numa fase precoce da doença de Huntington poderá constituir uma terapia promissora contra esta patologia.

Palavras-chave: Disfunção metabólica cerebral; doença de Huntington; liraglutida e grelina; murganhos R6/2; stress intracelular.

Abstract

Huntington's disease is a rare, autosomal dominant neurodegenerative disease, characterized by the expansion of the cytosine-adenine-guanine (CAG) repeat in the exon 1 of the huntingtin gene that results in a mutant huntingtin protein. Huntington's disease remains incurable and unpreventable, due to the lack of chronically effective drugs and biomarkers. However, Huntington's disease shares several molecular mechanisms with type 2 diabetes (namely peripheral and brain glucose dysmetabolism), suggesting that efficient anti-type 2 diabetes drugs may be also effective against Huntington's disease.

Following a recent study from our group, we hypothesized that chronic, peripheral co-administration of liraglutide (an anti-type 2 diabetes incretin drug with neuroprotective effects) and ghrelin (an orexigenic hormone that stimulates appetite and affects peripheral metabolism) recovers brain metabolic function and protects against intracellular stress upon Huntington's disease. Thus, in the present study we aimed to determine the molecular mechanisms underlying the neuroprotection mediated by the administration of liraglutide and/or ghrelin against Huntington's disease-associated brain metabolic dysfunction and intracellular stress in an animal model of the disease (the R6/2 mouse).

Early symptomatic, 9-week old R6/2 mice were daily, subcutaneously co-injected with liraglutide and/or ghrelin, for 2 weeks. The effect of liraglutide alone or together with ghrelin on the R6/2 mouse brain cortical glucose and mitochondrial metabolism were evaluated by colorimetric and fluorimetric techniques. Finally, we analyzed the effect of liraglutide and/or ghrelin on the R6/2 mouse brain cortical intracellular oxidative/nitrosative stress markers, by colorimetry and ELISA.

Subcutaneous administration of ghrelin *per se* or in combination with liraglutide promoted brain cortical glucose metabolism via the pentose phosphate pathway and tricarboxylic acid (TCA) cycle, and increased ATP formation through the phosphocreatine/creatine kinase system in early symptomatic R6/2 mice. Furthermore, ghrelin alone or combined with liraglutide promoted protective mechanisms against their brain cortical DNA oxidation. Finally, liraglutide *per se* or together with ghrelin recovered brain cortical mitochondrial respiratory chain complex IV activity and ketone bodies' production in early symptomatic R6/2 mice.

Altogether, the results indicate that the co-administration of liraglutide and ghrelin may benefit brain cortex during early symptomatic Huntington's disease, namely by promoting glucose metabolism, ketogenesis and phosphocreatine/creatine kinase system,

protecting against DNA oxidative damage and, ultimately, against Huntington's disease brain adverse effects. In this perspective, we can speculate that co-administration of ghrelin and liraglutide during an early stage of Huntington's disease may constitute a promising therapeutic approach against its pathology.

Keywords: Brain metabolic dysfunction; Huntington's disease; intracellular stress; liraglutide and ghrelin; R6/2 mice.

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3-NP	3-nitropropionic acid
3-NT	3-nitrotyrosine
8-OHdG	8-hydroxy- 2'-deoxyguanosine
α-KGDH	α-ketoglutarate dehydrogenase
$\Delta\Psi_m$	mitochondrial membrane potential
AcAc	acetoacetic acid
Acetyl-CoA	acetyl-coenzyme A
ACO	aconitase
ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-monophosphate
AMPK	5' AMP-activated protein kinase
AREs	antioxidant response elements
ATP	adenosine 5'-triphosphate
BBB	blood-brain barrier
BB-CK	creatine kinase brain isoenzyme
BDNF	brain-derived neurotrophic factor
BOH	3-hydroxybutyric acid
BSA	bovine serum albumin
Ca^{2+}	calcium ion
CAG	cytosine-adenine-guanine
cAMP	cyclic adenosine monophosphate
CaMKKβ	Ca^{2+}/calmodulin-dependent protein kinase kinase β
CNC	Center for Neuroscience and Cell Biology
CNS	central nervous system
CoA-SH	coenzyme A
CoQ_{10}	coenzyme Q10
CREB	cAMP response element binding protein
CS	citrate synthase
Cytc	cytochrome c
DCPIP	2,6-dichlorophenolindophenol
DNTB	5,5′-dithiobis 2-nitrobenzoic acid
DTT	dithiotreitol
EDTA	ethylenediamine tetraacetic acid
EGTA	ethylene glycol tetraacetic acid
$FADH_2$	flavin adenine dinucleotide

FUM	fumarase
GABA	γ-aminobutyric acid
G6PDH	glucose 6-phosphate dehydrogenase
GLP-1	glucagon-like peptide-1
GLUT	glucose transporter
HBDH	3-hydroxybutyrate dehydrogenase
HD	Huntington's disease
HO-1	heme oxygenase-1
Htt	huntingtin
IDH	isocitrate dehydrogenase
MDA	malondialdehyde
MDH	malate dehydrogenase
mtDNA	mitochondrial DNA
mHtt	mutant huntingtin
nDNA	nuclear DNA
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NAFLD	non-alcoholic fatty liver disease
NO_2^-	nitrite anion
NQO1	NAD(P)H:quinone oxidoreductase 1
Nrf-2	nuclear factor erythroid 2-related factor 2
O_2	molecular oxygen
$O_2^{\cdot -}$	superoxide anion
PDH	pyruvate dehydrogenase
PET	positron-emission tomography
PKA	protein kinase A
P_i	inorganic phosphate
PI3K	phosphoinositide 3-kinase
PMSF	phenylmethylsulfonyl fluoride
polyQ	polyglutamine
ROS	reactive oxygen species
RNS	reactive nitrogen species
SCOT	succinyl-CoA:3-ketoacid coenzyme A transferase
SDH	succinate dehydrogenase
T2D	type 2 diabetes
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactive substances

TCA	trichloroacetic acid
TCA cycle	tricarboxylic acid cycle
TEA	triethanolamine
TNB	5-thio-2-nitrobenzoic acid
TPP	thiamine pyrophosphate
VMAT-2	vesicular monoamine transporter-2

Chapter 1

Introduction

1.1. Huntington's Disease

1.1.1. Etiology

Huntington's disease (HD), first described by George Hutington in 1872, is an autosomal dominant neurodegenerative disorder with age-dependent complete penetrance, characterized by the expansion of the cytosine-adenine-guanine (CAG) repeat in exon 1 of the huntingtin (Htt) gene [Bates *et al.,* 2015; McColgan *et al.,* 2018; Wexler *et al.,* 2016]. This expansion triggers the elongation of a polyglutamine (polyQ) region present at the *N*-terminal domain of the translated huntingtin protein (Htt), that has been traditionally correlated with a gain of toxic function and protein aggregation (*i.e.*, with the formation of intracellular inclusion bodies, one of the main pathological features of HD [Bates *et al.,* 2015; Labbadia & Morimoto, 2013; Ross *et al.,* 2011]). The repeat length is inversely correlated with the age of onset [Andrew *et al.,* 1993; Lee *et al.,* 2012; Stine *et al.,* 1993], with individuals bearing 40 or more CAG repeats developing clinical symptoms at midlife (usually between the ages of 30 and 50 years - adult onset of HD) [Labbadia & Morimoto, 2013; McColgan & Tabrizi, 2018; Nopoulos, 2016]. Once the clinical symptoms appear, HD progresses inevitably to death within 15 to 20 years [Labbadia & Morimoto, 2013; Ross *et al.,* 2011; Schulte *et al.,* 2011].

Despite some controversy, HD can be divided into three phases: the pre-symptomatic, the prodromal and the manifest phases [Bates *et al.,* 2015; Durr *et al.,* 2012; Ross *et al.,* 2014, 2011]. In the pre-symptomatic phase, patients do not present symptoms or signs of HD, while in the prodromal phase there is a subtle appearance of some symptoms (motors or non-motors) prior to diagnosis [Bates *et al.,* 2015; Durr *et al.,* 2012; Ross *et al.,* 2014]. This diagnosis is based on several factors, including family history of the disease, genetic test for the CAG expansion and neurological evaluation with the manifestation of motor symptoms [Bates *et al.,* 2015; Craufurd *et al.,* 2015; Paulsen, 2011]. Finally, manifest HD is characterized by a slow progression of motor and cognitive impairment [Bates *et al.,* 2015; Durr *et al.,* 2012; Ross *et al.,* 2014].

Clinical HD symptoms include neuropsychiatric, cognitive and motor abnormalities, with neuropsychiatric and cognitive symptoms often appearing prior to motor ones [Ghosh & Tabrizi, 2018; McColgan & Tabrizi, 2018; Roth, 2019]. The later include hyperkinesia (that starts at early stages) with the presence of involuntary movements (*e.g.,* chorea), while at later stages patients tend to become hypokinetic, with bradykinesia (slowness of voluntary movement) and dystonia (sustained or repetitive

muscle contractions) [McColgan & Tabrizi, 2018; Nopoulos, 2016; Roth, 2019]. Cognitive symptoms starting at early stages of HD include the impairment of executive function (such as difficulties in decision making, organization, planning, psychomotor speed, visual attention) that, as disease progresses, exacerbates cognitive deficits towards a subcortical dementia [Ghosh & Tabrizi, 2018; McColgan & Tabrizi, 2018; Nopoulos, 2016; Roth, 2019]. Neuropsychiatric symptoms of HD include irritability, aggression, anxiety, impulsivity, depression, obsessive, compulsive behaviors and apathy, being consistent with frontal lobe dysfunction [Ghosh & Tabrizi, 2018; Martinez-Horta *et al.*, 2016; Nopoulos, 2016; Teixeira *et al.*, 2016].

1.1.2. Epidemiology

HD is a rare neurodegenerative disease, with a higher prevalence in Western populations (10.6-13.7 individuals per 100 000) [Evans *et al.*, 2013; Fisher *et al.*, 2014; McColgan & Tabrizi, 2018] than in Asian [McColgan & Tabrizi, 2018; Pringsheim *et al.*, 2012; Rawlins *et al.*, 2016] or African populations [Baine *et al.*, 2016, 2013]. This may result from the higher frequency of the CAG repeat expansion in the two haplotypes (A1 and A2) that are more common in Western populations [Baine *et al.*, 2013; Warby *et al.*, 2009].

1.1.3. Pathogenesis

HD pathogenesis involves the dysfunction and death of striatal ϒ-aminobutyric acid-ergic (GABAergic) medium spiny neurons [Ehrlich, 2012; Morigaki & Goto, 2017; Nopoulos, 2016; Rikani *et al.*,2014] and, to a lesser degree, of cortical pyramidal neurons [Cudkowicz & Kowall,1990; Hedreen *et al.*,1991; Macdonald & Halliday ,2002; Nana *et al.*, 2014; Thu *et al.*, 2010]. Recent studies also showed the loss of parvalbumin striatal interneurons [Reiner *et al.*, 2013] and cortical interneurons in HD brains [Kim *et al.*, 2014; Mehrabi *et al.*, 2016], with the subsequent abnormalities in cortical function/activity [Estrada-Sánchez *et al.*, 2013; Neuner *et al.*, 2018; Nopoulos *et al.*, 2010; Orth *et al.*, 2010; Schippling *et al.*, 2009] and metabolism [Ciarmiello *et al.*, 2006; Kuwert *et al.*, 1990; Martin *et al.*, 1992; Sampedro *et al.*, 2019; Shin *et al.*, 2013] constituting early events in HD (even in the pre-symptomatic phase). Indeed, increasing evidence suggests that cortical atrophy may contribute to the onset, progression and clinical heterogeneity of HD [Estrada-Sánchez & Rebec, 2013; Mehrabi *et al.*, 2016; Nana *et al.*, 2014] and, therefore, different patterns of cortical thinning may be associated

with both cognitive and motor phenotypes [Mehrabi *et al.,* 2016; Nana *et al.,* 2014; Rosas *et al.,* 2008, 2006, 2005]. This may somehow contribute to the loss of the corticostriatal functional network- an early event in HD that correlates with its clinical progression [Bunner & Rebec, 2016; Plotkin & Surmeier, 2015; Rebec, 2018; Veldman & Yang, 2018]. However, it remains unknown whether such loss of corticostriatal connectivity arises from the mutant Htt (mHtt)-induced dysfunction of medium spiny neurons or from its effect on the cortical input [Bunner & Rebec, 2016; Plotkin & Surmeier, 2015; Rebec, 2018; Veldman & Yang, 2018].

Even the role of (m)Htt on HD pathogenesis (especially the neurodegenerative process) remains a matter of debate. On one hand, Htt is a large (348 kDa) and highly conserved protein [Guo *et al.,* 2018b; Saudou & Humbert, 2016], ubiquitously and extremely abundant in the central nervous system (CNS) and testis [Landwehrmeyer *et al.,* 1995; Li *et al.,* 1993; Saudou & Humbert, 2016; Strong *et al.,* 1993]. It is necessary for embryonic development [Duyao *et al.,* 1995; Woda *et al.,* 2005; Zeitlin *et al.,* 1995] and neurogenesis [Haremaki *et al* , 2015;White *et al.,* 1997], and its functions appear to depend on the subcellular location (*i.e.,* it regulates nuclear transcription [Kumar *et al.,* 2014; Moumné *et al.,* 2013; Saudou & Humbert, 2016], the cytosolic vesicle and organelle trafficking [Caviston & Holzbaur, 2009; Chang *et al.,* 2006; Saudou & Humbert, 2016], endocytosis [Borgonovo *et al.,* 2013; Saudou & Humbert, 2016; Velier *et al.,* 1998] and autophagy [Martin *et al.,* 2015; Rui *et al.,* 2015; Saudou & Humbert, 2016]). Htt also interacts with other subcellular organelles, including mitochondria [Panov *et al.,* 2002; Reddy, 2014; Rockabrand *et al.,* 2007; Yano *et al.,* 2014], Golgi complex [Rockabrand *et al.,* 2007] and endoplasmic reticulum [Atwal & Truant, 2008; Rockabrand *et al.,* 2007]. This led some authors to postulate a role for the loss of normal wild-type Htt function [Paine, 2015; Saudou & Humbert, 2016; Schulte & Littleton, 2011] and of its beneficial effects (such as brain derived neurotrophic factor (BDNF) production [Park, 2018; Xie *et al.,* 2010; Zuccato & Cattaneo, 2009; Zuccato *et al.,* 2003], vesicle trafficking [Paine, 2015; Saudou & Humbert, 2016; Schulte & Littleton, 2011], autophagy [Gelman *et al.,* 2015; Rui *et al.,* 2015; Saudou & Humbert, 2016]) in HD-related neurodegeneration. On the other hand, numerous evidence point towards a role for the gain of toxic function by mHtt [Gipson *et al.,* 2013; Paine, 2015; Schulte & Littleton, 2011] (including transcriptional dysregulation [Kumar *et al.,* 2014; Moumné *et al.,* 2013; Saudou & Humbert, 2016], mitochondrial dysfunction [Carmo *et al.,* 2018; Farshbaf & Ghaedi, 2017; Guedes-Dias *et al.,* 2016], brain and systemic energy deficits

[Dubinsky, 2017; Mochel & Haller 2011b], oxidative stress [Kumar & Ratan, 2016], and intracellular protein aggregation and formation of inclusion bodies, *e.g.*, in cytoplasm, nucleus) [Arrasate & Finkbeiner, 2012; Davies *et al.*, 1997; DiFiglia *et al.*, 1997]. Other authors suggest that HD-associated neurodegeneration may involve both the loss of Htt function and the gain of toxic function by mHtt [Paine, 2015; Schulte & Littleton, 2011]. To further intricate this issue, mHtt-containing inclusion bodies were traditionally faced as the main toxic species in HD pathogenesis. But while the controversy persists on the toxic *versus* protective nature of inclusion bodies, nowadays, the soluble and diffusible mHtt oligomers are assuming a leading role in HD cytotoxicity [Arrasate & Finkbeiner, 2012; Kim *et al.*, 2016; Mitra & Finkbeiner, 2008; Wong *et al.*, 2008].

1.2. HD and Metabolism

1.2.1. HD as a disorder of brain glucose energy metabolism

The brain is a high energy demanding organ, whose neurons require large amounts of ATP for, *e.g.*, synaptic transmission, axonal transport. Hence, changes in the main metabolic pathways for ATP synthesis (glycolysis, tricarboxylic acid (TCA) cycle and oxidative phosphorylation) are harmful to these cells [Costa & Scorrano, 2012; Farshbaf & Ghaedi, 2017; Magistretti & Allaman, 2015]. As such, it is not surprising that changes in brain glucose metabolism are a common feature in HD. Indeed, striatal and cortical glucose hypometabolism were widely detected in manifest HD patients [Ciarmiello *et al.*, 2006; Kuhl *et al.*, 1982; Kuwert *et al.*, 1990; Martin *et al.*, 1992] and associated with motor and cognitive impairment [Berent *et al.*, 1988; Gaura *et al.*, 2017; Herben-Dekker *et al.*, 2014; Young *et al.*, 1986]. More specifically, Shin *et al.* (2013) found that the hypometabolism in frontotemporal and parietal cortices was higher in early stage manifest HD patients with a faster progression and, therefore, could constitute a marker for the rapid progression of the disease. Further studies demonstrated that this cortical and striatal hypometabolism was already present in premanifest HD patients [Antonini *et al.*, 1996; Ciarmiello *et al.*, 2006; Kuhl *et al.*, 1985; López-Mora *et al.*, 2016; Mazziotta *et al.*, 1987], preceding the onset of HD clinical symptoms [Ciarmiello *et al.*, 2012,2006; López-Mora *et al.*, 2016]. Positron-emission tomography (PET) analysis revealed that the efficiency of glucose metabolism towards ATP was maximal in brains of HD patients

during the resting state [Powers *et al.*, 2007], whereas under brain activity the elevation of this metabolic efficiency (and ATP production) was attenuated or even abrogated [Lou *et al.*, 2016; Mochel *et al.*, 2012b]. Similar findings were reported in the striatum and cortex of symptomatic R6/2 mice for HD, whose oxidative phosphorylation was normal at basal state, but upon a maximal stress their mitochondrial spare energy capacity (that gives the ability of mitochondria to generate energy beyond the cell's basal needs) was decreased [Lou *et al*., 2016]. This may result from an inhibition of the metabolic flux from, e.g., glycolysis or TCA cycle [Lou *et al.*, 2016].

1.2.1.1. Brain glucose uptake and glycolytic metabolism in HD

Starting from the beginning - the uptake of glucose from periphery towards the CNS-, studies in HD patients at early stages of striatal degeneration (grade 1) showed that, despite the decline in brain glucose utilization, the densities of membrane glucose transporter-3 and -1 isoforms (GLUT3 and GLUT1, the main neuronal and astrocytic isoforms of glucose transporter, GLUT [Morea *et al.*, 2017]) were maintained [Gamberino & Brennan Jr, 1994]. Conversely, at later stages of striatal degeneration (grade 3) GLUT1 and -3 densities were decreased [Gamberino & Brennan Jr, 1994]. This was reinforced by the normal levels of GLUT3 in $HD^{140Q/140Q}$ primary cortical neurons [McClory et al., 2014], while a reduction occurred in HD striatal and cortical neurons [Li *et al.*, 2012; McClory *et al.*, 2014]. This suggests that less glucose is taken up by HD neurons, probably due to an impairment in GLUT3 trafficking to cell surface [Li *et al.*, 2012; McClory *et al.*, 2014]. In fact, the upregulation of the gene SLC2A3 encoding for GLUT3 was able to delay the age of onset in HD patients, probably by increasing their neuronal GLUT3 levels, further highlighting the role of GLUT3 in HD pathogenesis [Vittori *et al.*, 2014].

In line with the lower glucose uptake, several authors demonstrated an early decrease of striatal glycolysis in HD patients [Powers *et al.*, 2007] and in primary striatal neurons from transgenic BACHD mice [Gouarné *et al.*, 2013], prior to any defects in the mitochondrial respiratory chain. This could indicate an astrocytic dysfunction or even death, thus involving astrocytes in HD-related metabolic defects [Powers *et al.*, 2007]. Accordingly, co-culture of wild-type neurons or BACHD neurons expressing the full-length mHtt with BACHD astrocytes resulted in a reduction in glucose uptake that, nonetheless, did not occur in primary astrocytes from BACHD mice [Boussicault *et al.*, 2014]. Hence, astrocytes may be a source of adverse effects on neuronal energy

metabolism [Boussicault *et al.,* 2014]. Other evidence for the decreased metabolic flux through glycolysis include the inhibition of the glycolytic enzyme phosphofructokinase in *postmortem* striatum of HD patients [Bird *et al.,* 1977]. Furthermore, glyceraldehyde-3-phosphate dehydrogenase is another glycolytic enzyme that binds both normal and mHtt, but preferably to the cleaved polyQ domain [Burke *et al.,* 1996]. This aberrant mHtt-glyceraldehyde-3-phosphate dehydrogenase interaction may in turn inhibit the enzyme, as observed in posterior brain homogenates (*e.g.* from striatum, hippocampus, thalamus) from late symptomatic N171-82Q HD mice [Oláh *et al.,* 2008]. In addition, the levels of the neuron-specific glycolytic enzyme enolase were decreased in both R6/2 mouse cortex and striatum concomitantly with increased protein carbonylation [Lou *et al.,* 2016]. Besides this, oxidation (and inactivation) of both neuronal and non-neuronal forms of enolase occurred in *postmortem* striatum from HD patients and R6/2 transgenic mice [Sorolla *et al.,* 2012, 2010, 2008].

1.2.1.2. Brain TCA cycle in HD

Pyruvate is the final product of glycolysis and can be converted into lactate by the reversible action of lactate dehydrogenase [Riske *et al.,* 2017] or metabolized by mitochondria towards acetyl-coenzyme A (acetil-CoA) and nicotinamide adenine dinucleotide (NADH), in a reaction catalyzed by pyruvate dehydrogenase [Patel *et al.,* 2014]. Acetyl-CoA is used by the TCA cycle to yield the reducing equivalents, NADH and flavin adenine dinucleotide ($FADH_2$), required for mitochondrial oxidative phosphorylation [Osellame *et al.,* 2012]. Hence, the compromise of glycolysis may condition the activities of TCA cycle enzymes, ultimately diminishing the reducing equivalents available for the oxidative phosphorylation to synthesize adenosine triphosphate (ATP) [Osellame *et al.,* 2012].

An inhibition of pyruvate dehydrogenase was found in *STHdh*$^{Q111/111}$ cells [Naia *et al.,* 2017], and in striatum (caudate and putamen) and hippocampus of *postmortem* HD brains [Butterworth *et al.,* 1985; Sorbi *et al.,* 1983]. This pyruvate dehydrogenase inhibition in caudate progressed with HD duration, possibly due to a progressive neuronal loss within this brain region [Butterworth *et al.,* 1985]. Such impairment in pyruvate dehydrogenase may lead to the accumulation of pyruvate and its conversion into lactate. In line with this, elevated lactate levels were detected in striatum and cortex of presymptomatic, early manifest and advanced stage HD patients, being also associated with a higher lactate/pyruvate in their cerebrospinal fluid [Jenkins *et al.,* 1998, 1993;

Koroshetz *et al.*, 1997; Reynolds *et al.*, 2005]. More specifically, this increase in lactate levels appeared to be correlated with the duration and severity of the disease, and with the number of CAG repeats [Jenkins *et al.*, 1998, 1993]. Similar increments in lactate levels occurred in basal ganglia from 3-nitropropionic acid (3-NP, an inhibitor of mitochondrial complex II activity) HD rats, thereby pointing to the subsequent defect in oxidative phosphorylation [Jenkins *et al.*, 1993]. In agreement with this, oral administration of coenzyme Q_{10} (an essential cofactor of the mitochondrial electron transport chain) to HD patients lowered their brain cortical lactate concentrations [Koroshetz *et al.*, 1997]. Alongside pyruvate dehydrogenase, HD affects other enzymes from brain TCA cycle, including α-ketoglutarate dehydrogenase [Klivenyi *et al.*, 2004], aconitase [Chen *et al.*, 2017; Sorolla *et al.*, 2012; Tabrizi *et al.*, 1999], citrate synthase [Sorolla *et al.*, 2012, 2010; Tabrizi *et al.*, 1999] and succinate dehydrogenase [Butterworth *et al.*, 1985, 1983; Damiano *et al.*, 2013; Gu *et al.*, 1996]. Surprisingly, a recent study showed the stimulation of brain cortical (but not striatal) pyruvate dehydrogenase, aconitase and succinate dehydrogenase in aged Q175 heterozygous knock-in mice, in contrast with their inhibition from late stage *postmortem* HD brains [Naseri *et al.*, 2015]. This suggested a compensatory mechanism in brain cortex during mid-stage HD [Naseri *et al.*, 2015].

Despite some controversies on the precise impact of HD in brain glycolysis and TCA cycle, it seems consensual that brain glucose dysmetabolism plays a pivotal role on HD pathogenesis, starting even before the onset of its clinical symptoms [Ciarmiello *et al.*, 2012, 2006; López-Mora *et al.*, 2016]. Nevertheless, given the ubiquitous expression of mHtt since embryonic development, it is plausible that some compensatory mechanisms may occur early during a rodent or human's lifetime, then becoming impaired and leading to HD signs and symptoms [Lou *et al.*, 2016; Saudou & Humbert, 2016].

1.2.1.3. Brain mitochondrial (energy) metabolism in HD

Mitochondria are key organelles in cellular homeostasis, that play numerous vital functions besides the most well-known ATP production (through the oxidative phosphorylation process): iron and calcium homeostasis, production of reactive oxygen species (ROS)/regulation of redox status, biosynthesis of macromolecules (including lipids, amino acids, nucleotides), antioxidant defenses, and regulation of apoptosis [Chandel, 2015; Herst *et al.*, 2017; Mena *et al.*, 2015]. As post-mitotic cells, neurons

require large amounts of energy [Area-Gomez *et al.*, 2019; Costa & Scorrano, 2012; Farshbaf & Ghaedi, 2017], being the ATP produced by oxidative phosphorylation crucial for proper neuronal activities, as previously referred [Farshbaf & Ghaedi, 2017; Pathak *et al.*, 2015; Rangaraju *et al.*, 2014].

Mitochondrial dysfunction is a well-known feature of HD [Carmo *et al.*, 2018; Farshbaf & Ghaedi, 2017; Guedes-Dias *et al.*, 2016]. Among such mitochondrial defects we may include alterations in: oxidative phosphorylation [Milakovic & Johnson, 2005], Ca^{2+} handling [Kolobkova *et al.*, 2017; Mackay *et al.*, 2018; Pchitskaya *et al.*, 2018], mitophagy [Guedes-Dias *et al.*, 2016; Khalil *et al.*, 2015], mitochondrial protein import [Napoli *et al.*, 2013;Yano *et al.*, 2014], mitochondrial fission/fusion [Franco-Iborra *et al.*, 2018; Guedes-Dias *et al.*, 2016; Reddy, 2014], mitochondrial trafficking [Guedes-Dias *et al.*, 2016; Li *et al.*, 2010b; Orr *et al.*, 2008], that culminate in the impairment of ATP synthesis [Dubinsky, 2017; Mochel & Haller, 2011b , Mochel *et al.*, 2012a].

Traditionally, HD has been associated with the inhibition of mitochondrial respiratory chain complex II, as reported in a yeast model of HD [Solans *et al.*, 2006], in mammalian cells expressing exogenous Htt exon 1 containing a pathogenic CAG repeat [Majumder *et al.*, 2007] or in primary striatal neurons expressing an *N*-terminal fragment of mHtt [Benchoua *et al.*, 2006; Damiano *et al.*, 2013]. More recent evidence suggests that, alongside the defects in complex II activity, changes in its expression and assembly caused by the *N*-terminal fragment of mHtt may account for the striatal neuronal death and ultimately the (preferential striatal) pathogenesis of HD [Damiano *et al.*, 2013]. Strikingly, succinate dehydrogenase was recently considered "the" preferential target of mHtt, since it links the TCA cycle to the mitochondrial respiratory chain precisely at the level of complex II and, thus, plays a crucial role within mitochondrial metabolism [Bezawork-Geleta *et al.*, 2017; Iverson *et al.*, 2012; Sousa *et al.*, 2018]. This is supported by studies demonstrating that the mitochondrial toxins 3-NP or malonate blunted succinate dehydrogenase activity, resulting in a decrement of striatal high energy phosphate metabolites (adenosine 5'-diphosphate (ADP), ATP and phosphocreatine) [Mochel *et al.*, 2012a], as well as in clinical and neurological characteristics of HD [Beal, 1994; Brouillet *et al.*, 2005; Greene *et al.*, 1993; Saulle *et al.*, 2004] (*e.g.*, striatal degeneration) both in non-humans primates [Brouillet *et al.*, 1995; Palfi *et al.*, 1996] and rodents [Brouillet *et al.*, 1998; Greene *et al.*, 1993; Pandey *et al.*, 2008]. Furthermore, a decreased expression of succinate dehydrogenase subunits (two small subunits (SDH-C and SDH-D), another constituted by iron sulfur clusters (SDH-B-Ip subunit) and the

fourth containing the flavin adenine dinucleotide (FAD) site (SDH-A-Fp subunit)) [Bezawork-Geleta *et al.,* 2017; Iverson *et al.,* 2012; Sousa *et al.,* 2018] was reported in the *postmortem* striatum of stages 1-3 [Benchoua *et al.,* 2006], suggesting that mitochondrial dysfunction may constitute an early pathogenic event in HD. Adding to this, Fp and Ip subunits of this enzyme were downregulated by posttranscriptional mechanisms in the striatum of HD R6/1 [Damiano *et al.,* 2013] and N171-82Q [Benchoua *et al.,* 2006; Damiano *et al.,* 2013] mice (expressing an *N*-terminal fragment of mHtt), thereby affecting their expression and assembly, ultimately impairing mitochondrial respiratory complex II activity [Benchoua *et al.,* 2006; Damiano *et al.,* 2013]. Accordingly, overexpression of succinate dehydrogenase Fp protein and, more specifically, of the Ip protein, restored the levels of complex II and mitochondrial function (including mitochondrial membrane potential, $\Delta\Psi_m$), preventing striatal neuronal death *in vitro* and *in vivo* [Benchoua *et al.,* 2006; Damiano *et al.,* 2013]. The HD-related changes in succinate dehydrogenase expression, alongside the reduced cAMP levels early in the Hdh^{Q111} mouse striatum, reinforce the hypothesis that bioenergetic dysfunction may be an early pathogenic event in HD [Gines *et al.,* 2003b]. Therefore, it is not surprising that succinate dehydrogenase has been one of the most explored therapeutic targets against HD.

Despite such well-described involvement of mitochondrial dysfunction in HD pathogenesis, it remains debatable whether mHtt impairs mitochondria directly or indirectly. Indeed, some authors postulated that mHtt binds to mitochondrial outer membrane, disrupting its ΔΨm [Choo *et al.,* 2004; Panov *et al.,* 2002; Rockabrand *et al.,* 2007]. This was supported by studies showing that brain mitochondria isolated from two lines of YAC72 mice expressing "low" and "high" levels of full length-mHtt exhibited a depolarized membrane [Panov *et al.,* 2002] and oxidative phosphorylation [Zorova *et al.,* 2018]. Alternatively, mHtt may interact with brain mitochondrial proteins (such as the TIM23 complex that transports nuclear-encoded mitochondrial proteins across the mitochondrial inner membrane), inhibiting protein import into mitochondria from R6/2 mice [Yano *et al.,* 2014]. This may occur early in disease process, even before mitochondrial respiratory dysfunction, and contribute to neuronal death [Yano *et al.,* 2014]. Furthermore, indirect effects of mHtt on mitochondria include the deregulation of nuclear transcription (several mitochondrial proteins are encoded by the nucleus) [Jin & Johnson, 2010; Kumar *et al.,* 2014; Moumné *et al.,* 2013].

In contrast with the above evidence, some authors observed that the activity of either cortical or striatal mitochondrial respiratory complexes remains functional in presymptomatic and early (grade 1) patients [Guidetti *et al.,* 2001; Powers *et al.,* 2007]. Similar findings were reported in striatal oxygen metabolism from early stage HD patients [Powers *et al.,* 2007], thus pointing towards unchanged mitochondrial respiratory chain activity in presymptomatic and early symptomatic patients (even in the presence of striatal lesions) [Guidetti *et al.,* 2001; Powers *et al.,* 2007], in contrast with the inhibition of striatal mitochondrial complexes II, III and IV from *postmortem* brains of symptomatic patients in an advanced neuropathological stage (grades 3 and 4, presenting strong striatal atrophy) [Browne *et al.,* 1997; Gu *et al.,* 1996; Tabrizi *et al.,* 1999]. If this holds true, then the reduction in mitochondrial respiratory complexes' activities could be a secondary pathophysiological event in HD (rather than its trigger) [Damiano *et al.,* 2010; Guidetti *et al.,* 2001; Polyzos & McMurray, 2017; Powers *et al.,* 2007]. However, this is still an intense matter of debate. First, because several studies failed to demonstrate a detrimental effect of mHtt on mitochondrial oxidative metabolism [Guidetti *et al.,* 2001; Hamilton *et al.,* 2017, 2016; Oliveira *et al.,* 2007; Pellman *et al.,* 2015]. For instance, despite the decreased mitochondrial respiration and ATP synthesis in STH*dh*$^{Q111/Q111}$ striatal cells expressing mHtt, no alterations were observed in their mitochondrial respiratory complexes I-IV [Lee *et al.,* 2007; Milakovic & Johnson, 2005; Seong *et al.,* 2005]. Therefore, full-length mHtt (or its fragments) could probably impair bioenergetics indirectly, through extra-mitochondrial mechanisms [Lee *et al.,* 2007; Milakovic & Johnson, 2005], namely oxidative stress [Ayala-Peña, 2013; Siddiqui *et al.,* 2012; Sorolla *et al.,* 2012; Zheng *et al.,* 2018], abnormal mitochondrial import [Napoli *et al.,* 2013; Yano *et al.,*2014], transcriptional dysregulation [Cui *et al.,* 2006; Jin & Johnson, 2010; Johri *et al.,* 2013; Tsunemi *et. al.,* 2012] and limited substrate availability [Dubinsky, 2017; Lou *et al.,* 2016; Perluigi *et al.,* 2005], culminating in a decrease in ATP production [Dubinsky, 2017; Milakovic & Johnson, 2005; Mochel *et al.* 2012a; Solans *et al.,* 2006]. In agreement with this, the iron-sulfur cluster-containing proteins from mitochondrial respiratory chain (*e.g.* succinate dehydrogenase B subunit of complex II and the Rieske protein of complex III) are highly vulnerable to oxidative stress and, therefore, the HD-related oxidative stress may exacerbate the damage and inactivation of these proteins [Mochel & Haller, 2011b].

In sum, direct or indirect action of mHtt leads to mitochondrial dysfunction [Carmo *et al.,* 2018; Farshbaf & Ghaedi, 2017; Guedes-Dias *et al.,* 2016], deficits in intracellular

quality control systems [Franco-Iborra *et al.*, 2018; Guedes-Dias *et al.*, 2016] and in mitochondrial transport and distribution [Guedes-Dias *et al.*, 2016; Li *et al.*, 2010b; Reddy & Shirendeb, 2012]. These may compromise neuronal function, culminating in neuronal degeneration and death in HD-affected brain regions [Guedes-Dias *et al.*, 2016; Reddy, 2014; Reddy & Shirendeb, 2012; Shirendeb *et al.*, 2011].

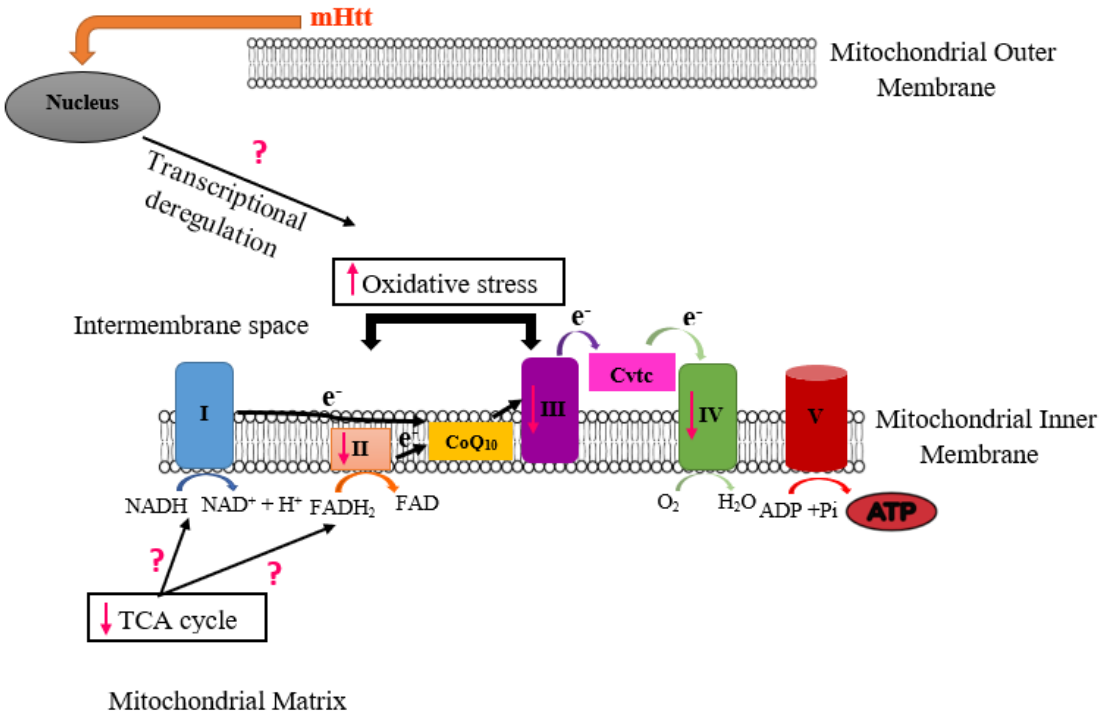

Figure 1: Impaired mitochondrial bioenergetics in HD. Electron transport chain is composed of four mitochondrial complexes, that transfer electrons from donors, such as NADH and $FADH_2$, to its final electron acceptor, O_2. During this process, the electron transport chain pumps protons into the intermembrane space, generating a gradient across the inner mitochondrial membrane that is used by F_0F_1 ATPase (complex V) for ATP synthesis. One of the main focus of mitochondrial deficits in HD brain is the inhibition of mitochondrial respiratory chain complex II, whose pharmacological inhibition recapitulates clinical and neuropathological features of the disease. However, inhibition of mitochondrial complexes III and IV was also found in HD brains. mHtt binds to the mitochondrial outer membrane, disrupting mitochondrial membrane potential ($\Delta\Psi_m$) and blunting mitochondrial respiratory chain function. Alternatively, mHtt may bind to mitochondrial TIM23 complex and impair brain protein import, even before the mitochondrial respiratory dysfunction. Moreover, mHtt may translocate to nucleus and interfere with transcriptional processes to blunt mitochondrial biogenesis and respiration. Furthermore, iron-sulfur cluster–containing proteins within mitochondrial respiratory chain may undergo oxidative damage by HD-related oxidative stress, thus becoming inactivated. Abbreviations: CoQ_{10}-coenzyme Q10; Cytc- cytochrome c; O_2-oxygen; H_2O- water; Pi-inorganic phosphate; ADP-adenosine 5'-diphosphate; ATP- adenosine 5'-triphosphate; $NADH/NAD^+$- nicotinamide adenine dinucleotide (oxidized/reduced); $FADH_2/FAD^+$-flavin adenine dinucleotide (oxidized/reduced); e^- - electrons; I- mitochondrial complex I; II- mitochondrial complex II; III-mitochondrial complex III; IV-mitochondrial complex IV.

1.2.1.4. Phosphocreatine/creatine kinase system and ATP regeneration in HD brain

As discussed in the previous section, STH*dh*$^{Q111/Q111}$ striatal cells had mitochondrial respiration deficits that yielded an inhibition of oxidative phosphorylation and the consequent reduction in mitochondrial ATP production and in ATP/ADP ratio [Milakovic & Johnson, 2005; Seong *et al.,* 2005]. A similar lower ATP/ADP ratio occurred in patient-derived lymphoblast cell lines (that mimic striatal cells expressing the full-length mHtt), being inversely correlated with the length of mutant polyQ [Seong *et al.,* 2005]. On the other hand, brains from late stage N171-82Q mice, Hdh$^{Q111/+}$ mice and R6/2 mice presented unaltered ATP levels, but higher phosphocreatine and creatine levels [Zhang *et al.,* 2011]. However, these apparently contradictory data may be due to the methodological approaches used, since ATP levels decrease dramatically immediately after the interruption of blood supply to the brain [Mochel *et al.,* 2012a]. This was supported by the reduction in brain ATP levels from early and late symptomatic R6/2 mice obtained using a different method [Mochel *et al.,* 2012a]. Prior to this ATP depletion, their striatal and frontal cortical phosphocreatine and creatine levels were already elevated, suggesting a compensatory mechanism towards the generation of ATP from phosphocreatine, via the phosphocreatine/creatine kinase system – a pathway that is activated in response to local energy demands to generate ATP faster than glycolysis or mitochondrial oxidative phosphorylation, through the reversible ATP-dependent phosphorylation of creatine into phosphocreatine catalyzed by creatine kinase [Kim *et al.,* 2010; Wallimann *et al.,* 2011; Zhang *et al.,* 2011]. In line with this, several authors observed that both creatine kinase isoforms expressed within the brain (the cytosolic and the ubiquitous mitochondrial creatine kinase - BB-CK and µMt-CK, respectively [Kim *et al.,* 2010; Schlattner *et al.,* 2013; Zhang *et al.,* 2011]) were downregulated in striatum and cortex of HD patients, R6/2 mice, N171-82Q mice and Hdh$^{Q111/+}$ mice [Kim *et al.,* 2010; Perluigi *et al.,* 2005; Sorolla *et al.,* 2010; Zhang *et al.,* 2011]. Regarding BB-CK inhibition by mHtt, it may occur at the transcriptional and post-transcriptional levels [Kim *et al.,* 2010; Lin *et al.,* 2013]. In addition, BB-CK protein levels were reduced in HD neuronal processes, possibly exacerbating energy defects in these cells [Lin *et al.,* 2013], since the phosphocreatine/creatine kinase system can deliver energy from its production sites to distal energy consumption ones [Wallimann *et al.,* 2011].

1.2.2. Alternative metabolic pathways in HD brain

1.2.2.1. Pentose phosphate pathway in HD

Alternative to glycolysis, glucose taken up into the CNS can be metabolized by the pentose phosphate pathway. This metabolic pathway is subdivided into two sequential reactions: the oxidative branch (whose rate-limiting enzyme is glucose-6-phosphate dehydrogenase) and the non-oxidative branch (whose key enzymes are transketolase and transaldolase) [Stanton, 2012; Stincone *et al.,* 2015]. The main products of the oxidative branch are nicotinamide adenine dinucleotide phosphate (NADPH) and ribose 5-phosphate, both essential for brain cell function [Stanton, 2012; Stincone *et al.,* 2015]. More specifically, brain NADPH plays a role in, *e.g.*, fatty acid and cholesterol synthesis, neurotransmitter synthesis and degradation. NADPH is also pivotal in the oxidative balance within brain cells, since it provides the reducing power for most antioxidant and redox regulatory enzymes (like thioredoxin/peroxiredoxin, glutathione redox cycle), alongside the detoxification of hydrogen peroxide (H_2O_2)), ultimately protecting against oxidative stress [Bolaños & Almeida, 2010; Fernandez-Marcos *et al.,* 2016; Stanton, 2012; Stincone *et al.,* 2015]. On the other hand, ribose 5-phosphate is a precursor for nucleotide biosynthesis [Stanton, 2012; Stincone *et al.,* 2015].

Mounting evidence suggest that the stimulation of the pentose phosphate pathway and the subsequently increased amount of NADPH may attenuate HD-related oxidative damage [Besson *et al.,* 2015; Wang *et al.,* 2012]. Indeed, knockdown of neuronal ribose 5-phosphate isomerase (an enzyme from the pentose phosphate pathway) resulted in a higher expression of glucose 6-phosphate dehydrogenase in young and aged *Drosophila* models of HD, with the subsequent attenuation of polyQ toxicity and oxidative stress, and increased lifespan [Wang *et al.,* 2012]. Conversely, downregulation of glucose 6-phosphate dehydrogenase abolished such protection against polyQ toxicity [Besson *et al.,* 2015; Wang *et al.,* 2012]. Although the mechanisms involved herein remain elusive, the authors hypothesized that ribose 5-phosphate isomerase knockdown triggers a shunt of ribulose-5-phosphate back to glucose-6-phosphate via the non-oxidative branch of pentose phosphate pathway, thereby stimulating glucose-6-phosphate dehydrogenase to enhance NADPH levels and the consequent protection against oxidative stress [Wang *et al.,* 2012]. This was in line with the stimulation of glucose-6-phosphate dehydrogenase in erythrocytes from human HD patients, which was in turn correlated with a higher hexokinase activity and, therefore, reinforces the notion that the resulting increment in

glucose 6-phosphate levels may then potentiate the pentose phosphate pathway [Zanella *et al.,* 1980]. Adding to this, an upregulation of transketolase expression (and of the non-oxidative branch of the pentose phosphate pathway) occurred in 2-week old R6/2 mice striatum [Zabel *et al.,* 2009] and in brain cortex of aged heterozygous Q175 mice [Naseri *et al.,* 2015]. However, others reported no alterations in glucose-6-phosphate dehydrogenase activity in the brains of human HD patients [Bird *et al.,* 1977] and cortex of 12-week old R6/2 mice [Choo *et al.,* 2005], or even its inhibition in HD cybrids [Ferreira *et al.,* 2011] and striatum of 12-week old R6/2 mice [Choo *et al.,* 2005], suggesting that eventual changes in this enzyme's activity may be region/tissue dependent.

In sum, despite the possible upregulation of the pentose phosphate pathway in HD [Naseri *et al.,* 2015; Zabel *et al.,* 2009; Zanella *et al.,* 1980] some controversy persists and, therefore, further studies are needed to evaluate its role on HD progression, including longitudinal studies.

1.2.2.2. Ketone bodies in HD brain

Ketone bodies are small lipid-derived molecules produced from β-oxidation of circulating free fatty acids within hepatic mitochondria [Newman & Verdin, 2014; Puchalska & Crawford, 2017]. Among the three types of ketone bodies, acetone, acetoacetic acid and 3-hydroxybutyric acid, the latter is the most abundant [Grabacka *et al.,* 2016; Newman & Verdin, 2014; Puchalska & Crawford, 2017]. Under physiological conditions, ketone bodies levels in blood are very low. However, they may increase upon, *e.g.,* insufficient glucose supply. Once their concentration rises, ketone bodies can cross the blood-brain barrier (BBB) through monocarboxylate transporters to be used as alternative fuel sources by the CNS [Grabacka *et al.,* 2016; Newman & Verdin, 2014; Puchalska & Crawford, 2017]. In line with this, *D*-β-hydroxybutyric acid (an optical isomer of 3-hydroxybutyric acid) exerted neuroprotective effects in the 3-NP murine model of striatal neuronal loss and in R6/2 mice [Lim *et al.,* 2011]. More specifically, *D*-β-hydroxybutyric acid delayed motor deficits and extended the lifespan of R6/2 mice, protected against striatal lesions and partially mitigated motor deficits induced by 3-NP in C57Bl/6 mice [Lim *et al.,* 2011]. Although still unclear, this may involve the sequential conversion of 3-hydroxybutyric acid into acetoacetic acid (catalyzed by the mitochondrial 3-hydroxybutyric acid dehydrogenase) and then to acetyl-CoA that will enter in TCA cycle. The resulting reducing equivalents (*e.g.,* NADH, $FADH_2$) will enter the

mitochondrial electron transport chain to generate ATP [Lim *et al.,* 2011]. In agreement with this, higher levels of products resulting from fatty acid cleavage were detected in plasma/serum of human gene carriers and symptomatic HD patients. Hence, β-oxidation shunt to TCA cycle may start at early stages of the disease [Cheng *et al.,* 2016; Underwood *et al,* 2006]. Administration of C7 fatty acid triheptanoin to HD patients improved their peripheral and brain energy metabolism [Adanyeguh *et al.,* 2015; Mochel *et al.,* 2010]. This points towards the use of C5 ketone bodies derived from triheptanoin as potential therapeutic strategies to overcome peripheral and brain glucose hypometabolism and ATP deficiency in HD [Adanyeguh *et al.,* 2015; Mochel *et al.,* 2010; Pagano *et al.,* 2016]. However, one possible limitation herein could be the hepatic mitochondrial dysfunction in premanifest and manifest HD patients, and the consequent deficits in ketone bodies' synthesis [Hoffmann *et al.,* 2014; Stüwe *et al.,* 2013]. This is reinforced by the downregulation of OXCT1 that encodes succinyl-CoA:3-ketoacid coenzyme A transferase (a pivotal mitochondrial matrix enzyme for ketone body utilization) in leucocytes from presymptomatic and symptomatic HD patients, and in the striatum of HD mice [Chang *et al*., 2012]. Hence, it is plausible that peripheral and CNS energetic supply from ketone bodies is affected in HD [Chang *et al.,* 2012; Hoffmann *et al.,* 2014; Stüwe *et al.,* 2013]. However, further studies are needed to clarify this issue.

1.2.3. Peripheral metabolic alterations in HD

Peripheral HD symptoms include altered body composition, progressive weight loss, endocrine and metabolic disturbances, alongside skeletal muscle wasting and atrophy [Mochel & Haller, 2011b; Mochel *et al.,* 2007; Saleh *et al.,* 2009; van der Burg *et al.,* 2009; Zielonka *et al.,* 2014]. Indeed, body weight loss is a hallmark of HD [Aziz & Ross, 2013; Aziz *et al.,* 2008; Mochel *et al.,* 2007; Robbins *et al.,* 2006; van der Burg *et al.,* 2017] that is inversely correlated with the number of CAG repeats [Aziz *et al.,* 2008]. Hence, body weight may constitute a biomarker for disease progression [van der Burg *et al.,* 2017]. This is further supported by evidence that individuals with a higher body mass index (BMI) had slower disease progression [Myers *et al.,* 1991; Süssmuth, *et al.,* 2015; van der Burg *et al.,* 2017]. Moreover, these changes in body weight are already detected in presymptomatic HD gene carriers [Djoussé *et al.,* 2002; Mochel *et al.,* 2007], but are more pronounced in later stages of the disease, leading to profound cachexia [Aziz & Ross, 2013; Robbins *et al.,* 2006; Stoy & McKay, 2000; van der Burg *et al.,* 2009]. Interestingly, this weight loss occurred prior to neurocognitive decline, suggesting that

the transport of substrates from the periphery may not be sufficient to overcome the energy deficits in HD brain, thus exacerbating neurological symptoms [Mochel *et al.*, 2007].

Although HD-related weight loss remains unexplained, among its possible causes we can include a reduced calorie intake and/or a higher energy expenditure due to hyperactivity and hypermetabolism [Djoussé *et al.*, 2002; Gaba *et al.*, 2005; Mochel *et al.*, 2007; Pratley *et al.*, 2000; van der Burg *et al.*, 2008], being the latter the most accepted one, since it is reported at early stages [Djoussé *et al.*, 2002; Mochel *et al.*, 2007; van der Burg *et al.*, 2008], even in presymptomatic HD mutations carriers [Mochel *et al.*, 2007]. Furthermore, studies showed that these patients even have a higher calorie consumption, possibly as a compensation for their hypermetabolic state [Mochel *et al.*, 2007]. This is further supported by the early increased oxygen consumption in R6/2 mice, prior to the weight loss [van der Burg *et al.*, 2008], as well as by the decrement in plasma levels of branched chain amino acids (valine, leucine and isoleucine) in HD patients since early disease stages, being correlated with the CAG repeat length, disease progression and weight loss [Cheng *et al.*, 2016; Graham *et al.*, 2016; Mochel *et al.*, 2011a, 2007]. Though this is still debatable, some authors hypothesized that the reduction in circulating branched chain amino acids levels may arise from their mitochondrial oxidation towards the formation of acetyl-CoA and succinyl-CoA to replenish brain TCA cycle [Andersen *et al.*, 2019; Mochel *et al.*, 2007]. In line with this, studies detected lower levels of branched-chain amino acids in brains from human HD patients [Andersen *et al.*, 2019; Patassini *et al.*, 2016].

From the above, HD appears to be associated with an early peripheral hypermetabolic state that, nonetheless, may culminate in body weight loss. Since such early catabolic state can precede the onset of HD symptoms, it is plausible that normalization of peripheral energy metabolism may be beneficial against HD progression [Goodman *et al.*, 2008; Mochel *et al.*, 2007; Underwood *et al.*, 2006; van der Burg *et al.*, 2008]. Indeed, weight loss is a pivotal feature that must be considered in the development of anti-HD therapies.

1.3. Intracellular Stress Mechanisms in HD

1.3.1. Brain oxidative/nitrosative stress in HD pathophysiology

Aerobic cells' metabolism generates ROS and reactive nitrogen species (RNS) that can be counteracted by their intracellular enzymatic and non-enzymatic antioxidant defenses (including free radical scavengers, antioxidant molecules and enzymes) to avoid the harmful effects of oxidative/nitrosative stress [Angelova & Abramov, 2018; He *et al.*, 2017; Mirończuk-Chodakowska *et al.*, 2018; Panigrahy *et al.*, 2017]. These arise from an imbalance between ROS/RNS production and antioxidant defenses [Angelova & Abramov, 2018; He *et al.*, 2017; Mirończuk-Chodakowska *et al.*, 2018; Panigrahy *et al.*, 2017], and has been involved in HD pathogenesis [Kumar & Ratan, 2016]. Indeed, high levels of ROS were found in the striatum of symptomatic R6/1 and R6/2 mice [Ellrichmann *et al.*, 2011; Pérez-Severiano *et al.*, 2004; Sadagurski *et al.*, 2011], as well as in neuronal and non-neuronal cells expressing mHtt [Bertoni *et al.*, 2011; Covarrubias-Pinto *et al.*, 2015; Firdaus *et al.*, 2006; Hands *et al.*, 2011; Li *et al.*, 2010a]. In addition, increased levels of the end-products of nitric oxide, nitrite and nitrate, were found in cerebrospinal fluid from HD patients [Boll *et al.*, 2008]. Despite the wide evidence for oxidative/nitrosative stress and their consequent damage to proteins, DNA and lipids in HD brain, cerebrospinal fluid and plasma/serum [Klepac *et al.*, 2007; Kumar & Ratan, 2016; Stack *et al.*, 2008; Túnez *et al.*, 2011], it is possible that brain constitutes the most affected tissue [Browne & Beal, 2006; Kumar & Ratan, 2016; Stack *et al.*, 2008]. This is due to its high metabolic rate, oxygen and energy consumption, elevated levels of polyunsaturated fatty acids and poor antioxidant defenses [Cobley *et al.*, 2018; Friedman, 2011; Salim, 2017]. Inside *postmortem* HD brain, striatum and cortex were the most affected by oxidative/nitrosative damage, as given by the higher accumulation of DNA oxidative markers (mainly 8-hydroxy-2'-deoxyguanosine (8-OHdG), a marker for nuclear and mitochondrial DNA oxidation) [Browne *et al.*, 1997; Polidori *et al.*, 1999], of cytoplasmic lipofuscin (a product of polyunsaturated fatty acids peroxidation) specifically within vulnerable cortical and striatal neurons [Braak & Braak, 1992; Browne & Beal, 2006; Tellez-Nagel *et al.*, 1974], of other macromolecules associated with oxidative modifications (namely 3-nitrotyrosine, a product of peroxynitrite-mediated protein nitration), malondialdehyde (MDA) and 4-hydroxynonenal (both markers for lipid peroxidation) [Browne & Beal, 2006; Kumar & Ratan, 2016; Stack *et al.*, 2008; Stoy *et al.*, 2005]. Accordingly, studies in R6/2 mice revealed an early increase in striatal

lipid peroxidation markers (*e.g.*, MDA, 4-hydroxynonenal and 8-iso-prostaglandin) that worsened with HD progression [Browne & Beal, 2006]. This was accompanied by their higher striatal carbonylation (a marker for protein oxidation) and subsequent inhibition of key enzymes (such as creatine kinase, aconitase and α/γ enolase) [Chen *et al.*, 2017; Perluigi *et al.*, 2005], alongside the accumulation of 8-OHdG in striatal microdialysates, isolated brain nuclear DNA, urine and plasma [Bogdanov *et al.*, 2001]. Elevated levels of DNA oxidative damage were also found in brains of HD mice [Browne & Beal, 2006; Johri & Beal, 2012].

Mitochondria is both a target and a source of ROS [Ayala-Peña, 2013; Johri & Beal, 2012]. At this respect, mHtt stimulates mitochondrial generation of ROS by, *e.g.*, affecting fission/fusion balance, deregulating calcium homeostasis and mitochondrial energy synthesis, promoting mitochondrial DNA damage/depletion and transcriptional deregulation [Zheng *et al.*, 2018]. In line with this, *STHdh*$^{Q111/Q111}$ cells showed an increment in basal production of mitochondria-generated superoxide anion ($O_2^{\bullet-}$), reduced spare respiratory capacity and increased mitochondrial DNA lesion [Siddiqui *et al.*, 2012]. Similarly, a progressive increase in mitochondrial (and, to a lesser extent, of nuclear) DNA oxidative damage occurred in the striatum and cerebral cortex of R6/2 mice [Acevedo-Torres *et al.*, 2009], as well as in *postmortem* striatum from late-stage HD patients [Siddiqui *et al.*, 2012]. This particular vulnerability of mitochondrial DNA may be due to its proximity with the respiratory chain, limited repair mechanisms, few non-coding sequences and lack of histones [Browne & Beal, 2006; Yang *et al.*, 2008]. Since mitochondrial and nuclear DNA encode components of the five mitochondrial respiratory chain complexes [Chinnery & Hudson, 2013; Mastroeni *et al.*, 2017], their increased oxidative damage upon HD, promotes DNA instability of both genomes, thus hampering mitochondrial bioenergetics and function upon mHtt exposure [Ayala-Peña, 2013; Browne & Beal, 2006]. Moreover, the increased carbonylation (and inhibition) of enzymes from the TCA cycle (like citrate synthase and aconitase), oxidative phosphorylation (*e.g.*, subunit 2 of the cytochrome b-c1 complex III and α-subunit of ATPase) and ATP production (*e.g.*, creatine kinase) in striatal mitochondria from HD patients [Sorolla *et al.*, 2012, 2010] may underlie their diminished ATP synthesis and increased ROS production, further exacerbating this mitochondrial injury and creating a vicious cycle of oxidative damage [Ayala-Peña, 2013; Browne & Beal, 2006; Sorolla *et al.*, 2012].

Under physiological conditions, increased nuclear DNA damage elicits DNA repair mechanisms to remove the oxidized bases and restore its normal structure and function [Ayala-Peña, 2013; Kumar & Ratan, 2016]. However, studies demonstrated that these mechanisms may promote tissue specific expansion of CAG repeats, thus inducing the selective vulnerability of neurons to HD (especially striatal and cortical ones, since the CAG repeat expansion affects post-mitotic neurons and a very large one occurred in cortex and striatum) [Jonson *et al.*, 2013; Kovtun *et al.*, 2007]. In addition, the repair mechanisms for nuclear and mitochondrial DNA are impaired in HD and may, therefore, contribute to the accumulation of DNA damage [Ayala-Peña, 2013; Kumar & Ratan, 2016; Massey & Jones, 2018; Siddiqui *et al.*, 2012]. *I.e.*, HD-related oxidative damage may arise from a higher oxidation of DNA and/or a lower DNA repair [Massey & Jones, 2018; Siddiqui *et al.*, 2012].

Surprisingly, high mitochondrial glutathione levels were detected in cortex and striatum from R6/2 mice [Choo *et al.*, 2005], indicating an adaptive mechanism towards the production of this non-enzymatic antioxidant to counteract oxidative/nitrosative stress and, thus, protect against HD-related neurodegeneration. Accordingly, an upregulation of the striatal and cortical antioxidants peroxiredoxins 1, 2, and 6, glutathione peroxidases 1 and 6, superoxide dismutase and catalase was found in HD patients [Sorolla *et al.*, 2008]. In fact, oxidative/nitrosative stress has been increasingly associated with neuronal loss, eventually constituting one of the primary events in HD neuropathology. However, it remains unclear whether oxidative/nitrosative stress is caused directly by mHtt or is a consequence neuronal death [Browne & Beal, 2006; Kumar & Ratan, 2016; Stack *et al.*, 2008].

1.3.2. Peripheral oxidative/nitrosative stress in HD pathophysiology

Importantly, oxidative/nitrosative markers were also detected at the periphery, especially in plasma/serum of symptomatic HD patients. These include 8-OHdG, MDA, thiobarbituric acid reactive substances (TBARS, a marker of lipid peroxidation) and advanced oxidation protein products [Chen *et al.*, 2007; Ciancarelli *et al.*, 2014; Hersch *et al.*, 2006; Klepac *et al.*, 2007; Peña-Sánchez *et al.*, 2015]. The increased plasma lipid peroxidation measured in asymptomatic HD gene carriers suggests its occurrence prior to clinical symptoms, being associated with disease progression [Klepac *et al.*, 2007]. Importantly, this correlation between peripheral oxidative damage and HD progression

[Túnez *et al.*, 2011] indicates that symptomatic HD patients may undergo a pro-oxidative state [Ciancarelli *et al.*, 2014].

From the above, it seems reasonable to hypothesize that oxidative stress and mitochondrial dysfunction are intertwined mechanisms involved in HD pathophysiology and, as such, blocking one should mitigate the other. In agreement with this, XJB-5-131 (a synthetic mitochondrially-targeted oxygen radical scavenger that crosses the BBB) attenuated or even reversed the deleterious effects of mHtt in Hdh^{Q150} knock-in mice, even when the treatment was initiated after disease onset [Polyzos *et al.*, 2016; Xun *et al.*, 2012]. But unfortunately, most clinical trials involving antioxidants performed to date in human HD patients were unsuccessful, since these molecules were not able to modify disease progression [Hersch *et al.*, 2017; Kumar & Ratan, 2016; McGarry *et al.*, 2017]. This led to the notion that oxidative stress may be a consequence of other pathological mechanisms in HD, namely transcriptional dysregulation [Johri *et al.*, 2013; St-Pierre *et al.*, 2006; Tsunemi *et al.*, 2012], mitochondrial dysfunction [Ribeiro *et al.*, 2014; Siddiqui *et al.*, 2012; Zheng *et al.*, 2018] or protein aggregation [Firdaus *et al.*, 2006; Hands *et al.*, 2011].

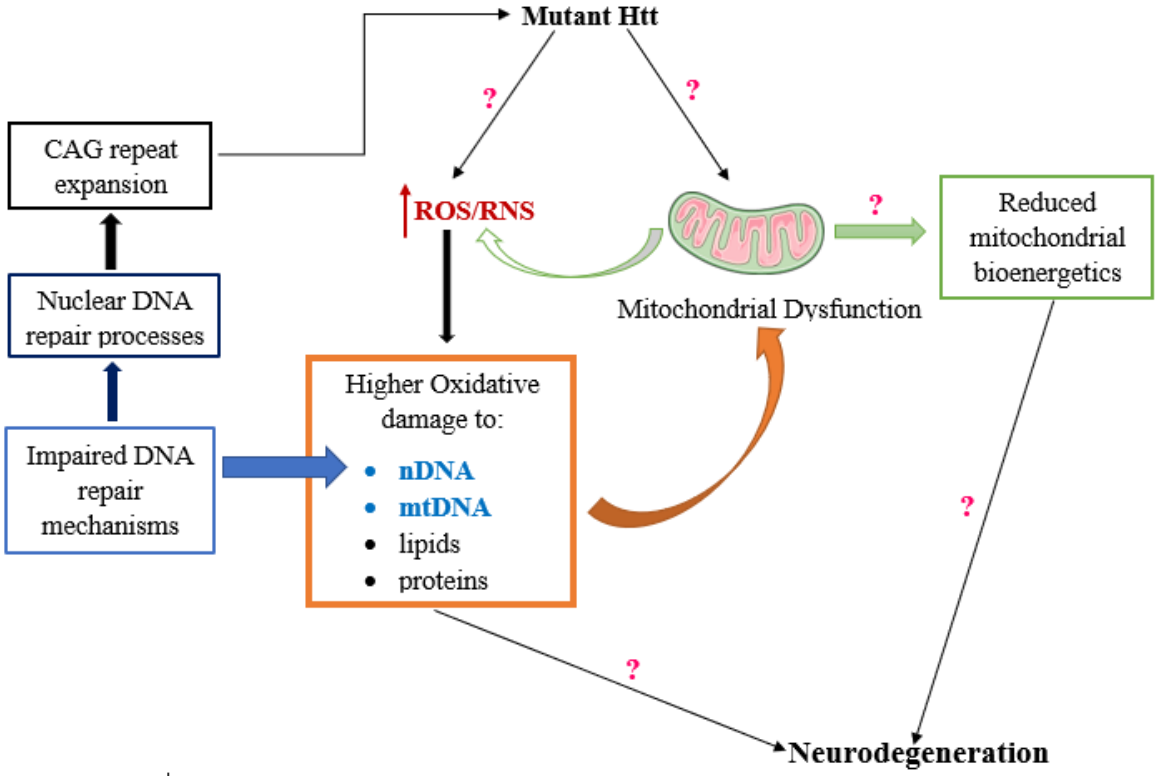

Figure 2: Vicious cycle of oxidative/nitrosative damage in HD. mHtt has been associated with high levels of ROS and RNS that may arise, e.g., from mitochondrial dysfunction. Indeed, mitochondria are among the major intracellular sources of ROS and RNS that, if not counteracted, lead to oxidative/nitrosative stress and damage to nuclear and mitochondrial DNA, proteins and lipids. In addition, mHtt can compromise the mechanisms that repair of both mitochondrial and nuclear DNA, further extending their own damage and exacerbating mitochondrial damage, hampering mitochondrial bioenergetics and function, and creating a vicious cycle of oxidative/nitrosative injury that may underlie HD pathophysiology. Of note, DNA repair processes activated in response to nuclear DNA damage can also promote CAG repeat expansion. Abbreviations: nDNA, nuclear DNA; mtDNA, mitochondrial DNA; ROS, reactive oxygen species; RNS, reactive nitrogen species.

1.4. Promising Therapeutic Strategies Against HD

Currently available treatments for HD are only symptomatic and aim to ensure a better quality of life to the patients [Frank, 2014; McColgan & Tabrizi, 2018; Pidgeon & Rickards, 2013]. The first anti-HD drug approved by US Food and Drug Administration (FDA) was tetrabenazine, a vesicular monoamine transporter-2 (VMAT-2) inhibitor used to manage chorea [Guay, 2010; Huntington Study Group, 2006; Yero & Rey, 2008]. However, tetrabenazine adverse effects include sleep problems, depression, anxiety and fatigue, being its administration not advised to patients with a history of psychiatric disorders [Coppen & Roos, 2017; Frank, 2014; McColgan & Tabrizi, 2018].

Afterwards, tetrabenazine was modified towards a molecule with a longer lifetime and less metabolic variability – deutetrabenazine [Coppen & Roos, 2017; Frank *et al.*, 2016] -, already accepted by FDA for the treatment of HD. A recent study demonstrated that deutetrabenazine significantly attenuates chorea with fewer adverse effects [Rodrigues *et al.*, 2017].

Controversy exists about possible treatments for HD-associated cognitive and psychiatric symptoms, and further studies are required [Frank 2014; McColgan & Tabrizi, 2018]. As such, and given the current lack of chronically effective anti-HD drugs, the goals of the currently available treatments are to delay the onset of symptoms and keep the patient autonomous and active for longer.

1.4.1. Liraglutide and ghrelin: a promising cocktail to treat HD?

As detailed elsewhere, although HD is a neurological disorder, the ubiquitous expression of mHtt outside the CNS results also in peripheral pathology, detected from the early stages of the disease (including, *e.g.*, impaired energy metabolism [Dubinsky, 2017; Mochel & Haller, 2011b] and weight loss [Mochel *et al.*, 2007; van der Burg *et al.*, 2017,2009]). Therefore, it is not surprising that, in HD, its CNS pathology can be modulated by treatments directed to the peripheral tissues – an important issue that opens a wider therapeutic window of opportunities, but also comprises the enormous challenge of developing drugs or strategies that impact the brain.

HD shares molecular mechanisms with type 2 diabetes, namely the impaired peripheral and brain glucose metabolism and cognitive decline [Candeias *et al.*, 2015, Martin *et al.*, 2009; Montojo *et al.*, 2017]. Moreover, abnormalities in glucose homeostasis and higher prevalence of *Diabetes mellitus* were reported in HD patients and mice [Björkqvist *et al.*, 2005; Hunt & Morton, 2005; Lalić *et al.*, 2008; Montojo *et al.*, 2017]. On the other hand, mHtt accumulation in β-pancreatic cells disrupts their function and decreases their number, ultimately affecting β-cell mass and insulin secretion [Björkqvist *et al.*, 2005; Hunt & Morton, 2005; Lalić *et al.*, 2008; Martin *et al.*, 2009; Montojo *et al.*, 2017]. Hence, among the promising therapeutic approaches against HD, we can find the long-acting, anti-type 2 diabetic drugs from the class of incretins/glucagon-like peptide-1 (GLP-1) analogues, exendin-4 [Martin *et al.*, 2012, 2009] and liraglutide [Duarte *et al.*, 2018], and the orexigenic hormone ghrelin [Sjögren *et al.*, 2017].

GLP-1 is an incretin hormone, synthesized by intestinal cells, that acts through the GLP-1 receptor (a G_S protein-coupled receptor, abundantly expressed in pancreas and CNS) to regulate glycemia and exert neuroprotective effects [Cabou & Burcelin, 2011; Donnelly, 2012; Rowlands *et al.*, 2018]. These beneficial effects, and particularly the glycemia management in a glucose-dependent manner that minimizes the risk of recurrent hypoglycemia episodes, rendered GLP-1-based drugs a very exciting and efficient option to treat type 2 diabetes. However, the therapeutic use of native GLP-1 is limited by its rapid degradation by dipeptidyl peptidase-IV and, thus, by its short half-life in circulation [Hinnen, 2017; Meier, 2012; Rowlands *et al.*, 2018]. Fortunately, several GLP-1 analogues have been developed and are used nowadays for the treatment of type 2 diabetes. Among them, exendin-4 and liraglutide are the most well-known, being very efficient, potent, with a longer half-life in circulation that induces a sustained therapeutic action with a minimum risk of hypoglycemia [Hinnen, 2017; Meier, 2012; Rowlands *et al.*, 2018].

Liraglutide was shown to protect, preserve and improve β-cell mass (possibly through anti-apoptotic effects) and function, improving insulin secretion and sensitivity, and enhancing glycemic control in type 2 diabetes [Candeias *et al.*, 2015; Davies *et al.*, 2011; Madsbad *et al.*, 2004; Shao *et al.*, 2014]. In line with this, we recently showed that liraglutide normalized peripheral glucose homeostasis, and rescued insulin resistance and pancreatic β-cell function in R6/2 mice [Duarte *et al.*, 2018]. Notably, liraglutide crosses the BBB and reaches the brain almost intact, whereby it activates GLP-1 receptor to exert beneficial effects against the neurodegeneration occurring in, *e.g.*, type 2 diabetes, Alzheimer's and Parkinson's diseases. Among such neuroprotective effects, liraglutide was shown to regulate cell metabolism, energy homeostasis, neuroinflammatory and stress responses, to inhibit apoptosis and promote neuronal survival [Candeias *et al.*, 2015; Gejl *et al.*, 2016; Rowlands *et al.*, 2018; Zhang *et al.*, 2018]. Thus, it is not surprising that several clinical trials are undergoing to evaluate the impact of liraglutide on human patients suffering from these diseases (*www.clinicaltrials.gov*: NCT02953665, NCT02889510, NCT03707171, NCT01843075) [Candeias *et al.*, 2015; Gejl *et al.*, 2016; Rowlands *et al.*, 2018; Zhang *et al.*, 2018]. This, together with the above-mentioned relation between HD and type 2 diabetes, led us to hypothesize that liraglutide may be also useful in HD treatment. In this perspective, in a recent study performed by our group liraglutide exerted beneficial effects in R6/2 mouse brains, possibly by activating GLP-1 receptor and adenylyl cyclase, with the subsequent increase in brain cyclic adenosine

monophosphate (cAMP) content, activation of protein kinase A (PKA), cAMP response element binding protein (CREB) and phosphoinositide 3-kinase (PI3K) [Duarte *et al.*, 2018]. This may in turn increase intracellular calcium levels and activate Ca^{2+}/calmodulin-dependent protein kinase kinase β (CaMKKβ), thereby stimulating AMP-activated protein kinase (AMPK) phosphorylation and activation [Shao *et al.*, 2015; Krasner *et al.*, 2014], known to inhibit ATP-consuming pathways (*e.g.*, protein synthesis) and stimulate ATP-generating pathways (*e.g.*, β-oxidation), ultimately regulating intracellular metabolism and energy homeostasis [Herzig & Shaw, 2018]. In addition, liraglutide normalized brain triglyceride levels in R6/2 mice, and as a result may decrease its use as brain alternative metabolite [Duarte *et al.*, 2018]. However, liraglutide is also an anorexigenic molecule that reduces food intake and body weight [Candeias *et al.*, 2015; Crane & McGowan, 2016]. Since progressive weight loss is a prominent feature in HD, this could pose serious questions to the administration of liraglutide alone to HD patients. To overcome this potential limitation, we hypothesized that liraglutide could be administered in association with orexigenic molecules, such as ghrelin.

Ghrelin is an orexigenic, gut peptide hormone with widespread effects in a multitude of tissues, mediated by the Gq-coupled growth hormone secretagogue receptor 1a (GHS-R1a, ubiquitously expressed within the CNS) [Albarrán-Zecker & Smith, 2013; Shi *et al.*, 2017; Stoyanova, 2014]. Ghrelin is abundant within the brain, either due to its local synthesis and/or to its ability to travel from the periphery towards the CNS [Cabral *et al.*, 2017; Rhea *et al.*, 2018], whereby it has been increasingly shown to exert protective effects (as well as at the periphery) against neurodegenerative diseases, such as Alzheimer's, Parkinson's and Huntington's diseases. These effects included the attenuation of weight loss, increased food intake and appetite, alleviation of inflammatory and oxidative stress mechanisms, regulation of cell metabolism and energy homeostasis, inhibition of apoptosis, and promotion of neuronal survival and function [Bayliss & Andrews, 2013; de Candia & Matarese, 2018, Shi *et al.*, 2017; Sjögren *et al.*, 2017; Stoyanova, 2014]. Although the precise mechanisms underlying such positive effects of ghrelin are under intense investigation, they may involve the stimulation of GHS-R1a-mediated intracellular signaling, with the consequent production of phospholipase C. The later cleaves the membrane lipid phosphoinositol 4,5 bisphosphate into inositol (1,4,5) trisphosphate and diacylglycerol [Schneeberger & Claret, 2012; Yin *et al.*, 2014] thereby increasing intracellular calcium levels and the activation of CaMKKβ that, in turn,

stimulates AMPK phosphorylation and activation [Andrews, 2011; Schneeberger & Claret, 2012; Yin *et al.*, 2014].

In terms of HD, ghrelin was recently shown to delay weight loss, reverse muscle loss, the catabolic phenotype caused by the accumulation of mHtt in myocytes, and behavioral changes in R6/2 mice [Sjögren *et al.*, 2017]. Furthermore, chronic treatment with ghrelin initiated in the early phase of HD normalized the expression of hypothalamic orexigenic peptides [Rudenko *et al.*, 2019]. This suggests that the attenuation of weight loss and enhancement of lean mass upon HD may arise from an increased food intake and decreased energy expenditure, thereby delaying the negative energy balance and ameliorating HD metabolic parameters (namely oxygen consumption) [Rudenko *et al.*, 2019]. However, data on ghrelin levels in HD patients remain controversial, since some studies showed no differences [Nambron *et al.*, 2016], while others described an increase in ghrelin content under these conditions [Popovic *et al.*, 2004; Wang *et al.*, 2014]. In addition, the efficacy of ghrelin treatment decreased with HD progression, most likely due to a decrease in expression of hypothalamic GHS-R1a and the subsequent ghrelin resistance condition [Rudenko *et al.*, 2019]. Nevertheless, we hypothesize that administration of ghrelin together with liraglutide may complement or even overcome their individual limitations and, therefore, constitute a promising therapy against HD. In agreement with this, a recent study from our lab showed that chronic, peripheral co-injection of ghrelin plus liraglutide improved peripheral metabolism (by recovering glucose homeostasis, insulin sensitivity and pancreatic β-cell function) and brain metabolism (by reducing brain insulin, lactate, and cholesterol levels) in early symptomatic R6/2 mice [Duarte *et al.*, 2018].

1.5. Animal Models of HD

Several genetic mouse models have been widely used to investigate the mechanisms underlying HD phenotype. Among such models, we can find: *i)* the transgenic truncated models, that express the N-terminal fragment of mHtt; *ii)* the transgenic full-length models, that express the complete mutant gene; and *iii)* knock-in models, resulting from the direct insertion of the CAG repeat expansion into the Htt gene [Cepeda *et al.*, 2010; Ehrnhoefer *et al.*, 2009; Pouladi *et al.*, 2013; Rangel-Barajas & Rebec, 2018]. Among the transgenic truncated mouse models, the most widely used are the R6/1 and R6/2 mice, as described below.

1.5.1. The R6/2 mouse model for HD

The R6/1 mouse usually expresses a mHtt with ~115 CAG repeats, its pathology usually starts at a later age and has a slower progression [Cepeda *et al.*, 2010; Ehrnhoefer *et al.*, 2009; Pouladi *et al.*, 2013; Rangel-Barajas & Rebec, 2018]. On the other hand, the R6/2 mouse traditionally expresses a mHtt with ~150 CAG repeats, its pathology often starts at an early age, has a rapid disease progression and short lifespan (13-18 weeks), being considered to mimic the juvenile onset of HD [Cepeda *et al.*, 2010; Ehrnhoefer *et al.*, 2009; Pouladi *et al.*, 2013; Rangel-Barajas & Rebec, 2018]. However, more recent studies showed that R6/2 mice exhibit a progressive HD-like behavioral and neuropathological phenotype, more similar to human HD than previously thought. Thus, the R6/2 mouse has been considered an appropriate model for studies evaluating the therapeutic potential of drugs against HD [Ehrnhoefer *et al.*, 2009; Stack *et al.*, 2005].

In the R6/2 mouse, mHtt aggregates are widespread throughout the brain since 3-4 weeks of age, with abundant intranuclear inclusions detected mainly within the neurons that are most affected by HD (*e.g.*, striatal GABAergic medium spiny neurons and cortical pyramidal neurons) [Davies *et al.*, 1997; Gutekunst *et al.*, 1999; Kosinski *et al.*, 1999; Meade *et al.*, 2002]. Their behavioral changes start as early as 4-5 weeks of age and worsen progressively with disease progression [Ehrnhoefer *et al.*, 2009; Rangel-Barajas & Rebec, 2018; Stack *et al.*, 2005]. Furthermore, a widespread brain atrophy is detected from an early until the end-stage of disease (*e.g.*, since 3 weeks old), namely in striatum, cortex, hippocampus and thalamus [Aggarwal *et al.*, 2012; Stack *et al.*, 2005; Zhang *et al.*, 2010]. Neurodegeneration is still controversial in R6/2 mice, since some authors only observed few signs of it in brains from late-stage animals (16–20 weeks old) with 150–250 CAG repeats [Davies *et al.*, 1997; Morton *et al.*, 2009; Yu *et al.*, 2003], whereas others reported striatal, cortical and hippocampal neurodegeneration in late-stage (12-week old) mice [Iannicola *et al.*, 2000; Kusakabe *et al.*, 2001; Zhang *et al.*, 2010]. These discrepancies may result either from the different methodologies used and/or from animals with distinct CAG repeat lengths. Indeed, instability in the CAG repeat length due to different breeding practices has been increasingly described, which may decrease or increase the average CAG repeat lengths between different colonies and, thus, result in differential onset and progression of HD phenotype [Cummings *et al*., 2011; Morton *et al*., 2009]. Accordingly, Morton *et al.* (2009) described that the expansion of CAG repeats beyond the range where formation of neuronal intranuclear inclusions was the

dominant event in HD pathology, slowed down the progression of neurological phenotype in R6/2 mice. This resulted in a more human adult onset HD-like brain pathology, accompanied by neurodegeneration but a longer life expectancy. *I.e.*, in R6/2 mice with <250 CAG repeats disease onset correlates with the occurrence of neuronal intranuclear inclusions, whereas in those with >300 CAG repeats there are non-nuclear inclusions, onset of symptoms is delayed (*e.g.* weight loss, motor dysfunction, neuronal intranuclear inclusions) and live longer [Dragatsis *et al*., 2009; Morton *et al*., 2009].

In sum, R6/2 mice recapitulate many features of human HD, including disturbed glucose metabolism [Björkqvist *et al*., 2005; Cepeda-Prado *et al*., 2012], mitochondrial dysfunction [Gizatullina *et al*., 2006; Lou *et al*., 2016; Tabrizi *et al*., 2000], transcriptional deregulation [Moumné *et al*., 2013; Zabel *et al*., 2009], skeletal muscle wasting [Zielonka *et al*., 2014], progressive weight loss [van der Burg *et al*., 2017] and brain atrophy [Aggarwal *et al*., 2012; Stack *et al*., 2005; Zhang *et al*., 2010], alongside the accumulation of mHtt aggregates in brain neurons [Davies *et al.*, 1997; Gutekunst *et al.*, 1999; Kosinski *et al.*, 1999; Meade *et al.*, 2002] and motor dysfunction [Ehrnhoefer *et al*., 2009; Rangel-Barajas & Rebec, 2018; Stack *et al*., 2005]. Furthermore, R6/2 mice possessing very long CAG repeats (as our model, described in the next sections) may mimic the adult onset of HD and live longer, facilitating the study of the early phases of HD [Dragatsis *et al*., 2009; Morton *et al*., 2009].

Chapter 2

Hypothesis and Aim

Considering that:

1) HD-related pathophysiological mechanisms remain poorly understood, but may involve, *e.g.*, mitochondrial dysfunction [Carmo *et al.*, 2018; Farshbaf & Ghaedi, 2017; Polyzos & McMurray, 2017], oxidative stress [Browne & Beal, 2006; Kumar & Ratan, 2016; Stack *et al.*, 2008], and neurodegeneration and death affecting primarily striatal and cortical neurons [Bunner & Rebec, 2016; Plotkin & Surmeier, 2015; Rebec, 2018];

2) HD shares similar characteristics to type 2 diabetes, especially the compromise in peripheral and brain glucose metabolism and cognitive decline [Candeias *et al.*, 2015; Martin *et al.*, 2009; Montojo *et al*., 2017];

3) both liraglutide and ghrelin can have neuroprotective effects against type 2 diabetes, Alzheimer's and Parkinson´s diseases, that involve the promotion of neuronal survival, regulation of energy homeostasis, and anti-inflammatory, -oxidative and -apoptotic actions [Candeias *et al.*, 2015; Shi *et al.*, 2017; Stoyanova, 2014; Zhang *et al.*, 2018];

4) we previously showed that a 2-week ghrelin administration *per se* reversed the expression of catabolic markers and morphological changes in skeletal muscle in R6/2 mice, alongside their recovery from altered nest construction behavior [Sjögren *et al.*, 2017];

5) and we recently demonstrated that chronic, peripheral co-injection of ghrelin with liraglutide improved peripheral and brain energy metabolism [Duarte *et al.*, 2018],

in the present study we hypothesized that the co-administration of liraglutide and ghrelin recovers brain glucose (energy) metabolism and protects against intracellular stress upon HD.

Hence, we aimed to study the molecular mechanisms underlying the neuroprotective role of a 2-week co-administration of ghrelin and liraglutide against brain metabolic dysfunction and intracellular stress in 12-week old, early symptomatic R6/2 mice. More specifically, we aimed to evaluate the effect of co-administration of ghrelin and liraglutide on brain cortices from early symptomatic R6/2 mice, in terms of their:

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

2) brain intracellular oxidative stress markers, with a special emphasis on DNA and lipid oxidation, RNS.

With this study, we expect to give novel insight about the beneficial effects of a peripheral co-administration of ghrelin plus liraglutide against brain cortical glucose (energy) dysmetabolism and oxidative/nitrosative stress in the early stages of HD. Moreover, this study may contribute to define the co-administration of ghrelin plus liraglutide as a promising preventive/therapeutic strategy against HD.

Chapter 3

Materials and Methods

3.1. Materials

Ghrelin (Rat, mouse) was obtained from Phoenix Pharmaceuticals (Belmont, CA, USA). Liraglutide [(Lys(γ-Glu-palmitoyl)26,Arg34)-GLP-1 (7-37)]) was obtained from Bachem AG (Bubendorf, Switzerland). Infinity Glucose Hexokinase Kit was bought to Thermo Scientific, Middletown (Middletown, VA, USA). Mouse Insulin ELISA Kit was purchased to Mercodia (Uppsala, Sweden). Bio-Rad Protein Assay was purchased to Bio-Rad Laboratories (Richmond, CA, USA). PicoProbe™ Fructose-6-Phosphate Fluorimetric assay was purchased to BioVision (Milpitas, CA, USA). EnzyChrom™ Ketone Body assay kit was purchased to Bioassay Systems LLC (Hayward, CA, USA). DNA/RNA Oxidative Damage ELISA Kit was purchased to Cayman Chemical (Ann Arbor, Michigan, USA). All other chemicals used were of the highest grade of purity commercially available.

3.2. Methods

3.2.1. Animal housing and treatment

The present study is a continuation from a recently published one that resulted from a collaboration between our group (*Metabolism, Mitochondria and Hormones in Brain Disorders*, at the CNC – Center for Neuroscience and Cell Biology, University of Coimbra) and the *Brain Disease Biomarker Unit* (Department of Experimental Medical Sciences, Wallenberg Neuroscience Center, Lund University, Lund, Sweden) [Duarte *et al.*, 2018]. All experimental procedures performed in mice were carried out in accordance with the approved guidelines in the ethical permit approved by The Malmö/Lund Animal Welfare and Ethics Committee (ethical permit number: M5-15).

Male transgenic R6/2 HD mice (expressing the exon 1 of the HD gene) [Rangel-Barajas & Rebec, 2018] and their wild-type (WT) littermates were used. Mice were obtained through crossing heterozygous R6/2 males with WT females (F1 of CBAxC57BL/6 J). Tail tips were sent on dry ice to Laragen (Laragen Inc., CA, USA) for CAG repeat length determination, by polymerase chain reaction assay. Similar to our previously published study, the R6/2 mice used in the present one had a CAG repeat size ranging from 275–312, which resulted in a disease progression slower than that of the R6/2 mouse with 150 CAG repeats, as described by Morton *et al.* (2009). In our colony at Lund University (where the *in vivo* experiments were performed), 12-week old R6/2 mice correspond to late premanifest disease (as described by Morton *et al.* (2009), while

18-week old R6/2 mice correspond to overt disease (with changes in striatal volume, body weight and neurofilament light chain levels in cerebrospinal fluid) [Soylu-Kucharz *et al.*, 2017]. Mice were housed in groups with *ad libitum* access to chow food and water under standard conditions (12h light/dark cycle, 22°C).

As previously described [Duarte *et al.*, 2018], ghrelin (Rat, mouse; 100 µL, 150 µg/kg) and/or liraglutide [(Lys(γ-Glu-palmitoyl)26,Arg34)-GLP-1 (7-37)]; 100 µL; 0.2 mg/kg) or sterile NaCl (vehicle; 100 µL) were injected subcutaneously (s.c.) once daily, for 2 weeks, from the age of 10 weeks onwards (prior to R6/2 weight loss). 150 µg/kg ghrelin has previously been shown to exert beneficial effects on body weight [Sjögren *et al.*, 2017]. At the end of the 2-week treatment, mice were fasted for ~6 h (starting late in the evening) and then euthanized, blood was collected, and brain was dissected. Serum obtained from blood samples was collected by centrifugation at 2000 ×*g*, for 10 min, at 4 °C, and immediately frozen to −80 °C. Tissue samples were snap-frozen in liquid nitrogen and stored at −80°C until further use.

3.2.2. Serum analyses

According to our previous study [Duarte *et al.*, 2018], fasting serum glucose levels were measured using the glucose oxidase method (Infinity Glucose Hexokinase Kit, Thermo Scientific), and fasting levels of insulin were determined by the Mouse Insulin ELISA Kit (Mercodia). The homeostatic model assessment (HOMA) was calculated to measure insulin resistance (IR) and pancreatic beta-cell function (β). HOMA-IR was calculated as follows: (Insulin x Glucose)/22.5, and HOMA-β as follows: (20 × Insulin)/(Glucose − 3.5) [Matthews *et al*., 1985].

3.2.3. Isolation and preparation of brain cortical homogenates

Mice were euthanized by decapitation, the brains were immediately removed, and brain cortices immediately dissected, snap-frozen at -80°C, properly packed and sent on dry ice to our lab at CNC, where they were stored at -80ºC for further studies.

Immediately before the experiments, brain cortices were homogenized, according to our previously described method [Duarte *et al.*, 2018], at 0–4 °C, in lysis buffer containing (in mM): 25 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2 $MgCl_2$, 1 ethylenediamine tetraacetic acid (EDTA), 1 ethylene glycol tetraacetic acid

(EGTA) (pH 7.4), supplemented with 2 mM dithiotreitol (DTT), 100 µM phenylmethylsulfonyl fluoride (PMSF) and commercial protease and phosphatase inhibitors cocktails. The homogenates were centrifuged at 17,968 ×*g* for 10 min, at 4°C, in a Sigma 2-16 K centrifuge (Sigma, Newton, UK), to remove the nuclei, and the resulting supernatant was collected. The pellet was resuspended again in supplemented buffered solution and centrifuged again at 17.968 ×*g* for 10 min, at 4°C. The resulting supernatant was added to the previously obtained one, and protein content was measured as described below.

3.2.4. Protein quantification by the Bio-Rad method

Protein content from the brain cortical homogenates was measured using the Bio-Rad Protein Assay [Bio-Rad Laboratories, 2010; Bradford 1976], according to manufacturer's protocol (Bio-Rad Laboratories). This method is based on the well-known Bradford dye-binding method, which relies on the binding of the Coomassie Brilliant Blue G-250 dye to the proteins [Bradford, 1976]. Under acidic conditions, it occurs in the doubly protonated red cationic form (maximum absorbance: 465 nm), but when bound to the protein it is converted to a stable unprotonated blue form (maximum absorbance: 595 nm). This blue protein-dye complex is detected spectrophotometrically at 595 nm (Fig. 3) [Bio-Rad Laboratories, 2010; Bradford, 1976].

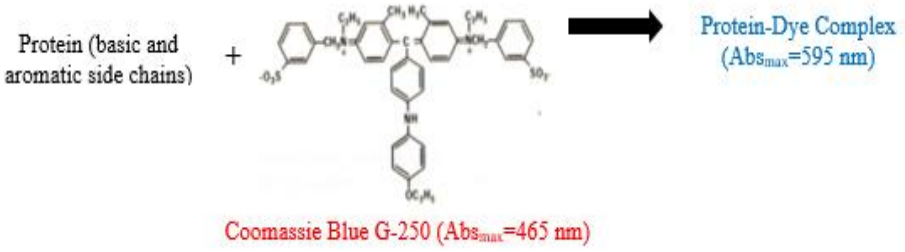

Figure 3: Schematic representation of the reaction principle of the Bio-Rad Protein Assay. Abbreviations: Abs_{max}, maximum absorbance.

Briefly, 1 µL of each brain cortical lysate were added, in a 96-well plate, to 79 µL of type 1 water (1:40 dilution). A standard curve was prepared using known concentrations of bovine serum albumin (BSA) 0.1% (Sigma-Aldrich, Missouri, USA): 0, 5, 10, 20, 30, 40 µg/µL, together with the supplemented lysis buffer used to prepare the samples. Bio-Rad reagent was diluted in a 1:3 with type 1 water and then 120 µL were added to each well. The plate was incubated at room temperature for 15 min, protected

from light, and then read in a SpectraMax Plus 384 spectrophotometer (Molecular Devices LCC, CA, USA), at 595 nm. Protein concentration was calculated by extrapolation from the standard curve and expressed in µg/µL.

Table 1. Protocol for determination of protein levels by the Bio-Rad colorimetric assay.

	BSA 0.1% (µg)	BSA 0.1 % (µL)	Supplemented lysis buffer (µL)	Type 1 H_2O (µL)	Sedmak Reagent (µL)
P0	0	0	1	79	120
P5	5	5	1	74	120
P10	10	10	1	69	120
P20	20	20	1	59	120
P30	30	30	1	49	120
P40	40	40	1	39	120
			Sample (µL)		
A1	?	-	1	79	120

3.2.5. Determination of brain markers for glycolysis and pentose phosphate pathway

Glycolysis and the pentose phosphate pathway are interconnected metabolic pathways that share three common intermediates: glucose 6-phosphate, glyceraldehyde 3-phosphate, and fructose 6-phosphate [Stincone *et al.*, 2015].

3.2.5.1. Determination of the rate of glucose 6-phosphate formation

The rate of glucose 6-phosphate production was determined by a colorimetric method previously described by Lamprecht *et al*. (1974), with some modifications. This method determines the rate of glucose 6-phosphate production using the glucose 6-phosphate dehydrogenase reaction with $NADP^+$ as coenzyme. Briefly, 5 µL of each brain cortical homogenate were incubated in 72.5 µL of triethanolamine (TEA) buffer, containing (in mM): 50 triethanolamine-hydrochloride and 22 NaOH (pH 7.5), supplemented with 0.2 mM β-$NADP^+$ sodium salt and 8.35 mM $MgCl_2$. Absorbance was continuously read at 339 nm, 37ºC, in a 96-well UV plate, for 2 min, with 30 s intervals, in a SpectraMax Plus 384 plate reader. Then, the reaction was initiated by the addition of 743.75 U/L glucose 6-phosphate dehydrogenase from baker's yeast (*S. cerevisæ*), type VII, ammonium sulphate suspension, and the absorbance continuously read at 339 nm, 37°C, for 3 min, with 30 s intervals. The rate of glucose 6-phosphate formation was

calculated by using an ε_{339nm} = 1 mol^{-1} mm^{-1}, from the extrapolation of absorbance, according the formula:

$\Delta A = A_2 - A_1$

where A_2 corresponds to the absorbance measured after the addition of glucose-6-phosphate dehydrogenase and A_1 corresponds to the basal reading. The rate of glucose 6-phosphate production was expressed as µmol/min/mg protein.

3.2.5.2. Determination of fructose 6-phosphate levels

Fructose 6-phosphate levels were measured by the PicoProbe™ Fructose-6-Phosphate Fluorimetric assay, according to manufacturers' instructions, with slight modifications. In this assay, fructose 6-phosphate is converted to glucose 6-phosphate that is subsequently oxidized with the formation of a fluorescent product, whose fluorescence intensity can be determined with an excitation and emission wavelengths of 535 nm and 587 nm, respectively. Briefly, 5 µL of each brain cortical homogenate were incubated in 45 µL of fructose 6-phosphate assay buffer. Then, the reaction was initiated by the addition of 50 µL of reaction mix and, after an incubation of 5 min at 37ºC, the fluorescence was measured in a SpectraMax Gemini EM multiplate fluorescence reader (Molecular Devices LCC, San Jose, California, USA), using an excitation and emission wavelengths of 535 nm and 587 nm, respectively. Fructose 6-phosphate levels were expressed as nmol/mg protein.

3.2.5.3. Determination of glucose 6-phosphate dehydrogenase activity

Glucose 6-phosphate dehydrogenase is the rate limiting enzyme of the oxidative branch of pentose phosphate pathway, that catalyzes the formation of 6-phosphogluconolactone from glucose 6-phosphate, at the expense of $NADP^+$ [Stanton, 2012; Stincone *et al.*, 2015] (Fig. 4). Glucose 6-phosphate dehydrogenase activity was determined by a method previously described by García-Nogales *et al.* (1999), with slight modifications. This method is based on the measurement, at 340 nm, of the change in absorbance resulting from the reduction of $NADP^+$ to NADPH.

Figure 4: Scheme of the reaction catalyzed by glucose 6-phosphate dehydrogenase. Abbreviations: glucose 6-phosphate dehydrogenase, G6PDH.

Briefly, 5 µL of each brain cortical lysate were incubated in 86.8 µL reaction buffer containing 50 mM Tris-HCl (pH 7.5), supplemented with 5 mM $MgCl_2$, 0.38 mM $NADP^+$ and 5 mM maleimide (an inhibitor of 6-phosphogluconate dehydrogenase). Basal absorbance was continuously read at 340 nm, 37ºC, for 3 min, with 30 s intervals, in a SpectraMax Plus 384 microplate reader. Then, the reaction was initiated by the addition of 3.3 mM glucose 6-phosphate, and the absorbance continuously read for 10 min, with 30 s intervals. Glucose 6-phosphate dehydrogenase activity was calculated through the mean of slopes of duplicates, obtained during the linear phase. Glucose 6-phosphate dehydrogenase specific activity was determined as the difference between basal activity in the absence and presence of 3.3 mM glucose 6-phosphate (specific substrate). An ε_{340nm} =6220 M^{-1} cm^{-1} and normalization to protein amount were applied. Glucose 6-phosphate dehydrogenase activity was expressed as nmol glucose 6-phosphate/min/mg protein.

3.2.6. Determination of enzymes' activities from TCA cycle

TCA cycle function was given by the activity of the enzymes citrate synthase, α-ketoglutarate dehydrogenase and malate dehydrogenase.

3.2.6.1. Determination of citrate synthase activity

Citrate synthase catalyzes the first reaction of the TCA cycle: the condensation of oxaloacetate and acetyl-CoA to yield citrate and free coenzyme A with thiol groups (CoA-SH) (Fig. 5.1) [Akram, 2014].

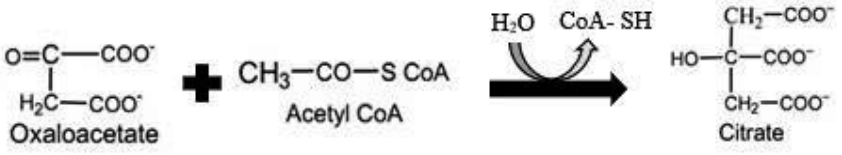

Figure 5.1: Scheme of the reaction catalyzed by citrate synthase.

The activity of citrate synthase was determined according to a method previously described by Coore *et al.* (1971), with slight modifications. This method is based on the reaction between 5,5′-dithiobis 2-nitrobenzoic acid (DNTB) and CoA-SH that results in formation of a yellow product (5-thio-2-nitrobenzoic acid, TNB) that absorbs at 412 nm (Fig. 5.2).

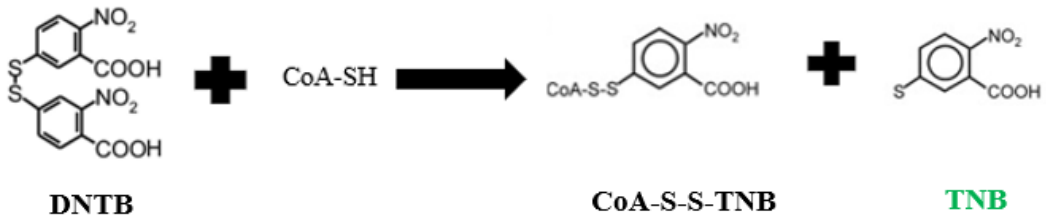

Figure 5.2: Principle of the colorimetric reaction used for the activity of citrate synthase.

Briefly, 5 µL of each brain cortical homogenate were added, in a 96-well plate, to 89 µL reaction buffer containing 200 mM Tris (pH=8.0), supplemented with 0.2 mM acetyl-CoA, 0.2 mM DTNB. Basal absorbance was continuously read at 412 nm, 37ºC, for 3 min, with 30 s intervals, in a SpectraMax Plus 384 microplate reader. Then, the reaction was initiated by the addition of 100 µM oxaloacetate, and the absorbance was read again for 6 min, with 30 s intervals. Finally, a negative control was performed with 0.1% Triton X-100 (a nonionic detergent that solubilizes mitochondria, inhibiting citrate synthase) [Gurtubay *et al.*, 1980], and the absorbance was read again for 6 min, with 30 s intervals. Citrate synthase activity was calculated through the mean of slopes of duplicates, obtained during the linear phase. Citrate synthase activity was determined as the difference between basal activity in the absence and presence of 0.1% Triton X-100. An ε_{412nm} =13.6 mM^{-1} cm^{-1} and normalization to protein amount were applied. Citrate synthase activity was expressed as µmol oxaloacetate/min/mg protein.

3.2.6.2. Determination of α-ketoglutarate dehydrogenase activity

α-Ketoglutarate dehydrogenase is the fourth enzyme of TCA cycle and catalyzes the conversion of α-ketoglutarate into succinyl-CoA, with the production of NADH (Fig. 6) that can subsequently provide electrons for the mitochondrial respiratory chain complex I [Osellame *et al.*, 2012; Vatrinet *et al.*, 2017].

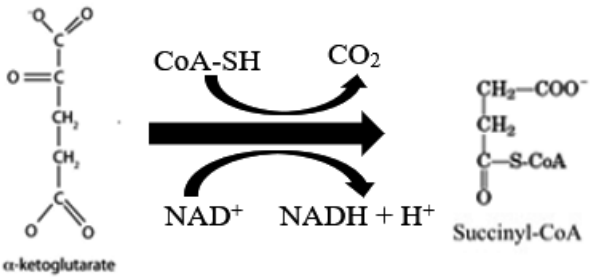

Figure 6: Scheme of the reaction catalyzed by α-ketoglutarate dehydrogenase.

α-Ketoglutarate dehydrogenase activity was determined according to a previously described method by Starkov *et al.* (2004), with some modifications. This method is based on the colorimetric detection of NAD^+ reduction, at 340 nm. Briefly, 5 µL of each brain cortical homogenate were incubated in 90 µL 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 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 in 96-well UV plates, at 340 nm, 37ºC, during 2 min, with 30 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 5 min, with 30s intervals. α-ketoglutarate dehydrogenase activity was calculated through the mean of slopes of duplicates, obtained during the linear phase. α-Ketoglutarate dehydrogenase specific activity was determined as the difference between basal activity in the absence and presence of 5 mM α-ketoglutarate. An $\varepsilon_{340nm} = 6220\ M^{-1}\ cm^{-1}$ and normalization to protein amount were applied. α-Ketoglutarate dehydrogenase activity was expressed as nM α-ketoglutarate/min/mg protein.

3.2.6.3. Determination of malate dehydrogenase activity

Malate dehydrogenase is the last enzyme of TCA cycle, that reversibly catalyzes the oxidation of malate to oxaloacetate, with the concomitant reduction of NAD^+ to NADH and its subsequent involvement in mitochondrial respiratory chain complex I [Dasikla, *et al.*, 2015; Osellame *et al.*, 2012] (Fig. 7).

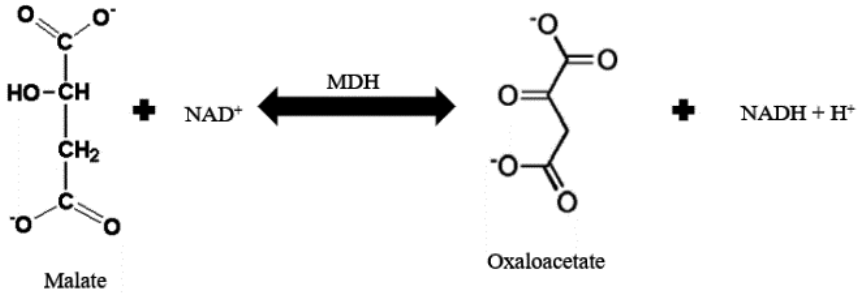

Figure 7: Scheme of the reaction catalyzed by malate dehydrogenase.

Malate dehydrogenase activity was determined according to a previously described procedure by Nulton-Persson & Szweda (2001), with some modifications. This method is based on the colorimetric evaluation of the reduction of NAD^+ at 340 nm. Briefly, 5 µL of each brain cortical homogenate were incubated in 74.27 µL lysis buffer, containing (in mM): 25 KH_2PO_4 (pH=7.25), 0.5 EDTA, 0.01% Triton X-100, supplemented with 40 µM rotenone, 5 mM $MgCl_2$, 1 U/mL citrate synthase, 0.3 mM acetyl-CoA, 10 mM NAD^+. Basal absorbance was continuously read 96-well UV plates at 340 nm, 37ºC, for 2 min, with 30 s intervals, in a SpectraMax Plus 384 microplate reader. The reaction was initiated upon the addition of 25 mM malate and absorbance read again for 20 min, with 2 min intervals. Malate dehydrogenase activity was calculated through the mean of slopes of duplicates, obtained during the linear phase. Malate dehydrogenase specific activity was determined as the difference between basal activity in the absence and presence of 25 mM malate (substrate). An $\varepsilon_{340nm} = 6220\ M^{-1}\ cm^{-1}$ and normalization to protein amount were applied. Malate dehydrogenase activity was expressed as nM malate/min/mg protein.

3.2.7. Determination of mitochondrial respiratory chain complexes I-IV activities

3.2.7.1. Determination of Complex I activity

Mitochondrial complex I (NADH-ubiquinone oxidoreductase) is the main entry point for the electrons from NADH in the respiratory chain to reduce ubiquinone towards ubiquinol and, therefore, it occupies a central role in energy metabolism [Mimaki *et al.*, 2012; Sharma *et al.*, 2009]. Complex I activity was determined by a method previously described by Long *et al.* (2009), with slight modifications. This method uses 2,6-dichlorophenolindophenol (DCPIP) as the final acceptor of NADH electrons (instead of

exogenous ubiquinone) [Long *et al.,* 2009], with the advantages of preventing the accumulation of ubiquinol and the subsequent inhibition of complex I [Bénit *et al*., 2008], together with the mitigation of the signal obtained from other unspecific dehydrogenases, since the absorbance is read at 600 nm (from DCPIP, instead of 340 nm, as the experimental procedures used to measure complex I activity in isolated mitochondria based on NADH oxidation) [Long *et al.,* 2009].

Briefly, 5 µL of each brain cortical homogenate were diluted in 91.83 µL reaction buffer containing (in mM): 25 KH_2PO_4 (pH 7.5), 5 $MgCl_2$, 0.3 KCN, 0.004 antimycin A, supplemented with 3 mg/mL BSA fatty acid-free, 60 µM coenzyme Q_1 and 160 µM DCPIP. Complex I activity was continuously measured at 600 nm, for 6 min, with 30s intervals, in a SpectraMax Plus 384 microplate reader, by following the decrease in absorbance of DCPIP at 37ºC, upon addition of 100 µM NADH, freshly prepared. Mitochondrial complex I activity was calculated through the mean of slopes of duplicates, 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). An ε_{DCPIP} =19.1 mM^{-1} cm^{-1} and normalization to protein amount were applied. The activity of complex I was expressed as mmol DCPIP/min/mg protein.

3.2.7.2. Determination of Complexes II/III activities

Mitochondrial complex II (also known as succinate dehydrogenase or succinate: ubiquinone oxidoreductase) has a pivotal position within mitochondrial metabolism, since it is involved in both TCA cycle and mitochondrial complex II, thereby promoting their crosslink for energy production [Iverson *et al.,* 2012; Sousa *et al*., 2018]. Similar to mitochondrial complex I, complex II reduces ubiquinone to ubiquinol that is subsequently oxidized by mitochondrial complex III, and its electrons transferred to cytochrome c [Iverson *et al.,* 2012; Sousa *et al*., 2018]. Activities of complexes II/III (succinate-cytochrome c reductase) were determined by a method previously described by Tisdale (1967), with slight modifications. Briefly, 5 µL of each cortical homogenate were pre-incubated at 37ºC, for 5 min, in 60 µL of phosphate buffer containing: 166 mM KH_2PO_4 (pH 7.4), supplemented with 1 mM KCN and 33.2 mM sodium succinate. The reaction was initiated upon the addition of 10 µL phosphate buffer, supplemented with 0.1 mM oxidized cytochrome c (substrate of complex III) plus 0.3 mM EDTA-$2K^+$ (a calcium

chelator that regulates its levels for a proper oxidative phosphorylation) [Glancy *et al.* 2013]. Mitochondrial complex II/III activities were measured by following the reduction of cytochrome c, at 550 nm, for 5 min with 30s intervals, using a spectrophotometer SpectraMax Plus 384. Mitochondrial complex II/III activities were determined through the mean of slopes of duplicates, obtained during the linear phase. Mitochondrial complex II/III specific activities were determined as the difference between basal activity in the absence and presence of 36.5 µM antimycin A (specific inhibitor of complex III). An ε_{550nm} = 19.1 mM^{-1} cm^{-1} and normalization to protein amount were applied. Mitochondrial complex II/III activities were expressed as nmol oxidized cytochrome c/min/mg protein.

3.2.7.3. Determination of Complex III activity

Mitochondrial complex III (cytochrome c reductase) catalyzes both the oxidation of ubiquinol and the reduction of cytochrome c [Chandel, 2010; Sousa *et al*., 2018], and its activity was determined according to a previously described method by Luo *et al.* (2008), with slight modifications. Briefly, 5 µL of each brain cortical homogenate were incubated in 87.62 µL reaction buffer containing 50 mM KH_2PO_4 (pH 7.5), 3.75 µM rotenone, 0.05% Tween-20 and 0.2 mM freshly-prepared decylubiquinone, at 37ºC, and basal enzymatic activity was read at 550 nm, for 3 min, with 30s intervals, in a spectrophotometer SpectraMax Plus 384. Then, complex III activity was measured by following the reduction of cytochrome c upon the addition of 75 µM oxidized cytochrome c, for 5 min, with 30s intervals. Complex III activity was calculated through the mean of slopes of duplicates, obtained during the linear phase. Mitochondrial complex III specific activity was calculated as the difference between basal activity in the absence and presence of 2.5 µM antimycin A (specific inhibitor of complex III). An ε_{550nm} = 19.6 mM^{-1} cm^{-1} and normalization to protein amount were applied. Mitochondrial complex III activity was expressed as nmol oxidized cytochrome c/min/mg protein.

3.2.7.4. Determination of Complex IV activity

Mitochondrial complex IV (cytochrome c oxidase) catalyzes the transfer of electrons from reduced cytochrome c to O_2 to yield H_2O, generating the electrochemical gradient necessary to the ATP synthesis [Sousa *et al.*, 2018]. Its activity was determined according to a previously described method by Brautigan *et al.* (1978), with slight modifications. Briefly, 5 µL of each brain cortical homogenate were incubated, at 37°C,

in 71.55 µL reaction buffer containing 75 mM KH_2PO_4 (pH 7.0), supplemented with 3.65 µM antimycin A and 0.05% n-dodecyl-β-*D*-maltoside. Basal enzymatic activity was read at 550 nm, for 3 min, with 30s intervals, using a spectrophotometer SpectraMax Plus 384. Then, we measured the enzymatic activity given by the oxidation of cytochrome c upon the addition of 7.2 µM of freshly prepared reduced cytochrome c, for 6 min, with 30s intervals. Complex IV activity was calculated through the mean of slopes of duplicates, 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 $\varepsilon_{550nm} = 19.6\ mM^{-1}cm^{-1}$ and normalization to protein amount were applied. Mitochondrial complex IV activity was expressed as nmol reduced cytochrome c/min/mg protein.

3.2.8. Evaluation of the phosphocreatine/creatine kinase system

Creatine kinase catalyzes the reversible ATP-dependent phosphorylation of creatine into phosphocreatine (Fig. 8), and since the phosphocreatine/creatine kinase system can generate ATP in response to local energy demands faster than oxidative phosphorylation and glycolysis, creatine kinase is an important regulator of energy homeostasis [Wallimann *et al.*, 2011; Zhang *et al.*, 2011].

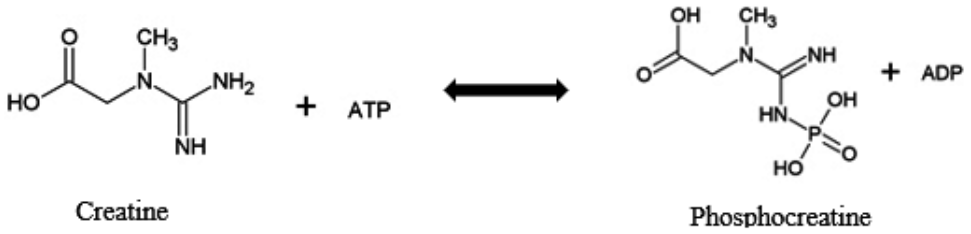

Figure 8: Schematic representation of the phosphocreatine/creatine kinase system.

3.2.8.1. Determination of the rate of ATP formation

The rate of ATP formation was determined similarly to the protocol described above to determine glucose 6-phosphate formation [Lamprecht *et al.*, 1974], by coupling the glucose 6-phosphate dehydrogenase and hexokinase reactions. Briefly, 5 µL of each brain cortical homogenate were incubated in a 96-well UV plate with 72.45 µL of reaction medium containing (in mM): 50 triethanolamine-hydrochloride and 22 NaOH, pH 7.5, supplemented with 0.2 mM β-$NADP^+$ sodium salt, 8.35 mM $MgCl_2$, 743.75 U/L glucose-

6-phosphate dehydrogenase and 41.75 mM *D*-glucose. Absorbance was continuously read at 37ºC, for 2 min, with 30 s intervals, at 339 nm, in a SpectraMax Plus 384 microplate reader. Then, the reaction was started by the addition of 595 U/L hexokinase from baker's yeast (*S. cerevisæ*), type F-300, sulfate-free, and the absorbance read again for 3 min, with 30 s intervals. The rate of ATP formation was calculated by using a ε_{339nm} = 1 mol^{-1} mm^{-1}, from the extrapolation of absorbance, according to the formula:

$$A_2 - A_1 = \Delta A$$

where A_2 was the reading after the addition of hexokinase and A_1 was the basal reading. Results were normalized to the amount of protein and expressed as µmol/min/mg protein.

3.2.8.2. Determination of the rate of phosphocreatine formation

The rate of phosphocreatine formation was determined similarly to the protocol described above to determine glucose 6-phosphate formation [Lamprecht *et al.*, 1974], by coupling the glucose 6-phosphate dehydrogenase, hexokinase and creatine kinase reactions. Briefly, 5 µL of each brain cortical homogenate were incubated in a 96-well UV plate with 72.45 µL of reaction medium containing (in mM): 50 triethanolamine-hydrochloride and 22 NaOH, pH 7.5, supplemented with 0.2 mM β-$NADP^+$ sodium salt, 8.35 mM $MgCl_2$, 743.75 U/L glucose-6-phosphate dehydrogenase from baker's yeast (*S. cerevisæ*), type VII, ammonium sulphate suspension, 41.75 mM *D*-glucose, 595 U/L hexokinase from baker's yeast (*S. cerevisæ*), type F-300, sulfate-free, and 0.18 mM ADP disodium salt. Absorbance was continuously read at 37ºC, for 2 min, with 30 s intervals, at 339 nm, in a SpectraMax Plus 384 microplate reader. Then, the reaction was started by the addition of 31.35 kU/L creatine kinase from rabbit muscle, and the absorbance read again for 3 min, with 30 s intervals. The rate of phosphocreatine formation was calculated by using a ε_{339nm} =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 normalized to the amount of protein and expressed as µmol/min/mg protein.

3.2.9. 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's instructions. The enzyme 3-hydroxybutyrate dehydrogenase (HBDH) catalyzes the conversion of acetoacetic acid to 3-hydroxybutyric acid and the reverse reaction, depending on the pH. Thus, the measurement of acetoacetic acid (AcAc) and 3-hydroxybutyric acid (BOH) levels is based on changes in the absorbance of NADH, at 340 nm, resulting from the action of this enzyme (Fig. 9).

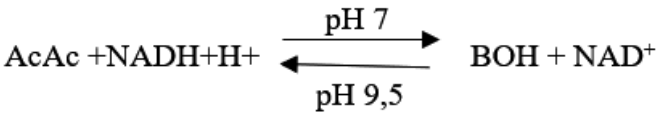

Figure 9: Scheme of the reactions catalyzed by 3-hydroxybutyrate dehydrogenase, showing the reagents and products, as well as the pH conditions for their activity.

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

3.2.10. Measurement of markers for oxidative/nitrosative stress

3.2.10.1. Determination of 8-OHdG levels

Levels of one of the most common DNA oxidative species, 8-OHdG, were determined using the DNA/RNA Oxidative Damage ELISA Kit, following manufacturers' instructions, with slight modifications. This kit is based on the competition between oxidatively damaged guanine species and the 8-OH-dG-acetylcholinesterase conjugate (a tracer for DNA/RNA oxidative damage), for a limited amount of monoclonal antibody against DNA/RNA oxidative damage. Then, this complex antibody-oxidatively damaged guanine binds to the goat polyclonal anti-mouse

IgG previously attached to the wells. The final product of this enzymatic reaction has a yellow color and its absorbance can be measured at 405 nm. Briefly, 5 µL of each brain cortical homogenate were incubated in 45 µL of ELISA buffer. Then, 50 µL of both the tracer and monoclonal antibody against DNA/RNA oxidative damage were added to the wells, and the plate was incubated for 18h, at 4ºC. Finally, the absorbance was measured after the addition of 200 µL Ellman's Reagent, at 405 nm, in a SpectraMax Plus 384 spectrophotometer. 8-OHdG levels were expressed as pg/mg protein.

3.2.10.2. Quantification of TBARS levels

TBARS assay is a simple and fast procedure to measure the extent of lipid peroxidation in cells, tissues and body fluids [Sochor *et al.*, 2012]. TBARS levels were measured according to a previously described method by Ernster & Nordenbrand (1967), with slight modifications. This assay is based on the ability of MDA (one of the most common products of lipid peroxidation) to react, under acidic conditions and high temperature, with thiobarbituric acid (TBA) to yield a pink MDA-TBA complex that is quantified spectrophotometrically at 530 nm (Fig. 10). Briefly, 10 µL of each brain cortical homogenate were boiled at 100°C, for 10 min, in 190 µL of reaction medium containing: 0.026 M TBA, 15% of trichloroacetic acid (TCA), 0.25 M HCl. Then, the samples were chilled on ice to stop the reaction, and centrifuged at 3000 rpm for 10 min, at 4°C, in a Sigma 2-16 PK centrifuge. The supernatant was collected, and the absorbance read at 530 nm, against a blank prepared under similar conditions, but in the absence of protein. The amount of TBARS formed was calculated using a $\varepsilon_{530nm} = 1.56 \times 10^5$ mol^{-1} cm^{-1}. TBARS levels were expressed as pmol/mg protein.

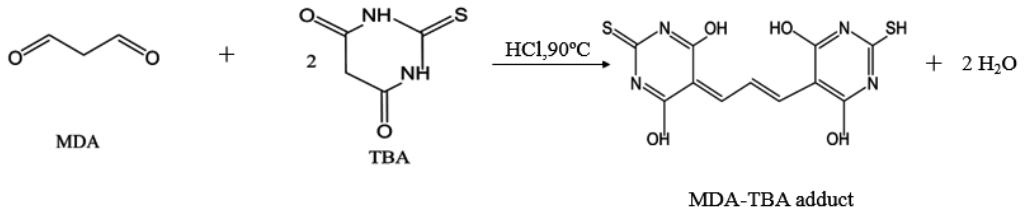

Figure 10: Scheme of the reaction between MDA and TBA, to a form a pink-colored MDA-TBA complex that is quantified spectrophotometrically at 530 nm.

3.2.10.3. Determination of nitrites levels

Nitrite (NO_2^-) is the final product of nitric oxide oxidation and its concentration is an indirect measure of nitric oxide production in biological systems [Giustarini *et al.*, 2008]. Nitrites levels were measured according to a previously described method by Green *et al.* (1981), with slight modifications. This assay is based on the reaction, under acidic conditions, of nitrite with sulfanilamide from the Griess reagent to produce a diazonium ion that is then coupled with *N*-(1-naphthyl) ethylenediamine to yield a chromophoric azo product that strongly absorbs at 550 nm (Fig. 11).

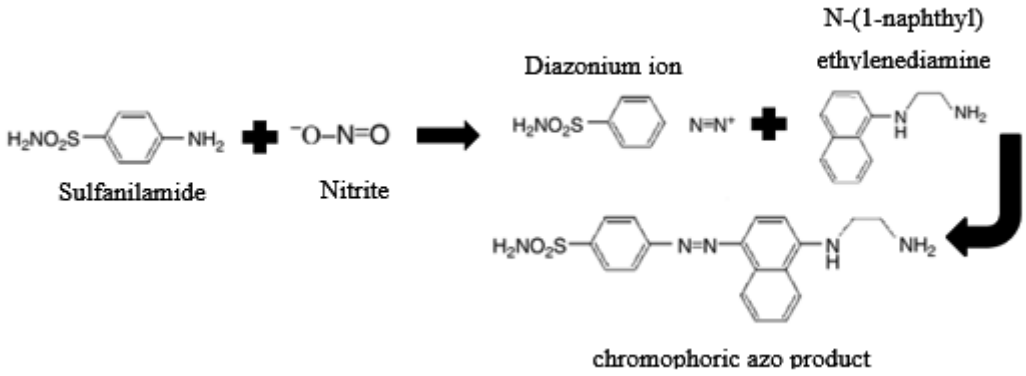

Figure 11: Diagram representing the principle of the Griess reaction used to measure nitrites levels.

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

3.2.11. Statistical analysis

Results were presented as scatter-dot plot with bar (mean ± SEM) of the indicated number of mice/group. Statistical analysis and graphic artwork were obtained using the GraphPad Prism 6.0 software. After identification of outliers with the ROUT test and the Kolmogorov-Smirnov normality test, statistical significance was determined using the one-way ANOVA test with Tukey or Sidak post-hoc tests for multiple comparisons (for a Gaussian distribution) or the Kruskal-Wallis test, with Dunn's post-test (for a non-

Gaussian distribution). Differences with a $P<0.05$ were considered statistically significant.

Chapter 4

Results

4.1. Peripheral Characterization of Early Symptomatic R6/2 Mice

According to our previously published peripheral characterization of the experimental groups used [Duarte *et al.*, 2018], at the end of treatment, 12-week-old, saline-treated R6/2 mice were hyperglycemic and liraglutide alone or together with ghrelin normalized their serum glucose levels (Table 2). In addition, HOMA-β and HOMA-IR indexes (two homeostasis models that assess β-cell function and insulin resistance, respectively [Duarte *et al*., 2018]) were significantly lower in saline-treated R6/2 mice compared to WT littermates, being rescued by liraglutide alone or together with ghrelin (Table 2). This suggested that both treatments may exert beneficial effects on the R6/2 mouse peripheral blood glucose homeostasis by ameliorating insulin resistance and β-cell function [Duarte *et al*., 2018].

Table 2. Effect of liraglutide plus ghrelin on blood biochemical features in 12-week old R6/2 mice.

	WT mice	R6/2 mice		
	+NaCl	+NaCl	+Liraglutide	+Liraglutide +Ghrelin
Serum glucose levels (mM, n = 10)	9.3 ± 0.47	15.11 ± 1.27**	8.72 ± 0.89^{ff}	9.89 ± 1.6^{f}
Serum insulin levels (mg/L, n = 8–10)	0.079 ± 0.0003	9.3 ± 0.47	15.11 ± 1.27**	0.081 ± 0.0011
HOMA-IR (n = 10)	0.81 ± 0.04	1.33 ± 0.11**	0.80 ± 0.095^{ff}	0.99 ± 0.15^{f}
HOMA-β (n = 9)	660.8 ± 36.8	339.8 ± 31.1*	927.1 ± 180.6^{f}	1202 ± 357.6^{ff}

Data are mean ± SEM of the indicated number of mice/group. Statistical significance: **P*<0.05, ***P*<0.01 *vs.* saline-treated WT mice; and ^{f}*P*<0.05, ^{ff}*P*<0.01 *vs.* saline-treated R6/2 mice, by one-way ANOVA, with Tukey or Sidak post-hoc tests for multiple comparisons (for a Gaussian distribution), or with the Kruskal-Wallis test, with Dunn's post-test (non-Gaussian distribution) [Adapted from Duarte *et al.*, 2018].

4.2. Ghrelin Alone Or In Combination With Liraglutide Slightly Stimulate Brain Cortical Pentose Phosphate Pathway In Early Symptomatic R6/2 Mice

Recently, we demonstrated that liraglutide *per se* or in combination with ghrelin decreased both brain cortical lactate and AMP levels, and improved its energy status in early symptomatic R6/2 mice, suggesting an attenuation in their brain cortical catabolic pathways [Duarte *et al*., 2018]. Others found an impairment of glycolysis in the striatum of HD patients and BACHD mice [Boussicault *et al*., 2014; Powers *et al*., 2007], while glucose 6-phosphate dehydrogenase (the rate-limiting enzyme of the pentose phosphate

pathway oxidative branch) remained unchanged in brains from HD patients and in the cortex from 12-week old R6/2 mice [Bird *et al.*, 1977; Choo *et al.*, 2005]. This suggested that the lower glycolytic flux in HD brain may not arise from a diversion of glucose-6-phosphate towards the pentose phosphate pathway. Hence, we next analyzed the effect of liraglutide alone or plus ghrelin on downstream markers for brain cortical glycolysis and pentose phosphate pathway (particularly on the formation of glucose 6-phosphate, fructose 6-phosphate and in the activity of glucose 6-phosphate dehydrogenase) in early symptomatic R6/2 mice (Fig. 12).

We observed that brain cortical glucose 6-phosphate formation was tendentiously higher (by 2.4-fold) in saline-treated R6/2 mice compared to WT littermates (Fig. 12A). Ghrelin administration *per se* or together with liraglutide slightly increased glucose 6-phosphate formation (by 1.5- and 1.4-fold, respectively), whereas liraglutide alone slightly decreased its formation (by 0.4-fold) in R6/2 mice compared to those injected with saline solution (Fig. 12A).

Similar to glucose 6-phosphate formation, brain cortical fructose 6-phosphate was 1.9-fold higher in early symptomatic saline-treated R6/2 mice than in age-matched WT littermates (P=0.003) (Fig. 12B). Ghrelin alone or together with liraglutide slightly decreased their fructose 6-phosphate levels (by 0.3-fold, P=0.09; and by 0.2-fold, respectively), while liraglutide did not affect brain fructose 6-phosphate content in R6/2 mice compared to saline-treated animals (Fig. 12B).

Despite no significant changes in brain cortical glucose 6-phosphate dehydrogenase activity between saline-treated R6/2 mice and WT littermates, ghrelin and liraglutide *per se* slightly increased its activity (by 5.7- and 1.7-fold, respectively) compared to saline-treated R6/2 mice (Fig. 12C). Co-administration of liraglutide and ghrelin also slightly stimulated (by 3.3-fold) glucose 6-phosphate dehydrogenase in R6/2 mice (Fig. 12C).

These results suggest that ghrelin and liraglutide *per se* or in combination tend to stimulate the metabolism of brain cortical glucose 6-phosphate via the pentose phosphate pathway in early symptomatic R6/2 mice.

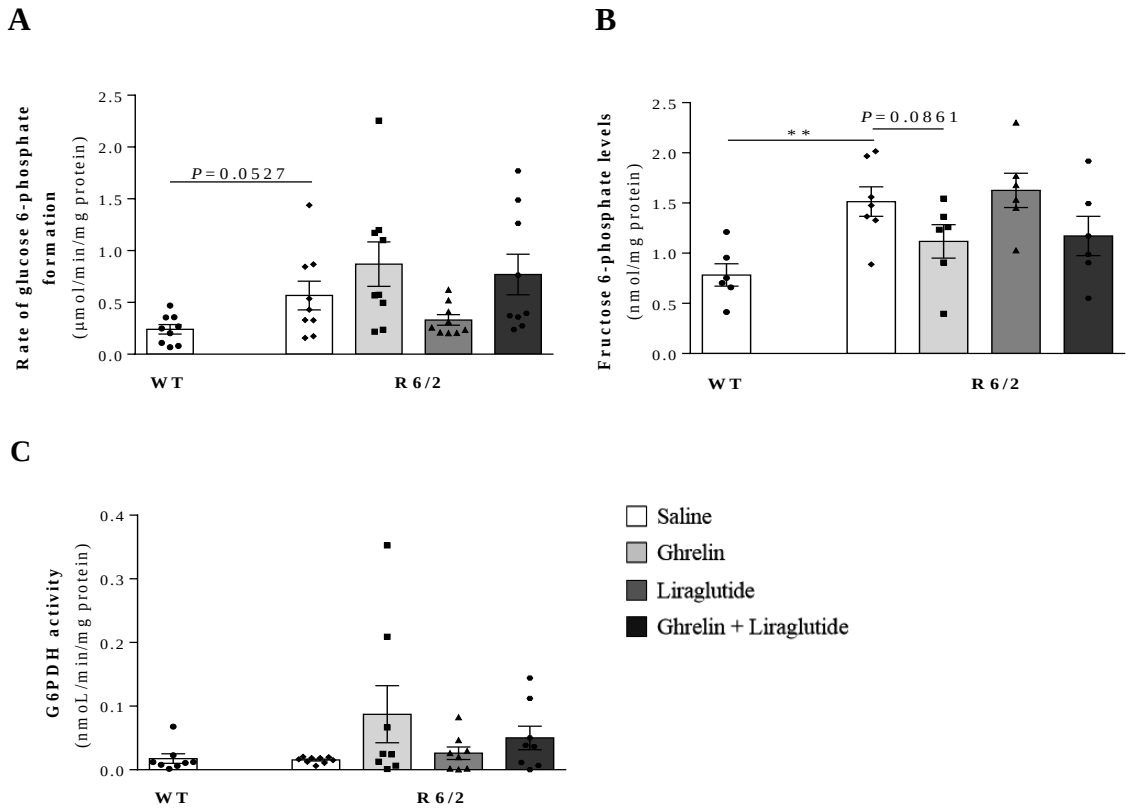

Figure 12: Effect of liraglutide plus ghrelin administration on brain cortical glycolytic and pentose phosphate pathway markers in early symptomatic R6/2 mice. Glycolysis markers included the rate of glucose 6-phosphate formation (A) and fructose 6-phosphate content (B), while pentose phosphate was given by the activity of glucose 6-phosphate dehydrogenase (G6PDH) (C) in brain cortical lysates from 12-week old R6/2 mice, treated with saline, ghrelin and/or liraglutide. Data are mean ± SEM of the indicated number of mice/group. Statistical significance: **P*<0.05, ***P*<0.01, by the Kruskal-Wallis test, with Dunn post-test (for a non-Gaussian distribution) (A, C), or by one-way ANOVA, with Tukey or Sidak post-hoc tests for multiple comparisons (for a Gaussian distribution) (B).

4.3. Ghrelin And/Or Liraglutide Tend To Stimulate Brain Cortical TCA Cycle In Early Symptomatic R6/2 Mice

Numerous studies showed that HD is associated with an inhibition of several enzymes from brain TCA cycle (including citrate synthase, aconitase, α-ketoglutarate dehydrogenase complex and succinate dehydrogenase) [Benchoua *et al.*, 2006; Butterworth *et al.*, 1985; Chen *et al.*, 2017; Damiano *et al.*, 2013; Klivenyi *et al.*, 2004; Sorolla *et al.*, 2010; Tabrizi *et al.*, 1999]. Therefore, we evaluated the effect of liraglutide alone or plus ghrelin on the enzymes for TCA cycle citrate synthase, α-ketoglutarate dehydrogenase and malate dehydrogenase in brain cortices from early symptomatic R6/2 mice (Fig. 13).

We observed that ghrelin and liraglutide *per se* significantly recovered (by 3.4-fold, *P*=0.026; and by 3.3-fold, *P*=0.016, respectively) the massive decrement (by 0.8-fold, *P*=0.0006) in brain cortical citrate synthase activity in 12-week old saline-treated R6/2 mice (Fig. 13A). Co-administration of liraglutide and ghrelin only tendentiously increased citrate synthase activity (by 1.8-fold) compared to saline-treated R6/2 mice (Fig. 13A).

Similar to citrate synthase, α-ketoglutarate dehydrogenase activity was massively downregulated (by 0.7-fold, *P*=0.021) in brain cortices from saline-treated R6/2 mice compared to WT littermates (Fig. 13B). Although ghrelin *per se* did not affect α-ketoglutarate dehydrogenase activity in R6/2 mice compared to saline-treated ones, liraglutide alone or together with ghrelin slightly augmented this enzyme's activity in R6/2 mouse brain cortices (by 2.8-fold; and by 4.2-fold, *P*=0.082, respectively) (Fig. 13B).

Brain cortical malate dehydrogenase activity was also slightly lower (by 0.7-fold) in saline-treated R6/2 mice compared to WT littermates, being reversed after ghrelin administration *per se* (by 4-fold, *P* =0.0043) when compared to saline-treated R6/2 mice (Fig. 13C). Interestingly, liraglutide alone or together with ghrelin only slightly enhanced malate dehydrogenase activity (by 1.3- and 1.8-fold, respectively) compared to vehicle treated R6/2 mice (Fig. 13C).

These results suggest that ghrelin and liraglutide *per se* or in combination tend to stimulate brain cortical TCA cycle in early symptomatic R6/2 mice.

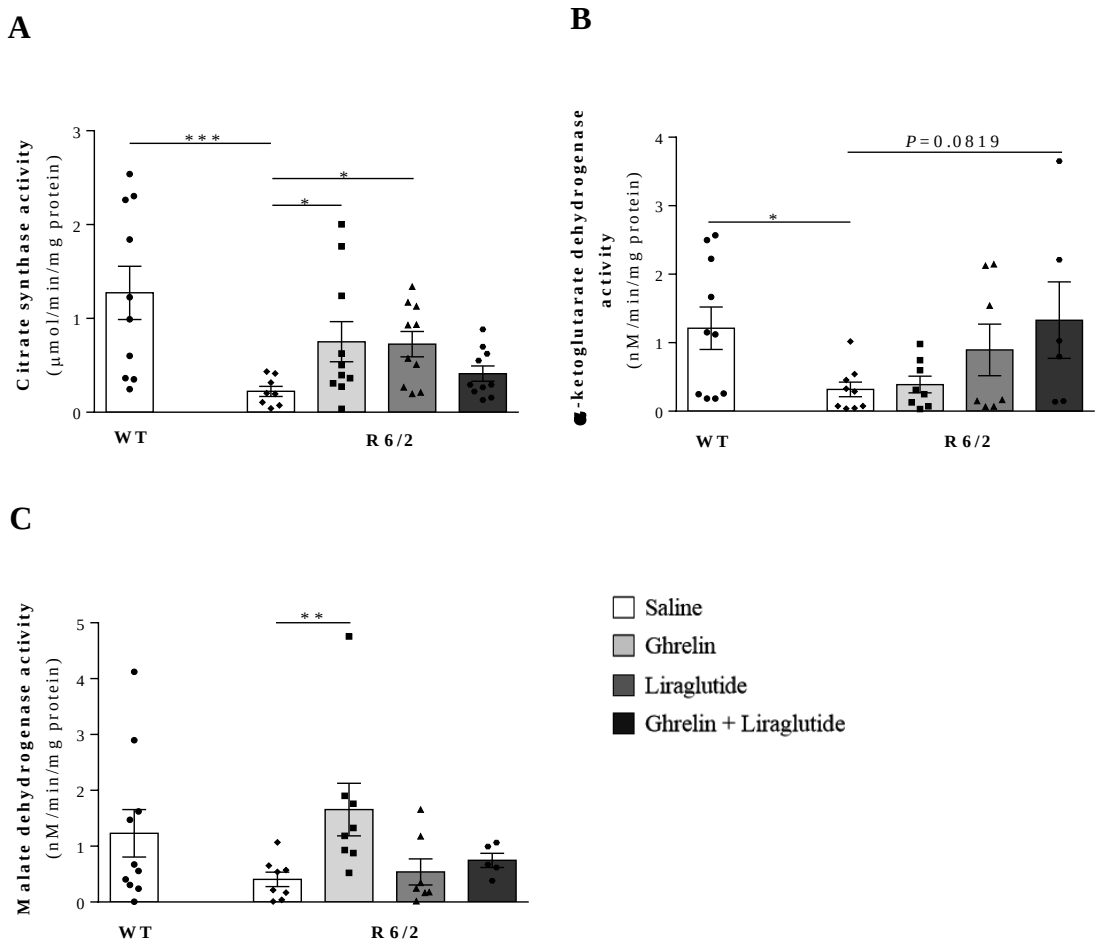

Figure 13: Effect of liraglutide plus ghrelin administration on brain cortical TCA cycle markers of early symptomatic R6/2 mice. TCA cycle markers included the activities of citrate synthase (A), α-ketoglutarate dehydrogenase (B) and malate dehydrogenase (C) in brain cortical lysates from 12-week old R6/2 mice, treated with saline, ghrelin and/or liraglutide. Data are mean ± SEM of the indicated number of mice/group. Statistical significance: **P*<0.05, ***P*<0.01, ****P*< 0.001, by the Kruskal-Wallis test, with Dunn post-test (for a non-Gaussian distribution).

4.4. Liraglutide Alone Or In Combination With Ghrelin Recovers The Activity Of Brain Cortical Mitochondrial Respiratory Chain Complex IV In Early Symptomatic R6/2 Mice

Increasing evidence point towards the inhibition of mitochondrial respiratory chain complexes II and III and, to a lesser extent, of complex IV in the striatum of HD patients at an advanced neuropathological stage [Browne *et al.*, 1997; Gu *et al.*, 1996; Tabrizi *et al.*, 1999], while others reported that cortical and striatal mitochondrial respiratory chain complexes remained unchanged in presymptomatic and early symptomatic human HD brains [Guidetti *et al.*, 2001; Powers *et al.*, 2007]. In this perspective, we analyzed the

role of liraglutide alone or plus ghrelin on the activities of mitochondrial respiratory chain complexes I-IV in brain cortices from early symptomatic R6/2 mice (Fig. 14).

We observed that mitochondrial respiratory complex I activity was slightly lower (by 0.6-fold) in the brain cortices from early symptomatic saline-treated R6/2 mice than in WT littermates (Fig. 14A). Although ghrelin alone did not alter mitochondrial complex I activity in R6/2 mice, liraglutide *per se* slightly stimulated (by 1.4-fold) and together with ghrelin tendentiously reduced (by 0.2-fold) its activity in these mice compared to saline-treated R6/2 mice (Fig. 14A).

Albeit brain cortical mitochondrial respiratory complexes II/III activities remained similar between saline-treated R6/2 mice and their WT littermates, and in ghrelin-treated R6/2 mice compared to saline-treated ones, liraglutide alone or together with ghrelin slightly inhibited mitochondrial respiratory complexes II/III activities (by 0.8- and 0.4-fold, respectively) in R6/2 mice (Fig. 14B).

Regarding brain cortical mitochondrial respiratory complex III activity, neither ghrelin nor liraglutide *per se* were able to overcome its massive inhibition (by 0.7-fold, P=0.042) in early symptomatic saline-treated R6/2 mice (Fig. 14C). Conversely, co-administration of liraglutide and ghrelin slightly stimulated (by 2-fold) the mitochondrial complex III activity in R6/2 mice compared to saline-treated ones (Fig. 14C).

Similarly, to mitochondrial respiratory complex III, the activity of complex IV was massively reduced (by 0.8-fold, P=0.0262) in saline-treated R6/2 mouse brain cortices compared to WT littermates (Fig. 14D). Ghrelin and liraglutide *per se* promoted (by 1.8-fold, not significant; and 4.8-fold, P=0.040) the activity of mitochondrial respiratory complex IV in brain cortices from R6/2 mice compared to saline-treated ones (Fig. 14D). Co-administration of liraglutide and ghrelin also recovered (by 5-fold, P=0.027) mitochondrial respiratory complex IV activity in R6/2 mouse brains (Fig. 14D).

These results suggest that liraglutide *per se* or in combination with ghrelin restores the activity of brain cortical mitochondrial respiratory complex IV in early symptomatic R6/2 mice.

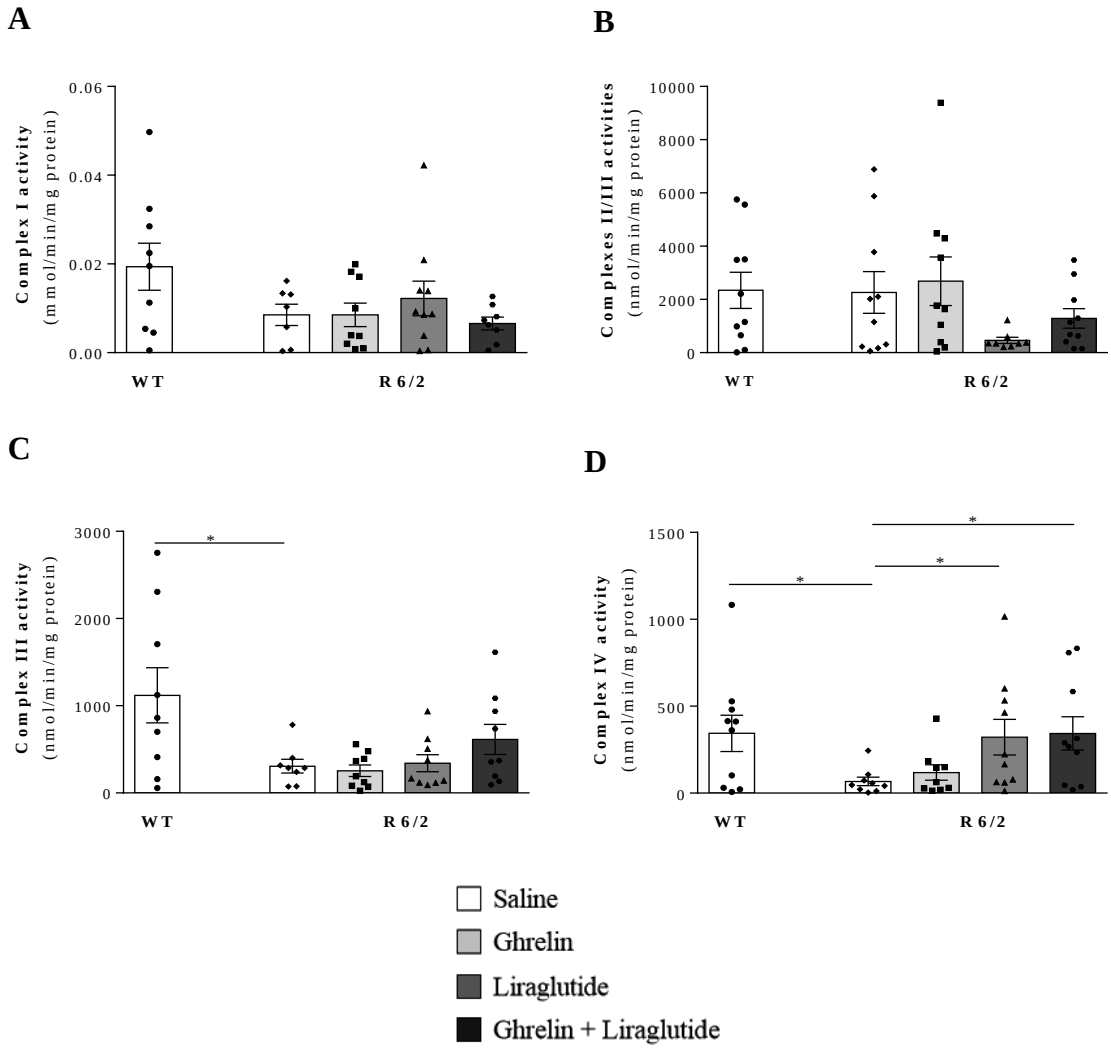

Figure 14: Effect of liraglutide plus ghrelin administration on brain cortical mitochondrial respiratory chain function in early symptomatic R6/2 mice. Activities of the mitochondrial respiratory chain complexes I-IV included the measurement of the enzymatic activities from Complex I (A), complexes II/III (B), complex III (C) and complex IV (D) in brain cortical lysates from 12-week old R6/2 mice, treated with saline, ghrelin and/or liraglutide. Data are mean ± SEM of the indicated number of mice/group. Statistical significance: **P*<0.05, by the Kruskal-Wallis test, with Dunn post-test (for a non-Gaussian distribution) (A-C); or by the one-way ANOVA, with Tukey or Sidak post-hoc tests for multiple comparisons (for a Gaussian distribution) (D).

4.5. Ghrelin Alone Or In Combination With Liraglutide Tend To Promote Brain Cortical ATP Formation Via The Phosphocreatine/Creatine Kinase System In Early Symptomatic R6/2 Mice

Besides glycolysis and oxidative phosphorylation at the mitochondrial respiratory chain, creatine kinase is an alternative source for ATP generation and maintenance of energy homeostasis via the phosphocreatine/creatine system. However, this system is compromised in HD brain [Kim *et al.*, 2010; Zhang *et al.*, 2011]. Therefore, we evaluated the role of liraglutide alone or plus ghrelin on the rates of brain cortical phosphocreatine and ATP formation from phosphocreatine in early symptomatic R6/2 mice (Fig. 15).

We observed that phosphocreatine was formed at a slightly higher rate (by 1.5-fold) in brain cortices from saline-treated R6/2 mice than in WT littermates (Fig. 15A). Conversely, ghrelin and liraglutide *per se* slightly attenuated (by 0.5- and 0.3-fold, respectively) the rate of phosphocreatine production in R6/2 mice compared to saline-treated ones (Fig. 15A). Co-administration of liraglutide and ghrelin slightly increased (by 1.3-fold) the rate of phosphocreatine formation in R6/2 mouse brains compared to saline-treated ones (Fig. 15A).

Albeit no significant alterations on the rate of brain cortical ATP formation from phosphocreatine in saline-treated R6/2 mice compared to WT littermates, ghrelin alone massively raised its values by 4.2-fold (P=0.002) compared to saline-treated R6/2 mice (Fig. 15B). In contrast, liraglutide *per se* slightly diminished (by 0.4-fold), while its co-administration with ghrelin slightly increased (by 1.6-fold) the rate of ATP formation in R6/2 mouse brains compared to saline-treated ones (Fig. 15B).

These results suggest that ghrelin alone or together with liraglutide tend to stimulate ATP generation through the phosphocreatine/creatine kinase system in brain cortices from early symptomatic R6/2 mice.

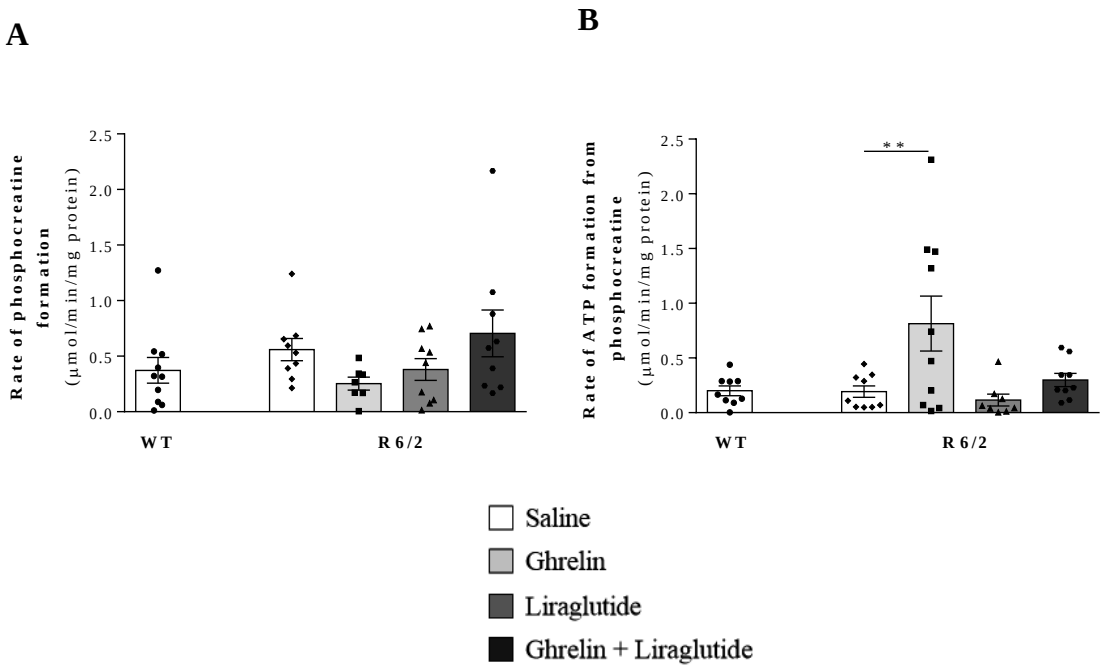

Figure 15: Effect of liraglutide plus ghrelin administration on brain cortical phosphocreatine/creatine system in early symptomatic R6/2 mice. The phosphocreatine/creatine system was given by the rates of phosphocreatine (A) and ATP formation (B) in brain cortical lysates from 12-week old R6/2 mice, treated with saline, ghrelin and/or liraglutide. Data are mean ± SEM of the indicated number of mice/group. Statistical significance: $^{**}P<0.01$, by one-way ANOVA, with Tukey or Sidak post-hoc tests for multiple comparisons (for a Gaussian distribution).

4.6. Liraglutide Alone Or In Combination With Ghrelin Increase Brain Cortical Levels Of Ketone Bodies In Early Symptomatic R6/2 Mice

Ketone bodies (acetone, acetoacetic acid and, particularly, the most abundant 3-hydroxybutyric acid) are alternative fuel sources to CNS that provide intermediates for the TCA cycle upon glucose deprivation [Newman & Verdin,2014; Puchalska & Crawford, 2017]. In addition, *D*-β-hydroxybutyric acid (an optical isomer of 3-hydroxybutyric acid) was neuroprotective in a murine model of striatal neuronal loss and in R6/2 mice [Lim *et al.*, 2011]. Hence, we determined the role of liraglutide alone or plus ghrelin on the brain cortical levels of acetoacetic acid and 3-hydroxybutyric acid in early symptomatic R6/2 mice (Fig. 16).

Brain cortical acetoacetic acid levels were 0.5-fold lower (P=0.018) in saline-treated R6/2 mice than in WT littermates (Fig. 16A). Ghrelin *per se* slightly increased (by 2-fold, P=0.066) their brain acetoacetic acid levels compared to saline-treated R6/2 mice.

Similarly, liraglutide alone or plus ghrelin augmented their levels by 2.2- and 2.6-fold (*P*=0.018 and *P*=0.005), respectively, compared to saline-treated R6/2 mice (Fig. 16A).

Similarly, 3-hydroxybutyric acid levels were slightly lower (0.5-fold, *P*=0.070) in brain cortices from saline-treated R6/2 mice compared to WT littermates (Fig. 16B). Ghrelin and liraglutide *per se* increased the levels of 3-hydroxybutyric acid by 3.1- and 2-fold (*P*=0.007 and *P*=0.038, respectively) in R6/2 mice compared to saline-treated ones (Fig. 16B). Surprisingly, co-administration of liraglutide and ghrelin only slightly increased (by 1.7-fold) the brain cortical levels of 3-hydroxybutyric acid in R6/2 mice (Fig. 16B).

These results suggest that ghrelin and liraglutide *per se* or in combination recover the brain cortical levels of the ketone bodies acetoacetic acid and 3-hydroxybutyric acid in early symptomatic R6/2 mice.

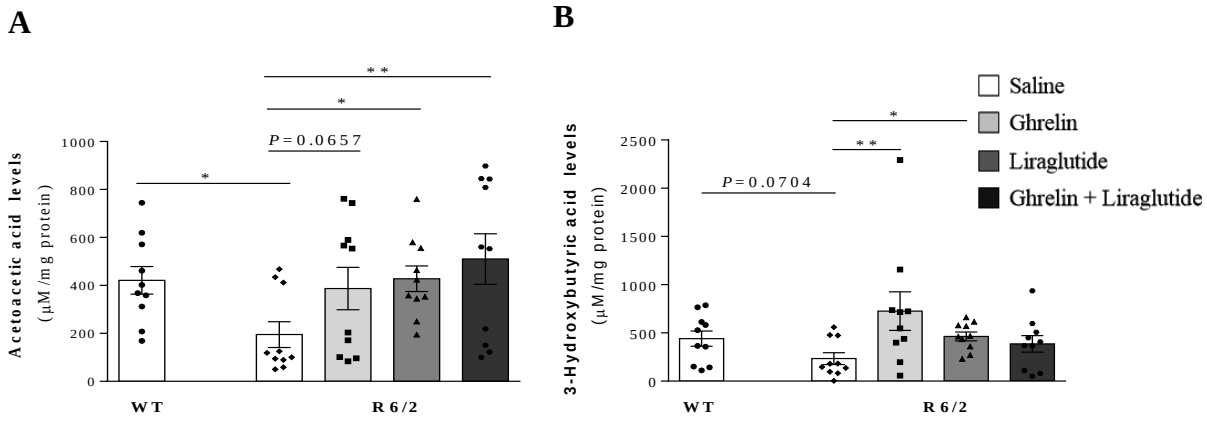

Figure 16: Effect of liraglutide plus ghrelin administration on brain cortical levels of ketone bodies in early symptomatic R6/2 mice. The ketone bodies acetoacetic acid (A) and 3-hydroxybutyric acid (B) were measured in brain cortical lysates from 12-week old R6/2 mice, treated with saline, ghrelin and/or liraglutide. Data are mean ± SEM of the indicated number of mice/group. Statistical significance: **P*<0.05, ***P*<0.01, by Kruskal-Wallis test, with Dunn post-test (for a non-Gaussian distribution).

4.7. Despite The Slight Increase In Lipid Oxidation Markers, Ghrelin *Per Se* Or In Combination With Liraglutide Tend To Reduce Brain Cortical Oxidative Damage To DNA And Nitrites Levels In Early Symptomatic R6/2 Mice

Oxidative stress may contribute to HD pathogenesis, most likely via the accumulation of oxidative markers in DNA bases (mainly 8-OHdG) [Browne *et al.*, 1997; Polidori *et al.*, 1999] and/or other oxidatively-modified macromolecules (*e.g.* 3-

nitrotyrosine, MDA or TBARS) [Ayala-Peña, 2013; Browne & Beal, 2006; Kumar & Ratan, 2016; Stack *et al.*, 2008;]. In this perspective, we determined the role of liraglutide alone or plus ghrelin on brain cortical levels of 8-OHdG, TBARS and nitrites in early symptomatic R6/2 mice (Fig. 17).

We observed that brain cortical levels of 8-OHdG were 2.2-fold higher (P=0.002) in saline-treated R6/2 mice than in WT littermates (Fig. 17A). Ghrelin *per se* or together with liraglutide slightly decreased (by 0.3-fold, P=0.067; and by 0.3-fold, respectively) brain 8-OHdG levels in R6/2 mice compared to saline-treated ones (Fig. 17A). Of note, liraglutide *per se* did not significantly affect the R6/2 mouse brain cortical 8-OHdG levels compared to saline-treated ones (Fig. 17A).

Brain cortical TBARS levels were slightly lower (by 0.5-fold) in saline-treated R6/2 mice compared to WT littermates (Fig. 17B). Ghrelin and liraglutide *per se* slightly increased (by 1.4-fold; and 2.2-fold, P=0.063, respectively) brain cortical TBARS levels in R6/2 mice compared to saline-treated ones (Fig. 17B). Co-administration of liraglutide and ghrelin slightly enhanced (by 2.3-fold, P=0.056) TBARS levels in the brain cortices from R6/2 mice compared to saline-treated ones (Fig. 17B).

Despite no significant alterations on brain cortical nitrites levels in saline-treated R6/2 mice compared to WT littermates, ghrelin alone slightly decreased its levels (by 0.5-fold) in R6/2 mice compared to saline-treated R6/2 mice (Fig. 17C). In addition, liraglutide *per se* diminished brain cortical nitrites levels in R6/2 mice by 0.7-fold (P=0.0317) compared to saline-treated ones (Fig. 17C). Interestingly, co-administration of liraglutide and ghrelin did not significantly affect the levels of nitrites in R6/2 mouse brain cortices compared to saline-treated ones (Fig. 17C).

These results suggest that, in spite of the slight increase in lipid oxidation markers, ghrelin *per se* or in combination with liraglutide tend to mitigate brain cortical oxidative damage to DNA in early symptomatic R6/2 mice.

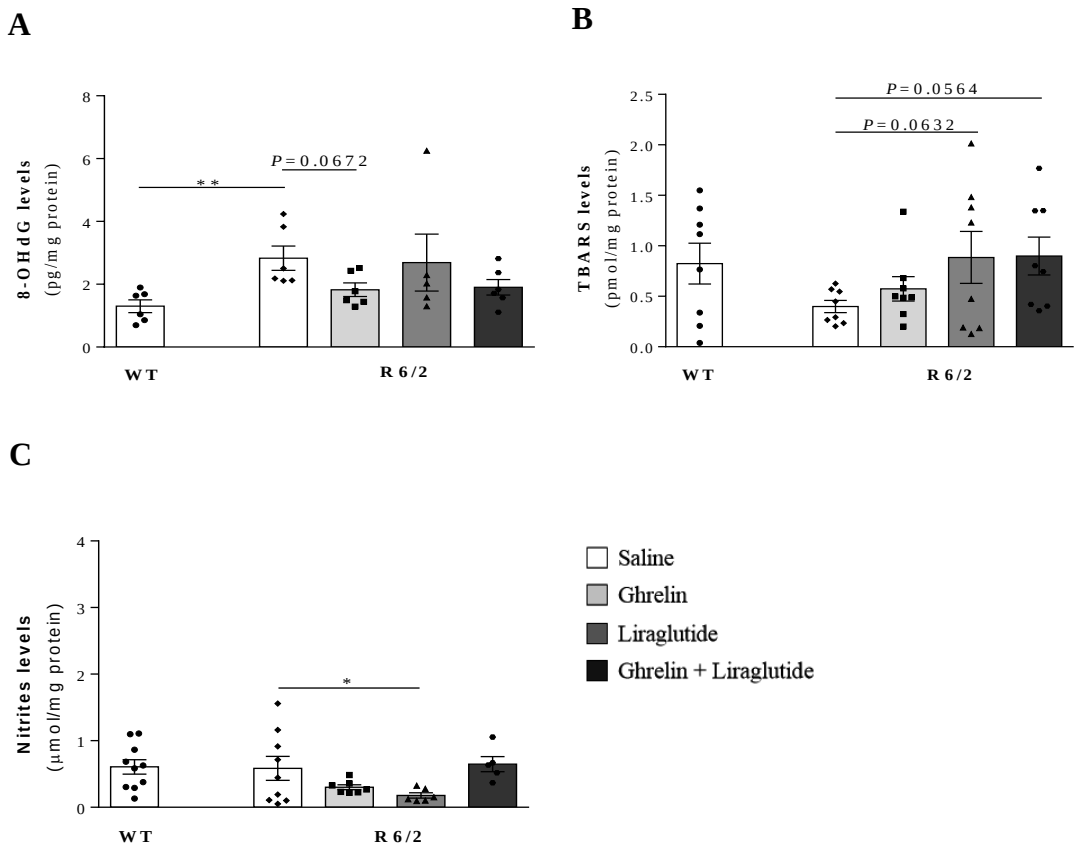

Figure 17: Effect of liraglutide plus ghrelin administration on brain cortical markers of oxidative stress in early symptomatic R6/2 mice. The levels of the marker for oxidative DNA damage 8-OHdG (A), for lipid oxidation TBARS (B) and of nitrites (C) were measured in brain cortical lysates from 12-week old R6/2 mice, treated with saline, ghrelin and/or liraglutide. Data are mean ± SEM of the indicated number of mice/group. Statistical significance: **P*<0.05, by Kruskal-Wallis test, with Dunn post-test (for a non-Gaussian distribution) (A), or with one-way ANOVA, with Tukey or Sidak post-hoc tests for multiple comparisons (for a Gaussian distribution) (B,C).

Overall, our results suggest that subcutaneous administration of ghrelin *per se* or in combination with liraglutide tend to promote brain cortical glucose metabolism via the pentose phosphate pathway and TCA cycle in early symptomatic R6/2 mice, thereby increasing ATP formation through the phosphocreatine/creatine kinase system. Furthermore, ghrelin alone or combined with liraglutide may also promote protective mechanisms against oxidative injury to DNA in such conditions. Importantly, liraglutide alone or together with ghrelin recovered brain cortical mitochondrial respiratory chain complex IV and ketogenesis in early symptomatic R6/2 mice.

Chapter 5

Discussion

To the best of our knowledge, this is the first study analyzing the role of co-administration of ghrelin and liraglutide against brain metabolic dysfunction and intracellular stress in 12-week old, early symptomatic R6/2 mice.

In the present study, we observed that the 2-week, peripheral administration of ghrelin *per se* or together with liraglutide slightly stimulate brain cortical glucose metabolism in early symptomatic R6/2 mice. This may occur via the pentose phosphate pathway and TCA cycle, promoting ATP formation by the phosphocreatine/creatine kinase system. Ghrelin *per se* or combined with liraglutide also protected against DNA oxidative damage in early symptomatic R6/2 mice. This may rely on a slight decrease in R6/2 mouse brain cortical levels of 8-OHdG. Conversely, liraglutide alone or together with ghrelin ameliorated brain cortical mitochondrial respiratory chain complex IV and ketone bodies production in early symptomatic R6/2 mice.

HD pathophysiology remains unclear, but may involve brain metabolic, mitochondrial and energy deficits that lead to neurodegeneration/death and motor-cognitive dysfunction [Duan *et al.*, 2014; Dubinsky, 2017; Johri *et al.*, 2013; Mochel & Haller, 2011b]. Studies performed in the last decade consistently reinforce that HD is a metabolic disorder affecting both brain and peripheral energy metabolism [Duan *et al.*, 2014; Dubinsky, 2017; Mochel & Haller, 2011b]. This dysmetabolism may constitute an early event in HD pathogenesis, detected already in presymptomatic individuals carrying the mutation [Duan *et al.*, 2014; Dubinsky, 2017; Mochel & Haller, 2011b; Mochel *et al.*, 2007]. Thus, targeting energy metabolism has been increasingly faced as a promising therapeutic or preventive strategy to slowdown the disease onset and progression [Goodman *et al.*, 2008; Mochel *et al.*, 2007; Underwood *et al.*, 2006]. Accordingly, several studies demonstrated that targeting peripheral energy metabolism attenuated both central and peripheral pathology in HD animal models [Ma *et al.*, 2007; Martin *et al.*, 2012, 2009].

HD treatment is only symptomatic, but this neurodegenerative disease shares peripheral and brain molecular mechanisms with type 2 diabetes [Martin *et al.*, 2012, 2009], and an increased prevalence of diabetes was reported in HD patients [Lalić *et al.*, 2008; Montojo *et al.*, 2017]. Thus, therapies used to treat type 2 diabetes may be beneficial in HD. In agreement with this, exendin-4 and liraglutide (two anti-type 2 diabetic drugs from the class of GLP-1 analogues) showed beneficial effects in this neurodegenerative disease [Chang *et al.*, 2018; Duarte *et al.*, 2018; Martin *et al.*, 2012, 2009]. However, their anoretic properties may be detrimental in HD [Li *et al.*, 2017;

Martin *et al.*, 2012, 2009; Wadden *et al.*, 2015], mostly due to its progressive weight loss [Aziz *et al.*, 2008; Mochel *et al*., 2007; van der Burg *et al.*, 2017]. Thus, to overcome this potential limitation, GLP-1 analogues should be administered together with a drug that minimizes weight loss, like the orexigenic hormone ghrelin [Morton *et al.*, 2014; Perry & Wang, 2012]. Accordingly, we showed recently that the subcutaneous co-administration of liraglutide with ghrelin was beneficial in early symptomatic R6/2 mice [Duarte *et al.*, 2018]. Ghrelin *per se* recovered body weight, energy metabolism in skeletal muscle and behavior [Sjögren *et al.*, 2017], while liraglutide alone or combined with ghrelin did not alter body weight, and restored peripheral glucose homeostasis and brain metabolic hormones in such conditions [Duarte *et al.*, 2018]. Since ghrelin and liraglutide *per se* can cross the blood brain barrier [Candeias *et al*., 2015; Rhea *et al.*, 2018], their co-administration may blunt the well-described deficits in R6/2 mouse brain energy metabolism [Cepeda-Prado E. *et al.*, 2012; Dubinsky, 2017; Mochel *et al*., 2012a], thus constituting a promising therapeutic strategy against HD progression.

At first sight, the increment in the formation of brain cortical glucose 6-phosphate and fructose 6-phosphate observed in early symptomatic, saline-treated R6/2 mice suggested a metabolic shift towards the pentose phosphate pathway. However, their unchanged glucose 6-phosphate dehydrogenase activity, together with the increased fructose 6-phosphate levels point towards the glycolytic conversion of glucose 6-phosphate into fructose 6-phosphate. This partially agrees with the upregulation of the canonical pathway WNT/β-catenin in HD [Dupont *et al*., 2012; Gines *et al.*, 2003a; Libro *et al*., 2016] and the subsequent upregulation of glycolytic enzymes (like hexokinase, pyruvate dehydrogenase kinase) – the so-called *Warburg effect* [Burns & Manda, 2017; Sameni *et al*., 2016; Vallée *et al*., 2018]. Nevertheless, our previous data showed that brain cortical levels of pyruvate and lactate were similar in saline-treated R6/2 and WT mice [Duarte *et al*., 2018], suggesting a slowdown in the glycolytic reactions downstream to fructose 6-phosphate. This was in line with the impaired expression and/or activity of phosphofructokinase (the gatekeeper of glycolysis that catalyzes the phosphorylation of fructose-6-phosphate into fructose-1,6-bisphosphate) [Komoda & Matsunaga, 2015; Mullarky & Cantley, 2015] observed in STH*dh*$^{Q111/Q111}$ striatal cells (expressing full-length mHtt) [Lee *et al*., 2007] and in *postmortem* striatum of HD patients [Bird *et al*., 1977]. Alternatively, such maintenance of pyruvate levels may be due to its generation from the amino acids alanine, cysteine, glycine, serine, threonine and tryptophan [Fluge *et al.*, 2016], from lactate (through the reversible lactate dehydrogenase) [Valvona *et al*.,

2016] and/or from its lower consumption by the pyruvate dehydrogenase complex [Naia *et al.*, 2017]. The expression of pyruvate dehydrogenase kinase (in particular its isoforms 1 and 3, the most active one in the brain) was higher in STH*dh*$^{Q111/Q111}$ striatal cells and in brain cortex from YAC128 mice, thus inhibiting mitochondrial pyruvate dehydrogenase and interfering with pyruvate conversion into acetyl-CoA to enter the TCA cycle [Naia *et al.*, 2017, 2016]. This may in turn explain the inhibition of citrate synthase, α-ketoglutarate dehydrogenase and malate dehydrogenase observed in brain cortices from saline-treated R6/2 mice in our experimental conditions. An immediate consequence of such α-ketoglutarate dehydrogenase and malate dehydrogenase inhibition is the downregulation of the mitochondrial electron respiratory chain complex I, due to the decrement in its substrate NADH (a product of the reactions catalyzed by those enzymes) [Dasika *et al*., 2015; Osellame *et al*., 2012; Vatrinet *et al*., 2017], as we observed in brain cortices from saline-treated R6/2 mice. Although no significant alterations were found in the activities of mitochondrial complexes II/III, since complex III *per se* was inhibited in saline-treated R6/2 mice, it is plausible that such maintenance in brain mitochondrial complexes II/III may arise from the activation of mitochondrial complex II. Similarly, previous studies reported the maintenance of striatal and cortical mitochondrial respiratory complexes II/III activities in late stage R6/2 mice [Seo *et al*., 2008; Tabrizi *et al.*, 2000]. Others observed that R6/2 mice were resistant to striatal lesions produced by the pharmacological inhibitors of succinate dehydrogenase/complex II activity - the mitochondrial toxins 3-nitropionoic acid (3-NP) and malonate [Hansson et al., 2001; Hickey & Morton, 2000]. This suggests that the maintenance of mitochondrial respiratory complexes II/III activities may constitute a compensatory mechanism to overcome such HD-related metabolic impairment [Hansson et al., 2001; Hickey & Morton, 2000]. Although we did not measure succinate dehydrogenase activity, others reported its stimulation in cortices from *postmortem* HD patients and in mid-stage heterozygous Q_{175} mice (a slower-onset HD model), further supporting the idea of a compensatory mechanism [Naseri *et al.*, 2015]. However, this compensation may not be sufficient, since the activities of the downstream mitochondrial respiratory complexes III and IV were decreased in saline-treated R6/2 mice. Similarly, a reduction of mitochondrial complex IV activity was found in the striatum and cerebral cortex of late stage R6/2 mice, together with the maintenance of mitochondrial complexes II/III activities [Tabrizi *et al.*, 2000].

Given the pivotal role of mitochondrial complexes I, III and IV in electron transfer from substrates (*e.g.*, NADH and $FADH_2$) to oxygen to generate the gradient across the inner mitochondrial membrane used for ATP synthesis at F_0,F_1-ATPsynthase (complex V) [Benard *et al*., 2011; Sousa *et al.*, 2018], one expected that the above impairment in brain mitochondrial complexes I, III and IV activities in saline-treated R6/2 mice compromised their ATP synthesis. However, we previously observed that their brain cortical ATP levels were maintained [Duarte *et al.*, 2018], similarly to other studies involving HD mice [Tkac *et al.*, 2012; Zhang *et al.*, 2011] and early stage human HD patients [Mochel *et al*., 2012a]. This may be explained by the decrement in brain ADP and AMP reported in our saline-treated R6/2 mice [Duarte *et al*., 2018]. Alternatively, the maintenance of ATP levels may arise from an increase in total creatine levels and the subsequent activation of the phosphocreatine/creatine kinase system [Mochel *et al.*, 2012a]. This constitutes an alternative source of ATP in response to a local energy demand, since it occurs faster than glycolysis or oxidative phosphorylation [Wallimann *et al*., 2011; Zhang *et al.*, 2011]. In line with this, the rate of phosphocreatine formation in saline-treated R6/2 mouse brains was tendentiously higher than in WT mice, whereas no significant alterations occurred in the rate of ATP formation from phosphocreatine. Importantly, these results agreed with the increment in cortical and striatal phosphocreatine and creatine levels widely reported in R6/2 mice, and that preceded their general ATP decrease and onset of motor symptoms [Mochel *et al*., 2012a; Tkac *et al.*, 2012; Zacharoff *et al.*, 2012]. Of note, the maintenance of brain cortical ATP levels in saline-treated R6/2 mice, alongside the decrement in their ADP and AMP content may ultimately contribute to their maintenance of brain cAMP [Duarte *et al.*, 2018], a pivotal second messenger for, *e.g.*, the regulation of both central and peripheral energy homeostasis [Newton *et al.*, 2016; Zhang *et al*., 2016]. Importantly, one cannot exclude that ATP can be released to the extracellular space to act as a neurotransmitter through P2X and P2Y receptors [Di Virgilio & Adinolfi, 2017; Puchałowicz *et al.*, 2014], or it can be sequentially dephosphorylated by a series of ectonucleotidases towards its ultimate product: adenosine [Barsotti & Ipata, 2004; Blum *et al.*, 2018]. Physiologically, adenosine is an important neurotransmitter that acts on four distinct receptors (A_1, A_{2A}, A_{2B} and A_3), being crucial for neuronal function and homeostasis (like modulation of synaptic activity and excitotoxicity, neurotransmitters release, and regulation of protein degradation mechanisms) [Blum *et al.*, 2018; Huang *et al.*, 2011; Kao *et al.*, 2017; Porkka-Heiskanen & Kalinchuk, 2011]. Although numerous studies showed that the

stimulation of neuronal activity increases energy consumption and extracellular adenosine concentrations within the central nervous system [Blum *et al.*, 2018; Huang *et al.*, 2011; Porkka-Heiskanen & Kalinchuk, 2011], others reported a decrement in extracellular adenosine in the striatum of the HD rodents zQ175 and Tg51 at an early stage, when motor dysfunction appears [Guitart *et al.*, 2016]. In line with this, elevation of adenosinergic tone increased brain proteasome activity, the levels of the neurotrophic factor brain-derived neurotrophic factor (BDNF) and promoted survival and motor function in R6/2 mice [Huang *et al.*, 2011; Kao *et al.*, 2017].

Concerning the possible involvement of ketone bodies' dysmetabolism in HD brain pathophysiology, to the best of our knowledge, this is the first study evaluating their levels in brain cortices from R6/2 mice. Nevertheless, at higher levels, they can cross the blood brain barrier via monocarboxylate transporters, ultimately entering in brain mitochondria, whereby 3-hydroxybutyric acid is metabolized by 3-hydroxybutyrate dehydrogenase to acetoacetic acid [Newman & Verdin, 2014; Puchalska & Crawford, 2017]. Then, acetoacetic acid is converted into acetoacetyl-CoA and succinate by succinyl-CoA:3-ketoacid coenzyme A transferase (SCOT). Acetoacetyl-CoA yields acetil-CoA (a substrate of TCA cycle), while succinate constitutes another substrate for the TCA cycle [Newman & Verdin, 2014; Puchalska & Crawford, 2017]. This renders ketone bodies important substrate providers for the TCA cycle [Newman & Verdin, 2014; Puchalska & Crawford, 2017]. In the present study, we observed that brain levels of acetoacetic acid and 3-hydroxybutyric acid were massively decreased in saline-treated R6/2 mice. Since brain ketone body metabolism depends, *e.g.*, on their blood content, on their transport across the blood brain barrier and into the cells, and/or on the activities of metabolizing enzymes [Morris, 2005], one may hypothesize that such decrement on brain cortical acetoacetic acid and 3-hydroxybutyric acid may arise from a compromise on their synthesis and/or uptake from the periphery, ultimately accounting (at least partially) for the inhibition of the TCA cycle described for saline-treated R6/2 mice. Importantly, to evaluate the impact of HD on ketone bodies synthesis, we should measure the activity and/or expression of enzymes from β-oxidation, ketogenesis and ketolysis (*e.g.* SCOT) in our experimental conditions.

Oxidative stress is well described in HD brain cortex and striatum [Kumar & Ratan, 2016; Sorolla *et al.*, 2012; Stack *et al*., 2008], especially in R6/2 mice [Bogdanov *et al*., 2001; Perluigi *et al.*, 2005; Sorolla *et al.*, 2012]. Among its markers, 8-OHdG arises from

the oxidation of guanine bases from nuclear and mitochondrial DNA by ROS [Ayala-Peña,2013; Browne *et al.*, 2006; Phaniendra, *et al.*, 2015], being the later more susceptible to such damage [Ayala-Peña *et al.*, 2013; Browne & Beal, 2006]. Indeed, studies demonstrated that oxidative injury to mitochondrial DNA damage may constitute an early biomarker for HD-associated neurodegeneration in R6/2 mice [Acevedo-Torres *et al.*, 2009], whereas nuclear DNA damage appears to occur at later stages of the pathology [Bogdanov *et al.*, 2001]. Our results demonstrate that brain cortical 8-OHdG levels were raised in early symptomatic saline-treated R6/2 mice. Since the inhibition of mitochondrial respiratory complexes (mainly complexes I and III) is an important source of ROS [Kalogeris *et al.*, 2014; Li *et al.*, 2003; Quinlan *et al.*, 2011; Wang *et al.*, 2015a] and enhanced levels of mitochondrial-generated superoxide ($O_2^{\bullet -}$) were reported in HD striatal cells [Ribeiro *et al.*, 2014; Ribeiro *et al.*, 2013; Siddiqui *et al.*, 2012], leading to DNA damage [Acevedo-Torres *et al.*, 2009; Ayala-Peña, 2013; Siddiqui *et al.*, 2012; Sorolla *et al.*, 2012], we hypothesize that the above-mentioned inhibition of mitochondrial respiratory complexes I, III and IV in the brain cortices from saline-treated R6/2 mice may account for their higher 8-OHdG content. In addition, we cannot exclude that oxidative stress targets primarily DNA due to an impairment in its repair mechanisms in HD [Ayala-Peña, 2013; Kumar & Ratan, 2016; Massey & Jones, 2018; Siddiqui *et al.*, 2012]. Although not expected, brain cortical TBARS levels were only slightly decreased in saline-treated R6/2 mice, in line with a previous study in striatum and brain cortex from 12-month old YAC128 mice [Brocardo *et al.*, 2016]. These data reinforce the notion that, in HD brains, oxidative stress may start in mitochondria and then progress towards the nucleus and membranes [Brocardo *et al.*, 2016]. Although, this is still controversial, since others failed to report alterations in mitochondrial oxidative metabolism and respiratory activity in striatal and cortical neurons from YAC128 mice up to the age of 12 months [Hamilton *et al.*, 2017; Pellman *et al.*, 2015]. Surprisingly, we did not find significant alterations in nitrites levels in brain cortices from saline-treated R6/2 mice. These are by-products of the oxidative cleavage of nitric oxide, and the accumulation of nitrite is a well-established indicator of nitric oxide production in biological systems [Gladwin *et al.*, 2005; Kim-Shapiro *et al.*, 2006]. Nevertheless, we cannot exclude that other reactive species, namely ROS, may play a role herein.

The slight increase in brain cortical glucose 6-phosphate formation and glucose 6-phosphate dehydrogenase activity observed after ghrelin administration *per se* to early

symptomatic R6/2 mice, alongside their slightly decreased fructose 6-phosphate content, suggests the shift of glucose 6-phosphate towards the pentose phosphate pathway. This agreed with a prior study showing an upregulation of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in ghrelin-treated rats [Sangiao-Alvarellos *et al.*, 2009]. This could be of the outmost relevance, since in one hand, these enzymes are the main cellular source of NADPH (a reducing equivalent for the vast majority of ROS-detoxifying enzymes) [Mullarky & Cantley, 2015; Stanton, 2012; Stincone *et al.*, 2015]. On the other hand, ghrelin increased the expression of the nuclear factor-erythroid 2-related factor 2 (Nrf2) that binds to antioxidant response elements (AREs) to regulate the transcription of genes involved in glutathione synthesis, NADPH regeneration, phase II detoxification and in antioxidant defense (*e.g.* catalase, peroxiredoxins, glutathione peroxidase and reductase, thioredoxin, heme oxygenase-1 (HO-1), NAD(P)H:quinone oxidoreductase 1 (NQO1)) [Allam & El-Gohary, 2017; Liu *et al.*, 2014; Yanaka, 2018], ultimately maintaining the redox balance [Buendia *et al.*, 2016; Gan & Johnson, 2014; Zhang *et al.*, 2013]. Accordingly, ghrelin upregulated HO-1 expression and protected gastric mucosa against oxidative stress induced by the anti-inflammatory indomethacin [Allam & El-Gohary, 2017]. It increased the activities of HO-1 and NAD(P)H:quinoneoxidoreductase 1, protecting lung against oxidative injury induced by the herbicide paraquat [Liu *et al.*, 2014]. In line with our results, ghrelin-mediated activation of the Nrf2-ARE pathway [Allam & El-Gohary, 2017; Liu *et al.*, 2014; Yanaka, 2018] increased glucose 6-phosphate dehydrogenase, redirecting glucose towards the pentose phosphate pathway to replenish cellular NADPH pools [Heiss *et al.*, 2013; Mitsuishi *et al.*, 2012]. In addition, ghrelin *per se* stimulated citrate synthase and malate dehydrogenase in R6/2 mouse brains, suggesting that this could potentiate their TCA cycle. Since previous studies showed that ghrelin promotes β-oxidation of fatty acids by activating AMPK in rat skeletal muscle [Kraft *et al.*, 2019], white adipose tissue [Sangiao-Alvarellos *et al.*, 2009], hepatocytes [Ezquerro *et al.*, 2016] and hypothalamus [Andersson *et al.*, 2004], thereby increasing the formation of ketone bodies [Foretz *et al.*, 2018; Herzig & Shaw, 2018], it is plausible that the observed increment in brain levels of acetoacetic acid and 3-hydroxybutyric acid upon ghrelin administration to R6/2 mice may also replenish the mitochondria with substrates for the TCA cycle (especially at the level of citrate synthase). Despite this, ghrelin only slightly increased brain cortical mitochondrial complex IV activity in R6/2 mice. Although in our previous study we did not measure their nucleotides levels after ghrelin administration *per se* [Duarte *et al.*,

2018], here we observed that the slightly decreased brain phosphocreatine levels in ghrelin-treated R6/2 mice were mirrored by a stimulation of ATP formation by the phosphocreatine/creatine kinase system. This may in turn provide an alternative energy source to the mitochondrial oxidative phosphorylation.

Previous studies showed that ghrelin reduces lipid peroxidation, with a special emphasis on the levels of MDA, in a human colorectal carcinoma cell line [Bułdak *et al.*, 2015], blood and brain upon hypoxia [Omrani *et al.*, 2015], rat testis [Kheradmand *et al.*, 2009] and in a mouse model of myocardial ischemia/reperfusion injury [Sun *et al.*, 2016]. Others also demonstrated a higher ghrelin-mediated downregulation in the expression of neuronal and inducible isoforms of nitric oxide synthase in mouse models of focal cerebral ischemia and myocardial ischemia/reperfusion injury that, in the former case, was accompanied by a reduction in 3-nitrotyrosine levels [Cheyuo *et al*., 2011; Sun *et al.*, 2016]. In accordance, we observed that ghrelin alone slightly reduced brain cortical 8-OHdG and nitrites levels in early symptomatic R6/2 mice, despite the slight increment in their TBARS levels. Though unexpected, this tendentious increase in brain lipid oxidation after peripheral ghrelin administration to R6/2 mice may arise from their higher brain cortical ketone bodies, since several authors correlated their levels (mainly acetoacetate) with an augmented lipid peroxidation in hepatocytes and endothelial cells [Jain & McVie, 1999; Jain *et al.*, 1998; Shi *et al.*, 2016]. However, in face of the wide evidence for an antioxidant role of ghrelin, namely by elevating total serum antioxidant capacity in human controls and undergoing hypoxia [Omrani *et al.*, 2015], by upregulating the expression and/or activities of glutathione peroxidase, superoxide dismutase or catalase in a mouse model of myocardial ischemia/reperfusion injury [Sun *et al.*, 2016], cardiomyocytes [Tong *et al.*, 2012], rat ovarian tissue [Kheradmand *et al.*, 2010] and human colorectal carcinoma cell line [Bułdak *et al.*, 2015], and by stimulating both glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in rat white adipose tissue and liver [Sangiao-Alvarellos *et al.*, 2009], it would be relevant to analyze the effect of ghrelin also in antioxidant defenses under our experimental conditions.

Similar to ghrelin, several studies demonstrated that liraglutide may promote the shift of glucose 6-phosphate towards the pentose phosphate pathway, namely by upregulating the Nrf2-ARE pathway and its downstream transcriptional targets, like HO-1 and NQO1 in the liver and brain nerve cells from Zucker diabetic fatty rats, reducing their hepatic oxidative stress and cerebral ischemia [Deng *et al.*, 2018; Guo *et al.*, 2018a]. Accordingly,

we observed that the tendentiously lower brain cortical glucose 6-phosphate formation in early symptomatic R6/2 mice was accompanied by a slight stimulation of glucose 6-phosphate dehydrogenase, indicating a possible metabolic shift towards the pentose phosphate pathway under such conditions. This was reinforced by the previously reported tendentious decrement in brain cortical lactate content in liraglutide-treated R6/2 mice [Duarte *et al.*, 2018]. However, we cannot exclude that this reduction in lactate may be caused by the metabolism of pyruvate through the TCA cycle (instead of lactate dehydrogenase), as given by the (at least tendentiously) increased activities of brain cortical citrate synthase, α-ketoglutarate dehydrogenase and malate dehydrogenase in liraglutide-administered R6/2 mice. This is indirectly supported by studies showing that liraglutide promotes β-oxidation of fatty acids [Foretz *et al.*, 2018; Herzig & Shaw, 2018], most likely by stimulating AMPK (as observed in mouse skeletal muscle [Li *et al.*, 2014], in neonatal type 1 diabetic rat cardiomyocytes [Zhang *et al.*, 2017], in visceral adipose tissue from the type 2 diabetic *db/db* mice [Shao *et al.*, 2015], and in non-alcoholic fatty liver disease (NAFLD) models [Wang *et al.*, 2015b; Yu *et al.*, 2019)-thus providing the TCA cycle with acetyl-CoA and succinate [Newman & Verdin, 2014; Puchalska & Crawford, 2017]. Accordingly, treatment with exendin-4 increased the levels of 3-hydroxybutyric acid in primary human hepatocytes loaded with the fatty acids palmitic or elaidic acid [Sharma *et al.*, 2011]. Adding to this, and despite the slight inhibition of brain mitochondrial complexes II/III after peripheral treatment of R6/2 mice with liraglutide, the (at least tendentiously) stimulated brain mitochondrial complexes I and IV may not be sufficient to replenish intracellular ATP stores, either produced through the mitochondrial oxidative phosphorylation (as described in our previous study [Duarte *et al.*, 2018]) or from the phosphocreatine/creatine kinase system. Indeed, liraglutide *per se* slightly reduced brain cortical phosphocreatine formation and the subsequent ATP regeneration from phosphocreatine in early symptomatic R6/2 mice.

As discussed above for ghrelin, studies reported that liraglutide diminishes MDA levels in liver, serum, heart and brains from rodent models of NAFLD, type 2 diabetes and myocardial ischemia reperfusion injury [Gao *et al.*, 2015; Guo *et al.*, 2018a; Huang *et al.*, 2019; Li *et al.*, 2016; Liu *et al.*, 2017], mitigating their lipid oxidation. It also lowered brain nitrites levels without affecting inducible nitric oxide synthase expression, suggesting that liraglutide's action may occur at the level of nitric oxide synthesis [Parthsarathy & Hölscher, 2013]. Accordingly, we observed that subcutaneous liraglutide injection reduced R6/2 mouse brain cortical levels of nitrites. However, it was not able to

protect against their higher 8-OHdG content and even tended to increase TBARS levels. These results suggest that peripherally-administered ghrelin was more efficient than liraglutide in counteracting brain oxidative stress markers in early symptomatic R6/2 mice.

Concerning the subcutaneous co-administration of liraglutide plus ghrelin, the tendentiously increased brain cortical glucose 6-phosphate levels and glucose 6-phosphate dehydrogenase activity in R6/2 mice suggests a metabolic shift of glucose 6-phosphate towards the pentose phosphate pathway, rather than to glycolysis. This is reinforced by their lower lactate content under these conditions, as described in our previous study [Duarte *et al.,* 2018]. Interestingly, this pattern of a metabolic shift to the pentose phosphate pathway induced by liraglutide plus ghrelin resembles more the one from ghrelin treatment alone (as discussed above) than the one from liraglutide *per se*, being also accompanied by a slight stimulation of the enzymes from the TCA cycle, citrate synthase, α-ketoglutarate dehydrogenase and malate dehydrogenase. Similar to the previously discussed increment in brain cortical ketone bodies levels upon liraglutide and ghrelin administration alone in early symptomatic R6/2 mice, we hypothesize that the AMPK-mediated stimulation of β-oxidation of fatty acids and synthesis of ketone bodies induced by ghrelin plus liraglutide may further replenish the TCA cycle with acetyl-CoA and succinate in our HD mice. Despite this, the co-injection of ghrelin and liraglutide was not able to recover from the inhibition of mitochondrial respiratory complex I in R6/2 mouse brains and even tended to further decrease their mitochondrial respiratory complexes I and II/III activities. At first sight, this pointed to antagonizing effects of liraglutide and ghrelin, as previously reported for the exendin-4-induced antagonism of ghrelin in rat hypothalamic metabolic regulation [Abtahi *et al.,* 2019, 2016]. However, the (at least slight) stimulation of both mitochondrial respiratory complexes III and IV upon ghrelin and liraglutide co-administration closely resembles the pattern observed for liraglutide treatment *per se* rather than ghrelin alone in early symptomatic R6/2 mice, whereas their slight stimulation of ATP formation via the phosphocreatine/creatine kinase system resembles the pattern observed for ghrelin treatment *per se*. This may in turn contribute for the maintenance of intracellular brain energetic pools (ATP and eventually also ADP), as described in our previous study [Duarte *et al.,* 2018], but also to the pivotal neurotransmitter role of these adenine nucleotides mediated by P2X and P2Y receptors [Di Virgilio & Adinolfi, 2017; Puchałowicz *et al.,* 2014]. Alternatively, ATP and ADP

can be further metabolized towards adenosine, another crucial neurotransmitter [Huang *et al.*, 2011]. This appears to be reinforced by the lower brain cortical AMP levels in ghrelin plus liraglutide-treated R6/2 mice [Duarte *et al.*, 2018], as well as by previous evidence that the crosstalk between GLP-1 and adenosine signaling promotes pro-survival mechanisms to counteract myocardial ischemia-reperfusion injury [Ihara *et al.*, 2015].

Interestingly, co-administration of ghrelin and liraglutide slightly decreased brain cortical 8-OHdG levels in R6/2 mice, in a pattern closely resembling that from ghrelin injection alone. As further detailed above, this ghrelin-mediated protection against DNA damage may either involve the stimulation of pentose phosphate pathway and/or their antioxidant defenses (namely superoxide dismutase, catalase) [Allam & El-Gohary, 2017; Kheradmand *et al.*, 2010; Liu *et al*., 2014; Omrani *et al.*, 2015; Sangiao-Alvarellos *et al.*, 2009; Tong *et al.*, 2012; Yanaka, 2018]. However, these may not be sufficient to counteract the slightly enhanced brain TBARS levels in R6/2 mice upon the co-injection of ghrelin plus liraglutide, despite their unaltered content in nitrites. A possible explanation for this apparent contradiction could lie on coenzyme Q_{10}, whose function as an electron carrier between mitochondrial complexes I and II to complex III may be augmented to stimulate mitochondrial complexes III and IV (and ultimately ATP production), in detriment of its role as a potent antioxidant [Johri *et al.*, 2012; Molyneux *et al.*, 2008; Stack *et al*., 2008]. Accordingly, administration of a high-dose of coenzyme Q_{10} increased brain ATP levels and lowered 8-OHdG levels in R6/2 mice [Smith *et al.*, 2006].

In sum, in the present study we demonstrated that the 2-week, subcutaneous injection of ghrelin *per se* or in combination with liraglutide to early symptomatic R6/2 mice tends to stimulate their brain cortical glucose metabolism. This appears to occur mainly through the pentose phosphate pathway and TCA cycle, allowing ATP synthesis via the phosphocreatine/creatine kinase system. Ghrelin *per se* or combined with liraglutide also protected these mice against oxidative damage to DNA, possibly by slightly mitigating their brain cortical levels of 8-OHdG. On the other hand, liraglutide alone or together with ghrelin recovered brain cortical mitochondrial respiratory chain complex IV and ketone bodies production in early symptomatic R6/2 mice.

Our results support a deeper analysis of brain cortical oxidative/nitrosative stress markers and antioxidant defenses, which may exert a pivotal role on HD pathophysiology

and progression. Evaluating the underlying effects of ghrelin and liraglutide administration in HD are warranted.

Chapter 6

Conclusions

In saline-treated early symptomatic R6/2 mouse brain cortices:

- Slowdown in the glycolytic metabolism downstream to fructose 6-phosphate.
- Impairment in TCA cycle and ketogenesis.
- Activities of mitochondrial respiratory chain complexes I, III and IV were impaired, but the activity of mitochondrial complexes II/III remained unaffected, suggesting a compensatory mechanism.
- Oxidative stress may start in mitochondria and ROS (rather than RNS) may play a role in oxidative damage.

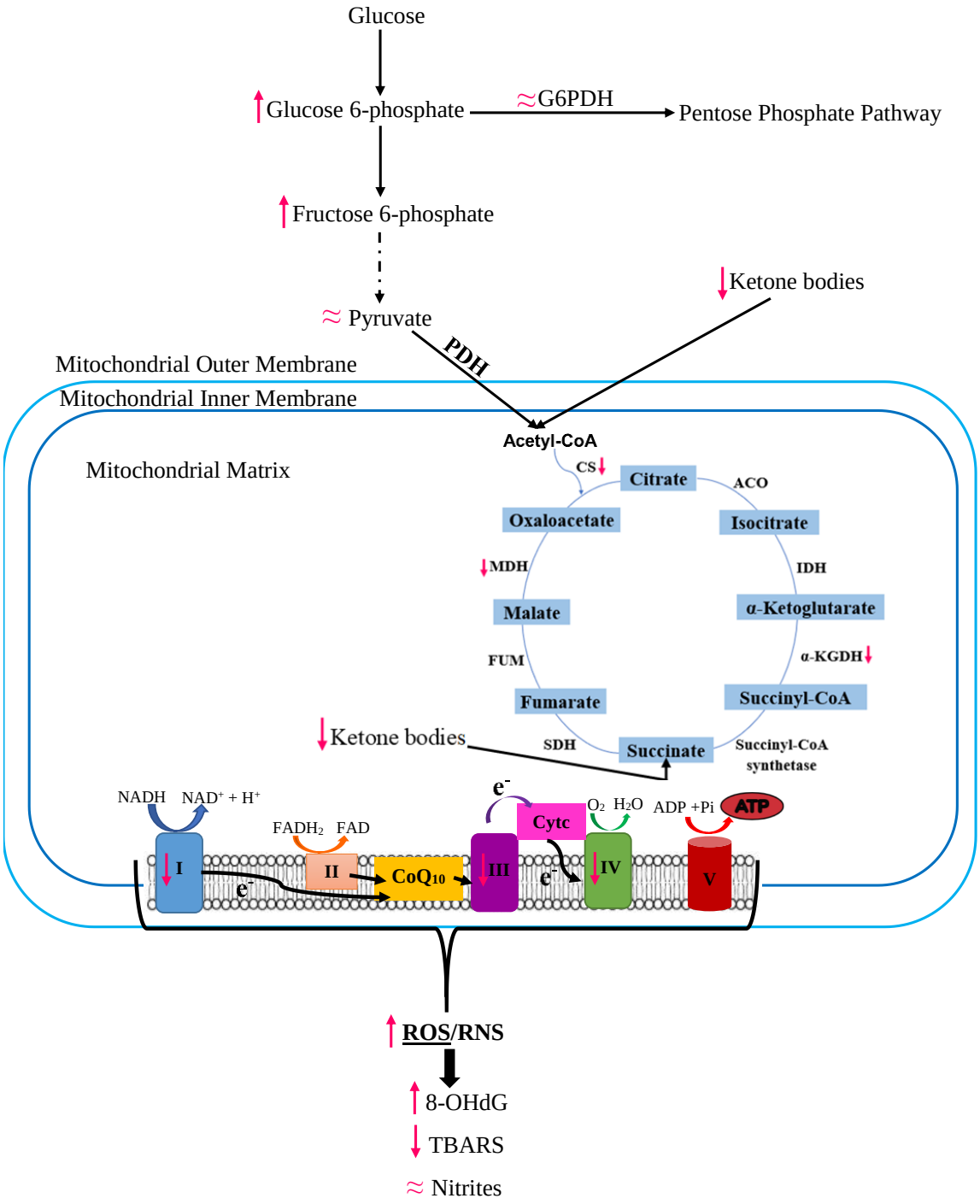

Figure 18: Possible mechanisms underlying HD-mediated alterations of brain cortical glucose (mitochondrial) metabolism and oxidative/nitrosative stress in early symptomatic R6/2 mice. Abbreviations: CoQ_{10}- coenzyme Q_{10}; Cytc- cytochrome c; O_2- oxygen; H_2O- water; Pi- inorganic phosphate; ADP- adenosine 5'-diphosphate; ATP- adenosine 5'-triphosphate; NADH/NAD^+- nicotinamide adenine dinucleotide (oxidized/reduced); $FADH_2$/FAD^+- flavin adenine dinucleotide (oxidized/reduced); e^- - electrons; I- mitochondrial complex I; II- mitochondrial complex II; III- mitochondrial complex III; IV- mitochondrial complex IV; PDH- pyruvate dehydrogenase; CS- citrate synthase; ACO- aconitase; IDH- isocitrate dehydrogenase; α-KGDH- α-ketoglutarate dehydrogenase; SDH- succinate dehydrogenase; FUM- fumarase; MDH- malate dehydrogenase.

In ghrelin-treated early symptomatic R6/2 mouse brain cortices:

- Slight stimulation of glucose 6-phosphate metabolism via the pentose phosphate pathway.
- Tended to stimulate brain cortical TCA cycle.
- Did not affect the activities of mitochondrial respiratory complexes I, II/ III and III, but slightly stimulated mitochondrial respiratory complex IV.
- Tended to promote ATP formation via the phosphocreatine/creatine kinase system.
- Increased ketone bodies levels.
- Tended to attenuate oxidative damage, despite a slight increase in lipid peroxidation markers.

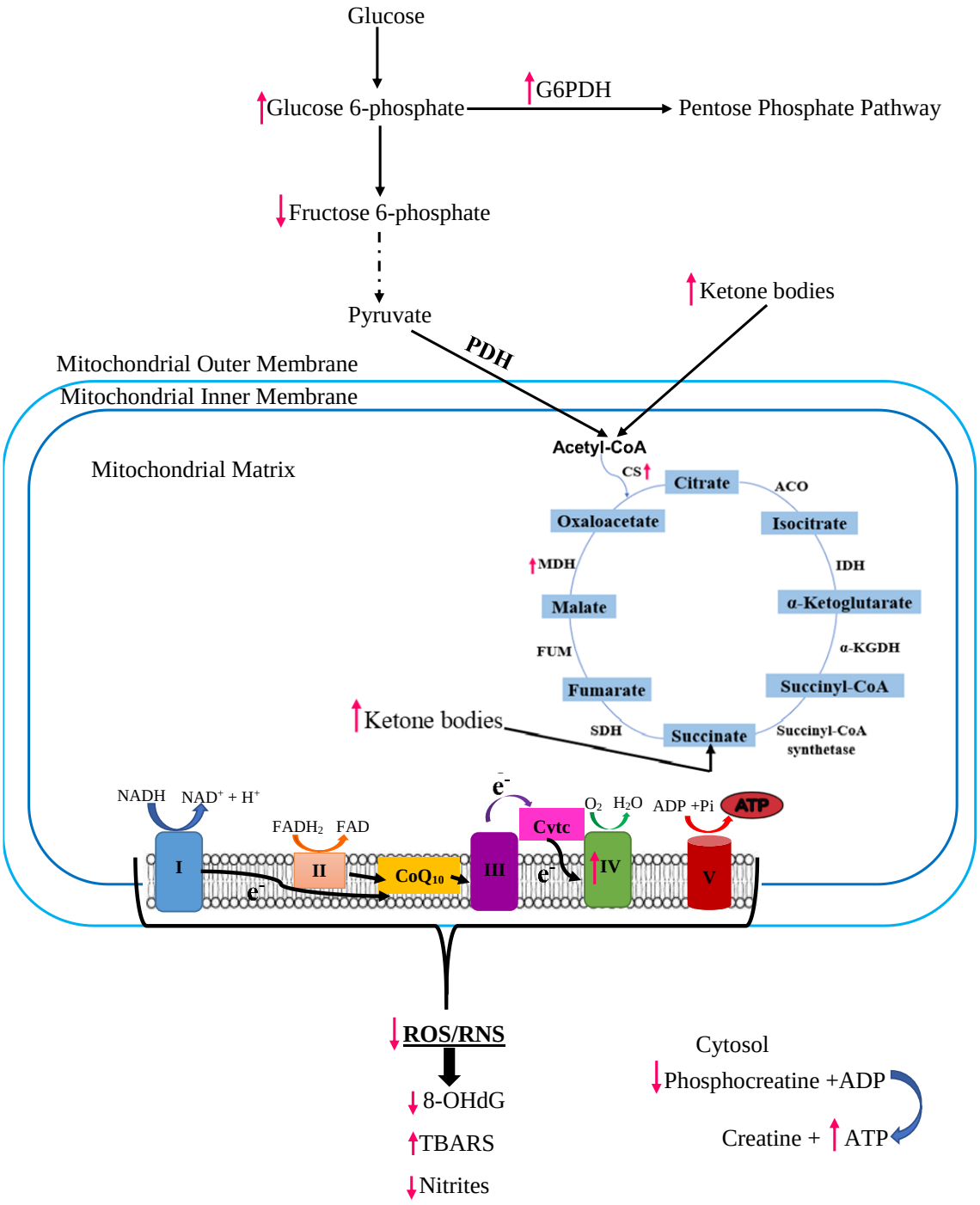

Figure 19: The role of peripheral ghrelin administration on brain cortical glucose (mitochondrial) metabolism and oxidative/nitrosative stress markers in early symptomatic R6/2 mice. Abbreviations: CoQ_{10}- coenzyme Q_{10}; Cytc- cytochrome c; O_2- oxygen; H_2O- water; Pi- inorganic phosphate; ADP- adenosine 5'-diphosphate; ATP- adenosine 5'-triphosphate; NADH/NAD^+- nicotinamide adenine dinucleotide (oxidized/reduced); $FADH_2$/FAD^+- flavin adenine dinucleotide (oxidized/reduced); e^- - electrons; I- mitochondrial complex I; II- mitochondrial complex II; III- mitochondrial complex III; IV- mitochondrial complex IV; PDH- pyruvate dehydrogenase; CS- citrate synthase; ACO- aconitase; IDH- isocitrate dehydrogenase; α-KGDH- α-ketoglutarate dehydrogenase; SDH- succinate dehydrogenase; FUM- fumarase; MDH- malate dehydrogenase.

In liraglutide-treated early symptomatic R6/2 mouse brain cortices:

- Slightly stimulated glucose 6-phosphate metabolism via the pentose phosphate pathway.
- Tended to stimulate TCA cycle.
- Stimulated the activities of mitochondrial respiratory complexes I and IV.
- Did not affect ATP formation via the phosphocreatine/creatine kinase system.
- Increased ketone bodies levels.
- Did not counteract oxidative damage.

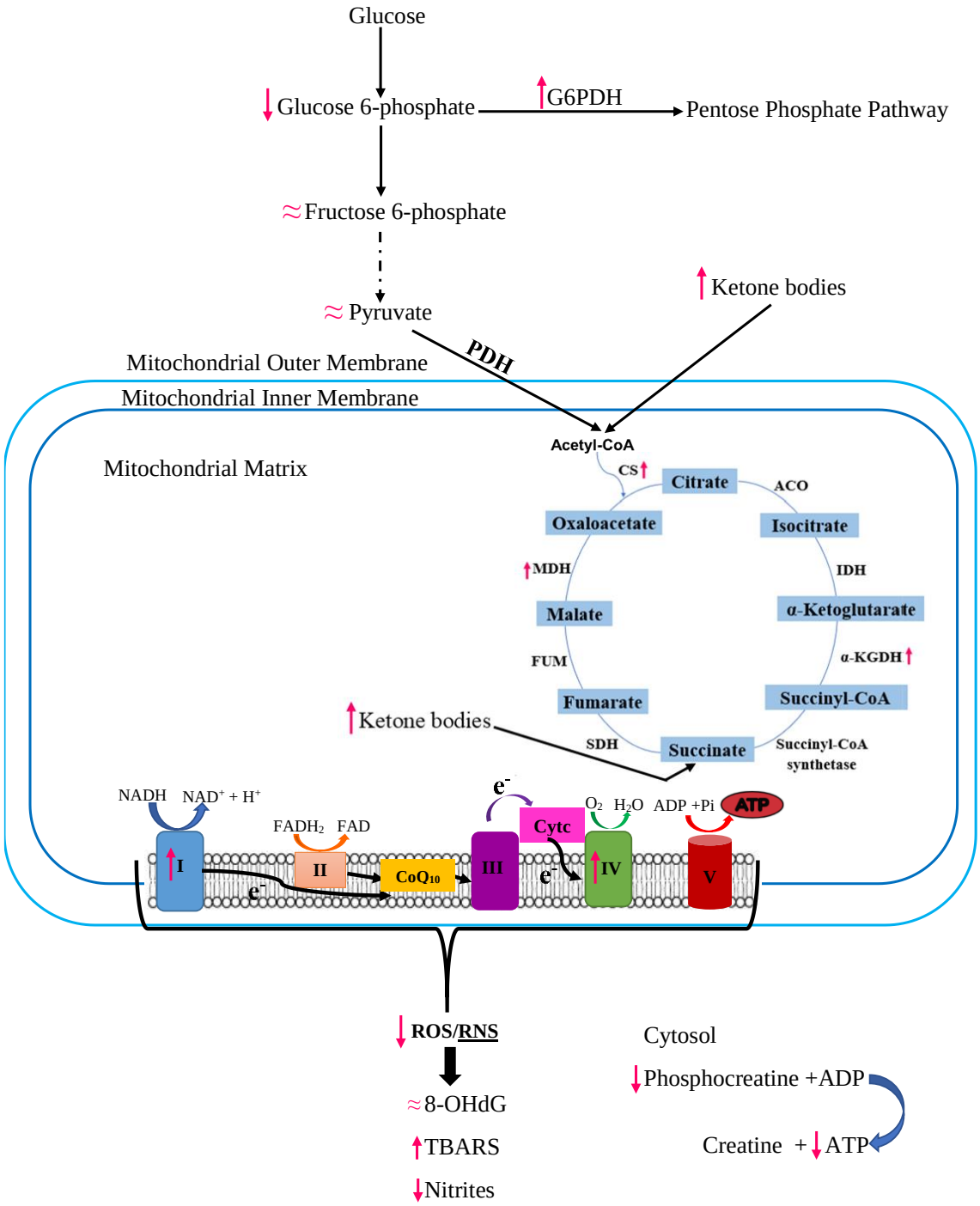

Figure 20: The role of peripheral liraglutide administration on brain cortical glucose (mitochondrial) metabolism and oxidative/nitrosative stress markers in early symptomatic R6/2 mice. Abbreviations: CoQ_{10}- coenzyme Q_{10}; Cytc- cytochrome c; O_2- oxygen; H_2O- water; Pi- inorganic phosphate; ADP- adenosine 5'-diphosphate; ATP- adenosine 5'-triphosphate; NADH/NAD^+- nicotinamide adenine dinucleotide (oxidized/reduced); $FADH_2$/FAD^+- flavin adenine dinucleotide (oxidized/reduced); e^-- electrons; I- mitochondrial complex I; II- mitochondrial complex II; III- mitochondrial complex III; IV- mitochondrial complex IV; PDH- pyruvate dehydrogenase; CS- citrate synthase; ACO- aconitase; IDH- isocitrate dehydrogenase; α-KGDH- α-ketoglutarate dehydrogenase; SDH- succinate dehydrogenase; FUM- fumarase; MDH- malate dehydrogenase.

In ghrelin plus liraglutide-treated early symptomatic R6/2 mouse brain cortices:

- Slightly stimulated the glucose 6-phosphate metabolism via the pentose phosphate pathway.
- Tended to stimulate TCA cycle.
- Restored the activity of mitochondrial respiratory complex IV.
- Tended to promote ATP formation via the phosphocreatine/creatine kinase system.
- Increased the levels of ketone bodies.
- Slightly reduced oxidative damage to DNA.

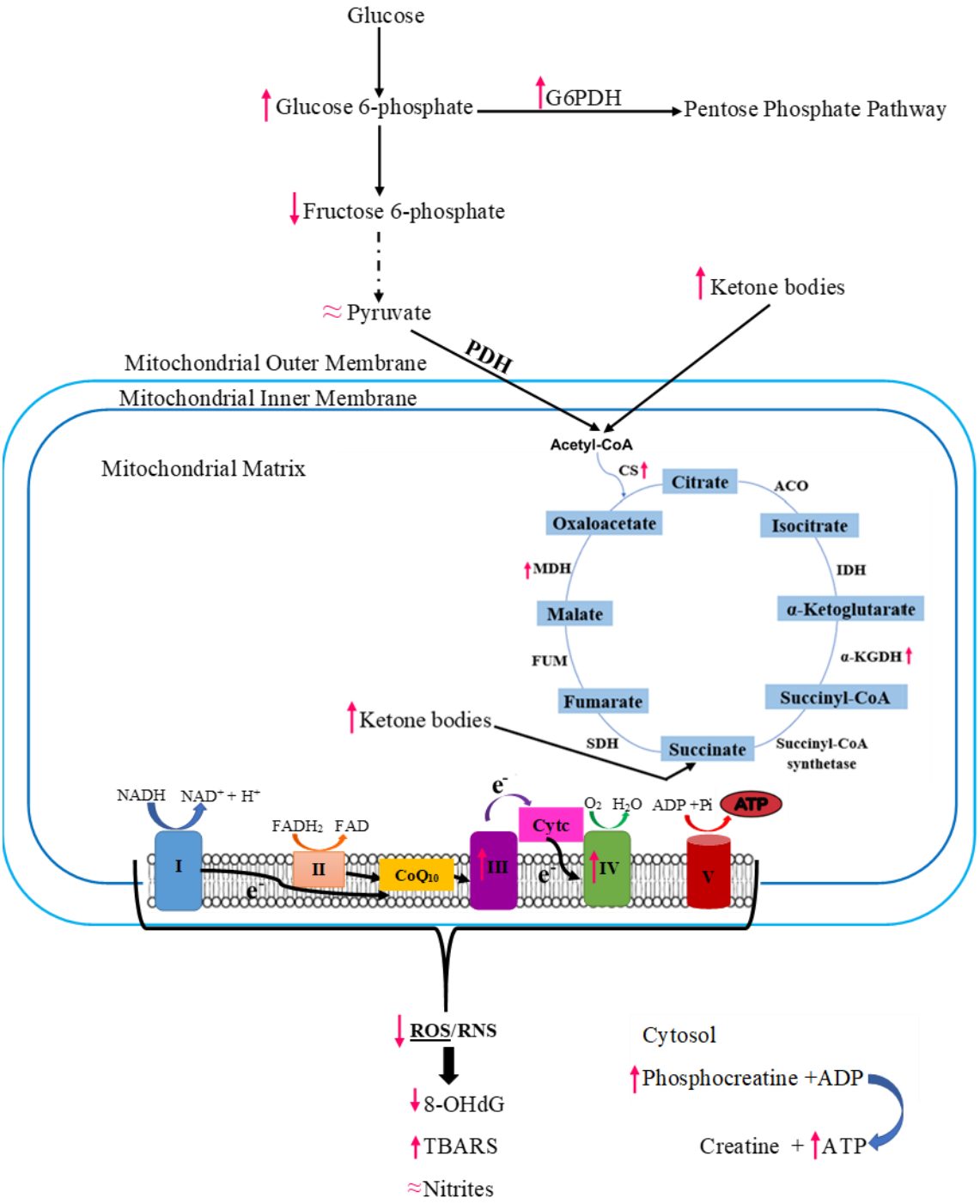

Figure 21: The role of peripheral co-administration of ghrelin and liraglutide on brain cortical glucose (mitochondrial) metabolism and oxidative/nitrosative stress markers in early symptomatic R6/2 mice. Abbreviations: CoQ_{10}- coenzyme Q_{10}; Cytc- cytochrome c; O_2- oxygen; H_2O- water; Pi- inorganic phosphate; ADP- adenosine 5'-diphosphate; ATP- adenosine 5'-triphosphate; NADH/NAD^+- nicotinamide adenine dinucleotide (oxidized/reduced); $FADH_2$/FAD^+- flavin adenine dinucleotide (oxidized/reduced); e^-- electrons; I- mitochondrial complex I; II- mitochondrial complex II; III- mitochondrial complex III; IV- mitochondrial complex IV; PDH- pyruvate dehydrogenase; CS- citrate synthase; ACO- aconitase; IDH- isocitrate dehydrogenase; α-KGDH- α-ketoglutarate dehydrogenase; SDH- succinate dehydrogenase; FUM- fumarase; MDH- malate dehydrogenase.

Chapter 7

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