NOVA MEDICAL SCHOOL

THE ROLE OF CAROTID BODY IN BRAIN INSULIN RESISTANCE AND NEURODEGENERATION

ADRIANA MARGARIDA MATEUS CAPUCHO

A dissertation submitted in partial fulfillment of the requirements for the Degree of Master's in Biomedical Research (Specialization Area: Ageing and Chronic Diseases) at Faculdade de Ciências Médicas | NOVA Medical School of NOVA University Lisbon

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THE ROLE OF CAROTID BODY IN BRAIN INSULIN RESISTANCE AND NEURODEGENERATION

Adriana Margarida Mateus Capucho
Supervisors: Sílvia Vilares Conde, Assistant Professor, Principal Investigator at NOVA Medical School
Hugo Vicente Miranda, Affiliate Assistant Professor, Principal Investigator at NOVA Medical School

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” Scientific research is one of the most exciting and rewarding of occupations”

-Frederick Sanger-

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Abstract

Recent epidemiological studies have shown that individuals who develop Type 2 Diabetes (T2D) at an early age have an increased risk to develop neurodegenerative disorders, as Alzheimer's (AD) and Parkinson's Disease (PD). A common characteristic between T2D and these neurodegenerative disorders is the development of an insulin resistance state. Considering that the therapeutics that exists to neurodegenerative disorders only attenuate the symptoms and have several side effects, it is important to search for novel disease modifying therapies. Recently, Conde's group showed that in states of insulin resistance, glucose intolerance and obesity, features associated with T2D, the carotid bodies (CBs) were overactivated. The CBs are peripheral chemoreceptors classically defined as oxygen sensors, and in the last years they have been described as metabolic sensors. Moreover, when CBs activity was abolished via the resection of the carotid sinus nerve (CSN), which transmits the information from the CBs to the brain, T2D dysmetabolic features were reverted. Considering that dysmetabolism is a risk factor to the development of neurodegenerative disorders, the main goal of this project was to investigate if the abolishment of CBs activity via bilateral resection of the CSN could prevent/ameliorate the neurodegenerative process, and cognitive impairment associated with brain insulin resistance.

For that, we have used an animal model of dysmetabolism, the high fat-high sucrose (HFHSu) diet animal and tested the effect of the abolishment of CBs activity, through the resection of the CSN, on whole-body glucose metabolism and on the behavioural activity of the animals. Moreover, the levels of insulin signalling- related proteins, synaptic and neurodegenerative markers in prefrontal cortex and in the hippocampus were evaluated.

We observed that the HFHSu animals exhibited impaired cognitive and olfactory functions evaluated by the y-maze and the block tests. In contrast, the CSN resection prevented this phenotype in the y-maze test. Additionally, HFHSu diet led to an increase in the levels of advanced glycation end products (AGEs), and the amyloid precursor protein (APP) in the hippocampus, while the CSN resection attenuating this second effect. In the prefrontal cortex, it was observed an increase in the levels of alpha-synuclein (aSyn), and APP, effects abolished by CSN resection.

In conclusion, this project demonstrate that CBs modulation might have a role in preventing neurodegenerative processes associated with insulin resistance.

Resumo

Estudos epidemiológicos recentes mostraram que pessoas com Diabetes Tipo 2 (DT2) em idade precoce têm um risco aumentado de desenvolver doenças neurodegenerativas, como a doença de Alzheimer (DA) e doença de Parkinson (DP). Uma característica comum entre a DT2 e doenças neurodegenerativos é o desenvolvimento de um estado de resistência à insulina. Considerando que as terapêuticas existentes para as doenças neurodegenerativas apenas atenuam os sintomas e apresentam vários efeitos secundários e/ou adversos, é importante o desenvolvimento de terapias que alterem a progressão destas doenças. O grupo da Professora Sílvia Conde tem-se dedicado a estudar a relação entre os corpos carotídeos (CCs) e o desmetabolismo, e demonstrou que em estados de resistência à insulina, intolerância à glicose e obesidade, características associadas à DT2, os CCs se encontram sobre-ativados. Os CCs são quimiorreceptores periféricos definidos como sensores de oxigênio, tendo nos últimos anos sido também descritos como sensores metabólicos. Além disso, quando a atividade dos CCs foi abolida através da ressecção do nervo do seio carotídeo (NSC), que transmite a informação dos CCs para o cérebro, essas características desmetabólicas da DT2 foram revertidas. Considerando que o desmetabolismo é um fator de risco para o desenvolvimento de doenças neurodegenerativas, o objetivo principal deste projeto foi investigar se a abolição da atividade dos CCs via ressecção bilateral do NSC poderia prevenir/melhorar o processo neurodegenerativo e o comprometimento cognitivo associado à resistência à insulina no cérebro.

Para tal, utilizámos um modelo animal de desmetabolismo, os animais *high-fat-high sucrose* (HFHSu) tendo-se testado o efeito da abolição da atividade dos CCs através da ressecção do NSC, no metabolismo e na atividade comportamental dos animais. Foram também avaliados os níveis de proteínas relacionadas com a via de sinalização da insulina, marcadores sinápticos e neurodegenerativos no córtex pré-frontal e no hipocampo. Observou-se que os animais HFHSu apresentaram alterações nas tarefas cognitivas e olfativas avaliadas pelos testes do labirinto y e do *block test*, respetivamente, e que a ressecção do NSC preveniu esse fenótipo no teste do labirinto y. Além disso, a dieta HFHSu levou a um aumento nos níveis de *Advanced glycation end products (AGEs)* e na proteína precursora de amiloide (APP) no hipocampo, tendo a ressecção do NSC diminuído esse segundo efeito. No córtex pré-frontal, observou-se aumento nos níveis de alfa-sinucleína (aSyn) e APP, efeitos estes abolidos pela ressecção do NSC.

Em conclusão, este projeto demonstrou que a modulação da atividade dos CCs pode ter um papel na prevenção de processos neurodegenerativos associados à resistência à insulina.

Index

Acknowledgements .... *VII*

Abstract .... *IX*

Resumo .... *XI*

Index .... *XIII*

List of figures .... *XVII*

List of tables .... *XIX*

List of Acronyms .... *XXI*

I. State of the art *3*

1.1 . Insulin and insulin signalling **3**

1.2 . Insulin action and glucose homeostasis **5**

1.3 . Alterations in insulin action: insulin resistance and metabolic disorders **6**

1.4 . Type 2 Diabetes *Mellitus* (T2DM) **8**

1.4.1. Criteria for diagnosing diabetes 9

1.5 . Role of insulin in the brain **11**

1.5.1. Insulin and cognitive function 13

1.6 . Effects of insulin resistance and T2D in the brain **15**

1.7 . T2D and Neurodegeneration **16**

1.7.1. Alzheimer's Disease (AD) and other Dementias 16

1.7.2. Parkinson's Disease (PD) 18

1.7.3. Glycation in Alzheimer and Parkinson' Diseases 20

1.8 . Therapeutic strategies to neurodegenerative disorders **21**

1.9 . The Carotid Bodies (CBs) **22**

II. Hypothesis and Objectives *27*

III. Materials and Methods *31*

3.1. Animals and experiments **31**

3.2. Metabolic profiling 32

3.2.1. Insulin tolerance test (ITT) 32

3.2.2. Oral glucose tolerance test (OGTT) 32

3.3. Behavioural Studies 33

3.3.1. Open field test (OF) 33

3.3.2. Y-maze test 33

3.3.3. Block test 34

3.4. *Ex vivo* analysis 35

3.4.1. Tissue lysate preparation 35

3.4.2. Immunoblot analysis 35

***3.5. Data analysis* 37**

IV. Experimental results 41

4.1. *In vivo* results 41

4.1.1. Metabolic profiling: Effect of HFHSu diet and CSN resection on weight gain and caloric intake 41

4.1.2. Metabolic profiling: Impact of HFHSu diet and CSN resection on glycaemia, insulin sensitivity and glucose tolerance 42

4.1.3. Behaviour phenotyping: Impact of HFHSu diet and CSN resection on locomotor activity 44

4.1.4. Behaviour phenotyping: Effect of HFHSu diet and CSN resection on spatial learning and memory capacity 46

4.1.5. Behaviour phenotyping: Effect of HFHSu diet and CSN resection on olfactory acuity 47

4.2. *Ex vivo* results: Biochemical Analysis 49

4.2.1. Impact of HFHSu diet and CSN resection on brain insulin signalling 49

4.2.2. Impact of HFHSu diet and CSN resection on brain glycation 50

4.2.3. Impact of HFHSu diet and CSN resection on synaptic markers 52

4.2.4. Impact of HFHSu diet and CSN resection on aSyn and APP 53

V. Discussion 57

5.1. CSN resection improves peripheral metabolic function 57

5.2. CSN resection prevents cognitive impairment induced by HFHSu diet, evaluated *in vivo* by the y-maze test 58

5.3. CSN resection decreased the levels of aSyn and APP proteins in the cortex and hippocampus of the HFHSu animals 61

VI. Concluding remarks 69

VII. Future work 73

VIII. References 77

List of figures

Figure 1. Schematic figure of insulin signalling pathways. 4
Figure 2. Regulation of glucose homeostasis by the liver and pancreas. 6
Figure 3. Natural history of Type 2 Diabetes. 9
Figure 4. Insulin and glucose transport to the brain. 12
Figure 5. Physiopathology of Alzheimer's disease. 17
Figure 6. Type 2 diabetes accelerates Alzheimer's disease pathology. 18
Figure 7. Physiopathology of Parkinson's disease (PD). 19
Figure 8. Glycation in metabolic and neurodegenerative disorders. 21
Figure 9. Carotid Bodies (CBs) anatomy. 24
Figure 10. Schematic representation of the in vivo studies performed in Wistar rats submitted either to a NC diet and or to a HFHSu diet. 34
Figure 11. Metabolic profiling of NC and HFHSu animals before and 5 weeks after CSN resection. 43
Figure 12. Effect of HFHSu diet and CSN resection on locomotor activity evaluated by the OF test, of NC and HFHSu animals before, 2- and 5-weeks post-surgery. 45
Figure 13. Effect of HFHSu diet and CSN resection on special memory and learning capacity evaluated by the y-maze test of both NC and HFHSu animals before, 2-, and 5- weeks post-surgery. 47
Figure 14. Effect of HFHSu diet and CSN resection on olfactory acuity evaluated by the bock test before surgery and, 2- and 5- weeks post-surgery. 48
Figure 15. Effect of HFHSu diet and CSN resection on the levels of proteins involved in insulin signalling in prefrontal cortex (A), and in the hippocampus (B), evaluated by Western Blot. 50
Figure 16. Effect of HFHSu diet and CSN resection on the levels of Glioxalase-1 (GLO-1) and Advanced Glycation End Products (AGEs) in prefrontal cortex (A) and in hippocampus (B), evaluated by Western Blot and DotBlot, respectively. 51
Figure 17. Effect of HFHSu diet and CSN resection on the levels of 3 different synaptic markers in prefrontal cortex (A), and in the hippocampus (B), evaluated by Western Blot. 53
Figure 18. Effect of HFHSu diet and CSN resection on the levels of alpha-synuclein (aSyn) (A), and Amyloid β (Aβ) precursor protein (APP) (B) in prefrontal cortex (A) and in hippocampus (B). 54

List of tables

Table 1. Criteria for the diagnosis of diabetes. 10
Table 2. Primary and secondary antibodies used for the proteins of interest in the present study, and its respective brand, specie, and concentration used. 36
Table 3. Effect of HFHSu diet and CSN resection on weight gain and caloric intake. 41

List of Acronyms

AD- Alzheimer's Disease

ADA- American Diabetes Association

ADTIQ- 1-acetyl-6,7-dihydroxy-1,2,3,4- tetrahydroisoquinoline

AGEs- Advanced Glycation End Products

AKT/PKB – Protein Kinase B

AMPARs- a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors

AMPK- Mitogen-activated protein kinase

aPKC- Atypical protein kinase C

APP- Amyloid Percursor Protein

AS160 – Akt substrate of 160 kDa

aSyn- Alpha synuclein

AT- Adipose Tissue

ATGL- Adipose triglyceride lipase

Aβ- Amyloid beta

BBB- Blood Brain Barrier

cAMP – Cyclic Adenosine Monophosphate

CBs – Carotid Bodies

CNS- Central Nervous System

CO_2- Carbon Dioxide

CoA – Acetyl coenzyme A

CREB – cAMP response element-binding protein

CRTC2 – CREB regulated transcription coactivator 2

CSF- Cerebrospinal fluid

CSN – Carotid Sinus Nerve

DA- Dopamine

DG- Dentate gyrus

DHAP- Dihydroxyacetone phosphate

DM- Diabetes *Mellitus*

DPP-4- Dipeptidyl peptidase 4

FFA – Free Fatty Acids

FOXO1 - Forkhead Box Protein O1

FPG- Fasting Plasma Glucose

GAP- Glyceraldehyde 3- phosphate

GIP- Glucose-dependent insulinotropic polypeptide

GlcNAc- β-N-acetylglucosamine

GLO-1- Glioxalase-1

GLP-1- Glucagon Like Peptide-1

GLUT4 – Glucose Transporter Type 4

GLUTs- Glucose Transporters

GSK3b – Glycogen Synthase Kinase 3 Beta

HbA1c- Haemoglobin A1c

HF – High Fat

HFHSu- - High fat- high sucrose

HFHSu den- High fat- high sucrose denervated

HSu – High Sucrose

IR- Insulin Receptor

IRS- Insulin Receptor Substrates

IRSp53- Insulin Receptor substrate p53

ITT – Insulin Tolerance Test

kDa- Kilodalton

K_{ITT}- Constant rate of glucose disappearance

LTD- Long Term Depression

LTP- Long Term Potentiation

MAPKS – Mitogen-activated Protein Kinase

MAPT- Microtubule-associated protein tau

MEK- Mitogen-activated protein kinase

mEPSCs- Miniature excitatory postsynaptic currents

MGO- Methylglyoxal

mPFC- Medial prefrontal cortex

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

mTORC1 – Mammalian Target of Rapamycin Complex 1

NC den- Normal Chow denervated

NC- Normal Chow

NFKB - Nuclear Factor Kappa-light-chain-enhancer of Activated B Cells

NFTs- Neurofibrillary tangles

NMDARs- N-methyl-D-aspartate receptors

NR2A- N-methyl-D-aspartate receptors 2A subunit

NR2B- N-methyl-D-aspartate receptors 2B subunit

O_2- Oxygen

OF- Open Field

OGTT – Oral Glucose Tolerance Test

PBS- Phosphate-buffered saline

PD- Parkinson's Disease

PDE3B- Phosphodiesterase 3B

PDK1 – Phosphoinositide-dependent Kinase-1

PDK2 – Pyruvate dehydrogenase kinase isoform 2

PFA- Paraformaldehyde

PFKFB3- 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3

PIP3 – Phosphatidylinositol 3,4,5-trisphosphate

PSD-95- postsynaptic density protein 95

RR- Respiratory Rate

SGLTs- Sodium–dependent glucose transporters

ShRNAs- Short hairpin RNAs

SNAP-25- Synaptosomal-associated protein 25

SNpc- Substantia nigra pars compacta

SNS- Sympathetic Nervous System

SOCS3 – Suppressor of cytokine signalling 3

SOS/RAS- Son of sevenless

Srepb1 – Sterol Regulatory Element-Binding Protein 1

T2D – Type 2 Diabetes

TNF-α- Tumor Necrosis Factor-α

UDP- uridine diphosphate

TV- Tidal volume

WB- Western Blot

WHO- World Health Organization

State of the Art

I. State of the art

1.1. Insulin and insulin signalling

One hundred years ago, in 1921, an important hormone - insulin - was discovered by Frederick G. Banting, Charles Best and John MacLeod in Canada. This event completely changed the lives of mankind, and since then thousands of lives have been saved [1].

Insulin is a 51 amino acids peptide hormone, organized in 2 polypeptides chains, A (21 amino acids) and B (30 amino acids), which are connected by disulphide bridges. Insulin production and release is carried out by pancreatic β-cells. These cells play a fundamental role in regulating fat and carbohydrate metabolism, promoting their conversion into storage macromolecules such as glycogen, proteins, and lipids [2], [3], [4].

Insulin is the most important anabolic hormone in the organism and plays a critical role in the regulation of different physiological processes, including metabolism, cell growth and differentiation [2],[5]. The effects of insulin are highly pleiotropic and differ according to the target insulin-sensitive tissue. Concerning the peripheral regulation of metabolism, the major tissues targeted by insulin include the skeletal muscle, adipose tissue (AT), and liver [6]. Insulin stimulates the uptake of glucose in the skeletal muscle and adipose tissue, inhibits hepatic glucose production, and promotes the storage of substrates in the adipose tissue, liver, and muscle by triggering lipogenesis, glycogen, and protein synthesis, and by inhibiting lipolysis, glycogenolysis and protein breakdown. Insulin also plays a role in the central nervous system (CNS), in which one of its main functions is to act on the brain centres responsible for controlling appetite and decreasing it [7].

Although insulin play several functions in the organism, it is widely known as a glucose homeostasis regulating hormone. The tight control of the plasma levels of glucose is driven by the balance between glucose absorption from the intestine after feeding, production by the liver and uptake and utilization by several peripheral tissues. An increase in the plasma glucose levels triggers insulin production in the pancreatic islets by β-cells.

Insulin acts by binding to its receptor, the insulin receptor (IR). IR is a glycoprotein that belongs to tyrosine kinase receptor family, being composed by an α extracellular and β transmembrane subunits. When insulin binds to the α subunit, it triggers the dimerization of the receptor, forming an $\alpha_2\beta_2$ complex, leading to the autophosphorylation of β subunit at Tyr^{1158}, ^{1162} and ^{1163}, the activation of IR phosphorylation cascade [5]. Activation of IR signalling pathway, leads to

the recruitment and phosphorylation of several proteins such as the insulin receptor substrates (IRS 1-4), that will induce the activation of intracellular pathways as phosphatidylinositide-3-kinase (PI3K) and the mitogen-activated protein kinase (MAPK) cascades [8], [5]. Insulin-induced activation of Ras →MAPK promotes the regulation of cell growth and mitogenesis, whereas PI3K activation generates phosphatidylinositol (3,4,5)-triphosphate (PIP3) that will activate phosphoinositide dependent protein kinase-1 and -2 (PDK1 and PDK2), which regulate the effect of insulin on metabolism and pro-survival. Also, PDK1 and 2, have an important function in the activation of Protein Kinase B (AKT/ PKB) [9], [5],[10] AKT has a key function in phosphorylating several downstream proteins, as glycogen synthase kinase b (Gsk3b), that inhibits glycogen synthesis [11], [9]. AKT also phosphorylates other mediators such as the activation of AKT substrate 160 kDa, (AS160) activating Rab10GTPase, leading to the translocation of glucose transporter type 4 (GLUT4) to the plasma membrane for glucose uptake [12]. Phosphodiesterase 3B (PDE3B), an enzyme that catalyses the degradation of cyclic adenosine monophosphate (cAMP) is also activated by AKT. Additionally, AKT inhibits cAMP response element-binding protein (CREB)- regulated transcription coactivator 2 (CRTC2), important to increase hepatic gluconeogenesis; AKT also triggers liver lipogenesis by phosphorylating sterol regulatory element-binding protein 1 (SREBP1) [13], and phosphorylates Forkhead box protein O1 (Foxo1), which inhibits its transcriptional activity, leading to a supressed liver glucose production[5], [11].

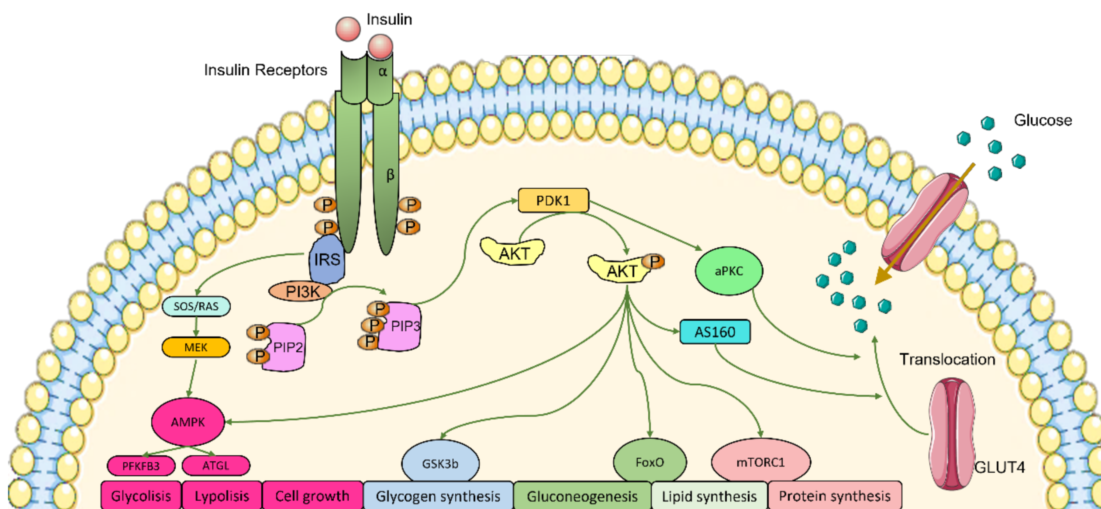


Figure 1. Schematic figure of insulin signalling pathways.

When insulin binds to its receptor (IR), there is the autophosphorylation of tyrosine kinase residues and the recruitment of various downstream regulatory proteins. The insulin signal is transduced among the target proteins/enzymes ending with the fusion of the GLUT4 vesicle with the cell plasma membrane and the placement of Glut4 transporters in the plasma membrane leading to the uptake of glucose. IRS, insulin receptor substrate; PI3K, phosphatidylinositide-3-kinase; PIP2, phosphatidylinositol (4,5)-biphosphate; PIP3, phosphatidylinositol (3,4,5)-triphosphate; PDK1, phosphoinositide dependent protein kinase-1; AKT, protein kinase B; AS160, Akt substrate of 160 kDa; aPKC, atypical protein kinase C; SOS/RAS, son of sevenless; MEK, mitogen-activated protein kinase; AMPK,

mitogen-activated protein kinase; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3; ATGL, adipose triglyceride lipase; GSK3b, glycogen synthase kinase b; FoxO, forkhead box protein O1; mTORC1, mammalian target of rapamycin complex 1; GLUT4, glucose transporter type 4.

1.2. Insulin action and glucose homeostasis

Glucose is the major source of energy of our body, being insulin the primary regulator of blood glucose concentrations. Together with insulin, glucagon also plays an important role in the regulation of glucose homeostasis, being secreted by the pancreatic α-cells [10], [14]. Whereas β-cells secret insulin to decrease blood glucose levels, α-cells secret glucagon to increase the levels of glucose in the blood to find a balance and meet the body's metabolic requirements [14].

Liver is responsible for 85% of endogenous glucose production, the other 15% are produced by the kidney. Additionally, both glycolysis and glycogenesis contribute equally to the basal rate of hepatic glucose production. In the postabsorptive state, the majority of total glucose disposal occurs in insulin dependent tissues. Therefore, since glucose is the main source of energy in the brain, approximately 50% of that is absorbed by the brain; 25% of glucose is used by the liver and gastrointestinal tissues, and the remaining 25% is absorbed by the muscle and in a less proportion by the AT [14].

After a meal, there is an increase in plasma glucose concentrations, that stimulate insulin production and release by the pancreatic β cells, and the inhibition of hepatic glucose production [15], [14]. Insulin as an antilipolytic hormone, is capable of inhibit lipolysis, leading to a decreased concentration of free fatty acids (FFA) in the circulation [10], [14], [16]. On the other hand, after a meal, approximately half of total hepatic glucose output is dependent on the basal levels of glucagon. The inhibition of glucagon production and release leads to a decrease in glucose production by the liver and a reduction in plasma glucose concentrations [14]. Other mediators that share glucose homeostasis control together with glucagon, include the incretins - glucagon-like peptide (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP). Incretins are neuropeptides secreted by the intestinal enteroendocrine cells in response to food. In fact, GLP-1 and GIP are the incretins that have higher effects in the production and release of glucose after eating a meal (about 90%) [14], [17], [18]. GLP- 1 has the ability to slowly empty stomach, it supresses glucagon secretion, and increases pancreatic insulin secretion, increasing insulin sensitivity in peripheral tissues. These findings suggest that GLP-1 also has a role in extra pancreatic tissues participating in overall glucose homeostasis [19], [20]. In fact, several studies have shown that GLP-1 increases glycogen synthesis and glucose transport by activating PI3K

and increasing phosphorylation of AKT, in skeletal muscles and the liver. Moreover, it was also demonstrated that GLP-1 stimulates glucose uptake, lipid synthesis and lipolysis in adipocytes isolated from either normal rats or streptozotocin-induced diabetic rats [21]. GIP is a hormone produced in the enteroendocrine K-cells and released into the bloodstream in response to nutrient stimulation. GIP can increase insulin production, which is dependent on the circulating concentration of glucose. The GIP action is greater when plasma glucose concentration is high and almost null when the plasma glucose concentration returns to basal levels [14], [22]. When insulin fails to control glucose homeostasis, the functions of all these proteins are also affected, leading to the development of dysmetabolic states.

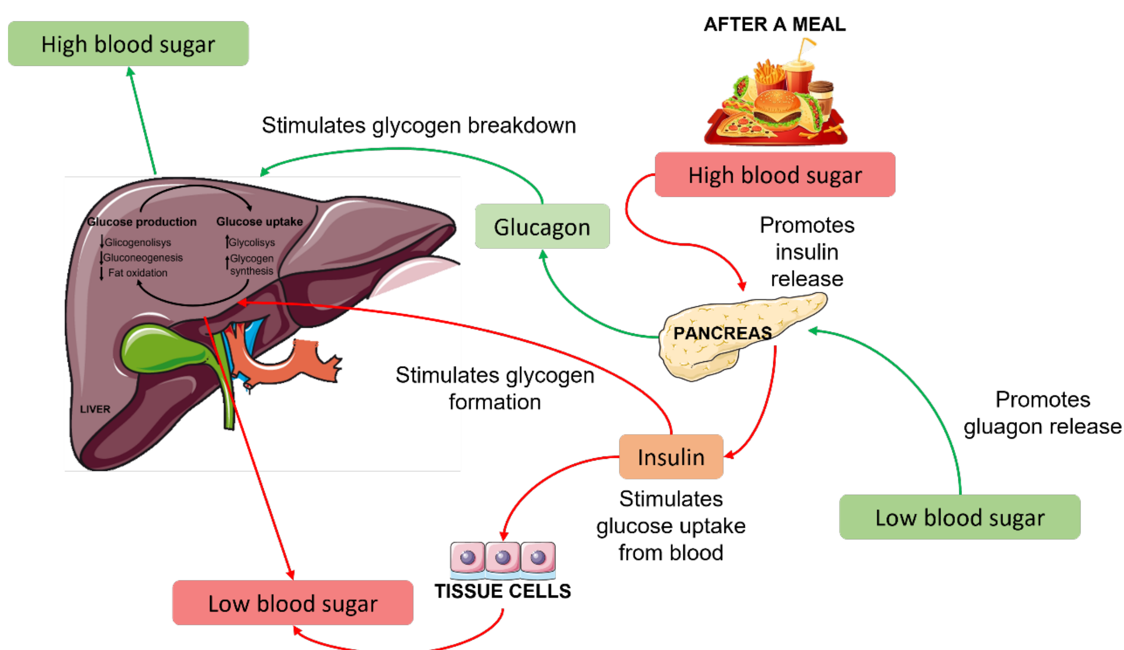


Figure 2. Regulation of glucose homeostasis by the liver and pancreas.
After a meal, blood glucose levels rise promoting insulin release by the pancreas. Insulin stimulates glucose uptake from blood to tissues, and promotes glucagon formation by the pancreas, leading to a decrease in blood glucose levels. On the other hand, when blood glucose levels are low, the pancreas promotes glucagon release which triggers glycogen breakdown by the liver culminating in an increase in blood glucose levels.

1.3. Alterations in insulin action: insulin resistance and metabolic disorders

The primary role of insulin in our body is to promote glucose uptake from the blood to tissues, among other functions [10]. When insulin becomes incapable of exerting adequately its effects on insulin sensitive tissues, a state of insulin resistance occurs [23], [24]. The mechanisms involved in the development of insulin resistance remain largely unknown. Nevertheless, a wide range of factors have been considered to contribute for this phenomenon such as: hyperinsulinemia, associated with Type 2 Diabetes *Mellitus* (T2DM), obesity, inflammation,

among others [24], [9]. The link between insulin resistance and hyperinsulinemia works in both ways with insulin resistance being one of the factors contributing to hyperinsulinemia also to the development of insulin resistance. In response to a lack of insulin action in the insulin sensitive tissues, the pancreatic β-cells increase their production of insulin aiming the normalization of glucose levels. However, the continuous overstimulation of pancreatic β-cells for long periods of time, decrease the capacity to produce high amounts of insulin and insulin secretion become impaired. Once insulin secretion by pancreatic β -cells drops to levels that are not sufficient to maintain normal blood glucose levels, people become insulin dependent [23], [24]. In fact, pre-clinical studies in animal models (rats) show that the administration of high doses of insulin induce insulin resistance [25]. In humans it was also demonstrated that even 40 hours after insulin infusion, plasma insulin levels are still high which leads to a decrease in glucose uptake, indicating an impairment in insulin signalling [25], [26]. Both studies support the idea that hyperinsulinemia at levels similar to those observed in many insulin-resistant states contribute for the development of insulin resistance. Remarkably, obesity and aging are the major risk factors for the development of insulin resistance and other metabolic diseases [24].

Obesity is defined by the World Health Organization (WHO) as an excessive adipose tissue accumulation, sufficient to impair health, being associated with several comorbidities [27]. Associated with obesity there is an increased inflammatory process which starts in the AT and in the liver with infiltration of macrophages and levels of proinflammatory cytokines as tumour necrosis factor-α (TNF-α) and leptin, among others [28]. This inflammatory state is capable of inhibiting insulin signalling in the AT and in hepatocytes through the inhibition of IRS-1 and insulin receptor levels [29], [30]. TNF-α also induces insulin resistance by phosphorylating IRS-1, and by increasing lipolysis and the secretion of FFAs to the plasma which inhibit and reduce the levels of GLUT4, decreasing glucose uptake [31], [32], [33].

On the other hand, leptin, an adipokine secreted mainly by the AT, has a crucial role in controlling energy homeostasis. Among its several functions, leptin is mainly known as the satiety hormone, since reduces appetite and increases energy expenditure by acting on its receptors in the hypothalamus, the major neuronal centre that controls satiety [34], [35]. It leads to the activation of the sympathetic nervous system towards the AT aiming to decrease AT mass and body weight [34]. In fact, in recent years the view regarding leptin functions in the organism has changed. It was considered an anti-obese hormone however later studies have found that in obese individuals this correlation was not demonstrated. Obese individuals show a correlation between body fat mass index and serum leptin levels meaning that an endogenous

leptin-resistant mechanism occur in obesity, this being reflected in an hyperphagic behaviour [34], [7]. Although obese individuals present increased plasma levels of leptin, they also have reduced levels of circulating adiponectin. Adiponectin is produced by adipocytes but in much higher concentrations. When adiponectin binds to its receptors: AdipoR, specially to AdipoR1 and AdipoR2 it regulated fatty acid oxidation and insulin sensitivity, among others, playing a key role in the pathogenesis of metabolic disorders. In fact, lower circulating levels of adiponectin, are inversely correlated with states of insulin resistance. Some studies have shown that mice with deficiency in adiponectin levels without affect leptin levels, are insulin resistance and more prone to develop metabolic disorders. Moreover, when these animals were submitted to hypercaloric diets, they become even insulin-resistant [36].

1.4. Type 2 Diabetes *Mellitus* (T2DM)

Metabolic disorders are mainly associated with a modern lifestyle, characterized by physical inactivity, sedentarism and with increased intake of hypercaloric diets [37], [38].

Diabetes *Mellitus* (DM) is a chronic metabolic condition characterized by high blood glucose levels due to the inability of the body to produce enough insulin to reduce glucose levels or due to the inefficient of insulin a phenomenon designated as insulin resistance [14],[39].

Currently, it is estimated that 463 million people aged between 20-79 years suffer from diabetes, representing 9.3% of world's population within this range of ages. In 2030 it is estimated that this number will rise to 578 million people (10.2%) and 700 million (10.9%) in 2045. Even more worrying is that in 2019, the estimated number of deaths resulting from diabetes and its complications were of 4.2 million people worldwide [23].

In accordance with the American Diabetes Association (ADA) classification, there are four different types of diabetes: Type 1 Diabetes, Type 2 Diabetes (T2D), Gestational Diabetes, and specific types of diabetes due to other causes [39]. T2D typically begins with the development of a state of insulin resistance. During this period, the pancreatic β-cells are stimulated to increase insulin production and secretion to maintain normal blood glucose concentration. Upon diagnosis of T2D, about 40-50% of the β-cells are already dysfunctional and no longer able to compensate the high levels of circulating glucose. This phenomenon results in a state of glucose intolerance, which ultimately leads to a state of fasting hyperglycaemia (figure 3) [14], [40].

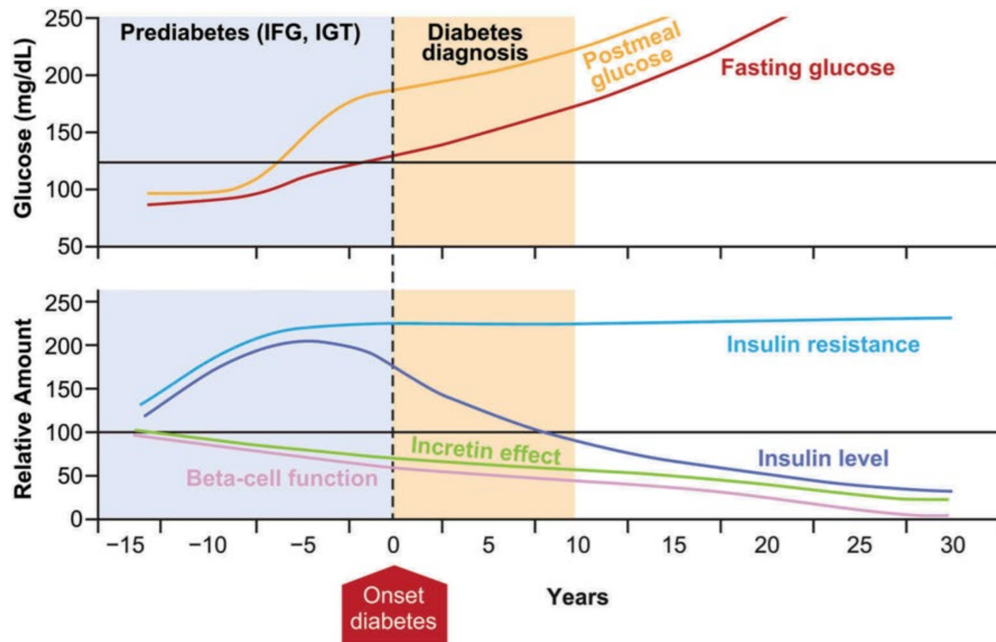


Figure 3. Natural history of Type 2 Diabetes.

The graph on the top represents the evolution of blood glucose levels from pre-diabetes to the type 2 diabetes stage. The graph below shows the decrease in beta cell function that are accompanied by a decrease in the incretin effect and an increase in insulin resistance. Note also, that in an initial stage of insulin resistance, the pancreas increases insulin secretion aiming to maintain glucose levels within the normal range. However, when the pancreas is not able to maintain the high secretion of insulin, insulin levels drop leading in a first stage to a post-prandial glucose intolerance and then to fasting hyperglycaemia [40].

T2D is most frequently observed in older adults, however its prevalence is increasing in children and in young people due to the increased prevalence of obesity and physical inactivity.

In fact, obesity is the most frequent metabolic disorder in the world and is believed to be a major cause of T2DM [37], [23], [41]. According to the National Diabetes Statistics Report (2017) [42], 87.5% of diabetic adults are overweight/obese. However, the 18.4 million people who are overweight/ obese and have diabetes only represent 13.8% of these individuals in 2016 in USA [43].

1.4.1. Criteria for diagnosing diabetes

Usually, it is not possible to determine the exact time when diabetes started, and sometimes the disease is detected late. T2D is often detected at an advanced stage when complications related to it begin to appear, such as retinopathy, diabetic foot, hypertension, among others. The most effective criterion for detecting diabetes is the measurement of blood glucose levels and of glucose tolerance. Both, WHO and ADA propose multiple ways to perform a correct diagnosis of the disease, namely the assessment of fasting plasma glucose (FPG), the oral

glucose tolerance test (OGTT), the assessment of random plasma glucose, and or the measurement of Haemoglobin A1c (HbA1c- glycated haemoglobin) [23], [39].

The FPG is used to assess blood glucose levels, assessing the rate of glucose in the bloodstream after a fasting period of 8 to 12 hours. The OGTT is a two-hours test to evaluate blood glycaemic levels before and two hours after the oral administration of 75 grams of glucose. This test provides information on how the body metabolizes circulating glucose[44]. HbA1c is an indicator of the average values of glycaemia during the last 2-3 months [45]. Generally, FPG, OGTT and A1c are equally suitable for the diagnosis of T2DM. The random glycaemia test is used when symptoms of hyperglycaemia are identified (see criteria for the diagnosis of diabetes in the table 1).

After diagnosis, the most appropriate therapeutic approach must be applied to avoid the progression and the development of comorbidities. One of the major and first strategies to control T2D is to combine a heathy diet with physical exercise to decrease weight and obesity, and consequently prevent the development and progression of T2D [46].

Apart from peripheral comorbidities, diabetes is also associated with several pathologies related with the CNS [47]. It is known that diabetes is associated with an increased risk to develop conditions as psychiatric and neurodegenerative disorders [48], [49]. As such, the control of peripheral and central insulin and glucose levels can be an important approach to prevent the development of those pathologies.

Table 1. Criteria for the diagnosis of diabetes.
FPG ≥126 mg/dL (7.0 mmol/L). Fasting is defined as no caloric intake for at least 8 h. OR 2-h PG ≥200 mg/dL (11.1 mmol/L) during OGTT. * OR HbA1C ≥6.5% (48 mmol/mol). OR in a patient with classic symptoms of hyperglycemia or hyperglycemic crisis, a random plasma glucose ≥200 mg/dL (11.1 mmol/L). FPG, fasting plasma glucose; OGTT, oral glucose tolerance test; 2-h PG, 2-h plasma glucose [44].

Criteria for the diagnosis of diabetes
FPG ≥126 mg/dL (7.0 mmol/L). Fasting is defined as no caloric intake for at least 8 h.*
OR
2-h PG ≥200 mg/dL (11.1 mmol/L) during OGTT. The test should be performed as described by WHO, using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water.*
OR
A1C ≥6.5% (48 mmol/mol). The test should be performed in a laboratory using a method that is NGSP certified and standardized to the DCCT assay.*
OR
In a patient with classic symptoms of hyperglycemia or hyperglycemic crisis, a random plasma glucose ≥200 mg/dL (11.1 mmol/L).

DCCT, Diabetes Control and Complications Trial; FPG, fasting plasma glucose; OGTT, oral glucose tolerance test; WHO, World Health Organization; 2-h PG, 2-h plasma glucose. *In the absence of unequivocal hyperglycemia, diagnosis requires two abnormal test results from the same sample or in two separate test samples.

1.5. Role of insulin in the brain

Apart from exerting its effect on peripheral insulin-sensitive tissues, insulin also plays a key role in the CNS. Insulin plays very important functions at the brain level such as the control of neuronal transmission and survival, neurogenesis, plasticity, and memory and cognition. The best-studied role of insulin in brain function is related to the control of glucose homeostasis, which is deeply related to the regulation of eating behaviour, satiety, and reward pathways [9], [8], [50].

Glucose is the main source of energy in the brain. It reaches this organ via its transportation by a specific family of proteins - the glucose transporters (GLUTs) [51]. GLUTs have numerous isoforms, and some of them are known to be expressed in the brain having a role in neuronal homeostasis and function [52]. In fact, they can be divided into 2 different types: sodium–dependent glucose transporters (SGLTs) and facilitated diffusion glucose transporters (GLUTs). There are 14 different classes of GLUTs, 13 of them are constitutively expressed in the cell membrane. Without insulin stimulation, the density of GLUT4 in the membrane is extremely low, being present in cytoplasmic vesicles [53]. In fact, GLUT4 moves to cell membrane to promote glucose uptake upon insulin binding to insulin receptor. The binding of insulin to its receptor triggers the activation of downstream protein kinases (AKT, AMPK, among others) that phosphorylates putative effectors that modulate GLUT4 translocation to the membrane [53], [54]. The effect of insulin and its relevance on glucose uptake in the brain is not consensual and it was only more recently that some authors showed that insulin might play a role in regulating global brain glucose uptake. In fact, several studies from the 80's that focused on the effect of insulin in brain glucose uptake showed that brain glucose metabolism was unaffected by insulin. Goodner et al. measured glucose uptake in fasting rats 30 minutes after 0.1U insulin administration [55] and observed that the brain did not increase the rate of glucose uptake concluding that the brain was "insensitive to insulin" [56]. More recently, using 18-fluorodeoxyglucose positron emission tomography, it was shown that insulin increases brain glucose uptake in humans, mostly marked in cortical areas [57],

Insulin reaches the brain from circulation by crossing the blood-brain barrier (BBB), a barrier which has an important function in controlling ions, and molecules transport between the bloodstream and the CNS [8], [58], [59]. Like other proteins, insulin cross the BBB through saturated transport [60], although this process in not yet fully described and well-known. What is well established and reported is that there are some factors that can influence the rate of insulin transport to the brain. For example, it is known that hypercaloric diets impair insulin

transport to the brain [61]. However, Elizabeth M. Rhea et al., showed that IR inhibition with the selective antagonist S961, did not modify insulin transport across the BBB, suggesting that insulin is able to cross this barrier in an IR signalling independent manner, or that the antagonist is unable to block all the receptors and therefore insulin could still bind to some [62].

However not all insulin that exists in the brain comes from the periphery. It was found that insulin can be synthetized in the brain by neurons and astrocytes in relative high amounts [63], [64] a process that may be independent of insulin peripheral concentrations, since the levels of insulin in the hypothalamus do not correlate with pancreatic insulin secretion during fasting [65].

Insulin in the brain acts through the binding to its receptors. In the brain, two different isoforms of the IR are described: a long isoform, IR-B and a short isoform, IR-A. Although IR-A is predominantly expressed in the brain [63], insulin has similar affinity and potency for both isoforms [66].

Insulin receptors are present in numerous brain regions, with higher expression at the olfactory bulb, cerebral cortex, hippocampus, and the hypothalamus [67].

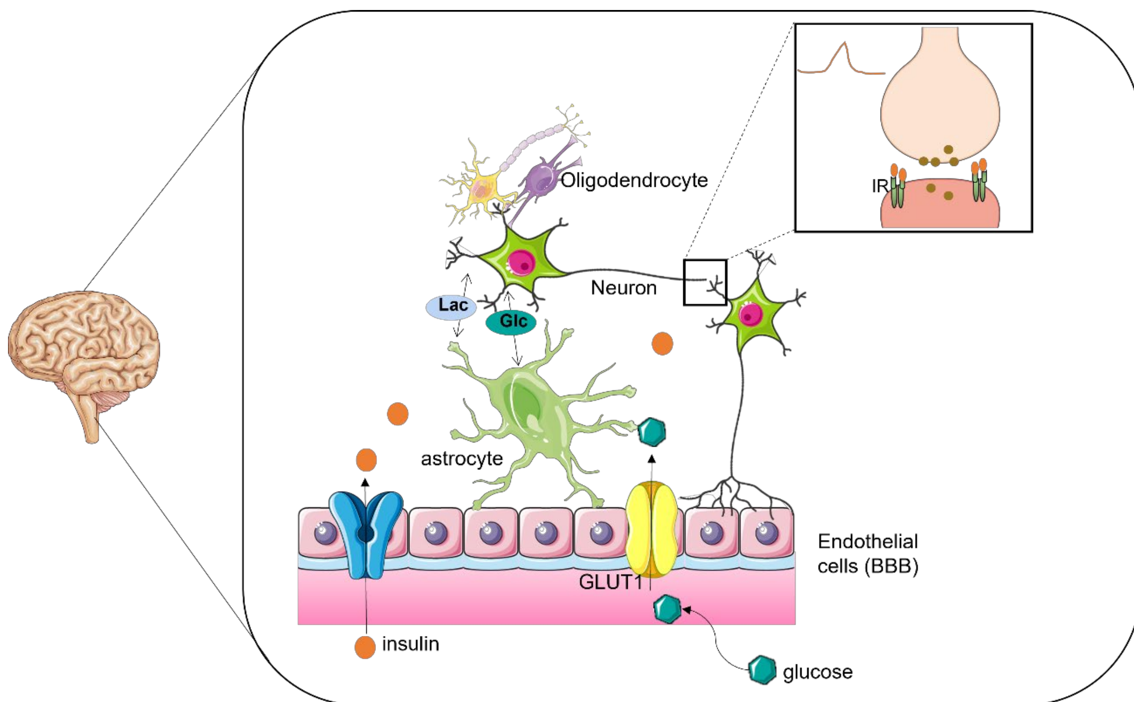


Figure 4. Insulin and glucose transport to the brain.
Glucose (Glc) is the main source of energy to the brain. Glucose reaches the brain from the bloodstream by crossing the blood brain barrier (BBB) through glucose transporter 1 (GLUT1). Glucose provides energy to the neurons helping in neurotransmission processes. Insulin crosses the BBB by saturated transport and binds to its receptors mainly located post- synaptically.

1.5.1. Insulin and cognitive function

The role of insulin in the brain was first studied in the context of energy homeostasis, a process mainly regulated by the hypothalamus [68]. More recently, the role of insulin in other brain functions such as memory and cognition, neuronal development, and plasticity [52], [69] have been explored. Particularly its action in the hippocampus, a brain region that expresses high levels of insulin receptor [68].

The hippocampus is a complex structure in the brain located in the temporal lobe. It forms part of the limbic system, being an extension of the cerebral cortex, with a major role in learning and memory [70]. The hippocampus is a very plastic region, with a key role in memory formation, organization, and storage. The hippocampus converts short-term memory into long-term memory, solves spatial memory, and recollects the past experiences of places. It also plays a pivotal role in emotions and behaviour of a person [71]. Different parts of the hippocampus have distinct functions in certain types of memory: spatial memory, mainly processed by the rear part of the hippocampus; memory consolidation, a process by which the hippocampus does the organization of stored information in the neocortex; and memory transfer, since long term memories are not stored in the hippocampus [71], [72]. Information in the hippocampus travels along a unidirectional trisynaptic pathway originating from the entorhinal cortex and projecting to the dentate gyrus (DG), then to area CA3 and finally to area CA1 of the horn of Ammon [73],[74]. These areas of the hippocampus have different functions, being composed of different types of neurons: the granular cells in the DG and pyramidal cells in the areas CA1 and CA3 of the horn of Ammon with a vast network of interneurons [74]. In recent years, special attention has been paid to the CA3 region for its specific role in memory formation and neurodegeneration [75]. CA3 receive excitatory input from the pyramidal cells and then inhibitory feedback they will inhibit the pyramidal cells. This recurrent inhibition is a simple feedback circuit that can dampen excitatory responses in the hippocampus, being involved in memory formation processes [76], [77].

The hippocampus has also an important role in brain plasticity. It underlies learning and memory depends both on the activity and the number of synapses. Synapses may be modulated via potentiation or depression, processes that may regulate the formation of new dendritic spines promoting tasks, learning, and consolidating behavioural alterations [52] [78].

Considering the higher mRNA levels of insulin receptors in dendrites and synapses, IR may be involved in the regulation of synaptic plasticity mechanisms and memory formation [68] [79]. Hippocampal neurons treated with insulin for 48h show an increase in the frequency of

miniature excitatory postsynaptic currents (mEPSCs), while upon downregulation with short hairpin RNAs (ShRNAs), fewer dendritic spines are formed, therefore reducing the frequency of mEPSCs [52], [80]. Insulin was also shown to regulate neuronal plasticity by controlling long-term potentiation (LTP) and long-term depression (LTD) [81]. Moreover, IR substrate p53 (IRSp53) interacts with the postsynaptic density protein 95 (PSD-95), present at excitatory synapses, therefore regulating a variety of receptors and channels, and increasing dendritic spine formation [82], [52], [52]. Insulin further plays a role on glutamatergic response by increasing the recruitment of the N-methyl-D-aspartate receptors (NMDARs) to the membrane and by enhancing the phosphorylation of NR2A and NR2B subunits [83]. In agreement, IRS2 knockout mice showed lower activation of NR2B subunits and a decrease in the LTP at CA3-CA1 synapses, however with higher density of CA1 dendritic spines [84], [83], [85]. Additionally, downregulation of a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) activity of CA1 neurons in the hippocampus is crucial to insulin-induced LTD, an important feature to process memory formation [86].

The hippocampus is not the single player in the generation and regulation of memory and cognition. The prefrontal cortex is known to mediate decision making being involved in the retrieval of remote long-term memory and supporting memory and consolidation in a time scale ranging from seconds to days [87]. In fact, they work together to support the rapid encoding of new information, consolidation, and organization of memory networks. The cerebral cortex is known to process information about objects and events that we experience, and about the places where occur. Additionally, the ventral hippocampus (in the rat) and the anterior hippocampus (in humans), sends information to the medial prefrontal cortex (mPFC), suggesting that the mPFC could accumulate interrelated memories. The information of mPFC is sent back to another cortex regions cortex, however the mPFC may bias or select the retrieval of event information in the 'what' stream. Therefore, interactions between these 2 brain regions can support the ability to create contextual representations that are associated with recent memories and use them to remember memories that are appropriate within a given context [88].

Considering the key role of cortex in memory and decision making, it was reported that patients with frontal lobe damage exhibit inappropriate social behaviours and memory decline [89], suggesting that these dysfunctions can result from an impairment of memory storage in the prefrontal cortex.

1.6. Effects of insulin resistance and T2D in the brain

The impairment of insulin signalling not only affects peripheral tissues, but also has a negative impact in some neuronal functions. Defects in insulin action in the CNS represent a possible relationship between metabolic and cognitive disorders [90], [91].

Interestingly, it was reported that T2D patients and/or obese people exhibit decreased concentrations of insulin in the cerebrospinal fluid (CSF), having higher insulin levels in plasma [92], [93]. Additionally, insulin-mediated transport to the brain relates to peripheral insulin sensitivity since low CSF insulin concentrations were observed in insulin-resistant individuals. It was also observed that this phenomenon is independent of body weight, and these findings suggest that insulin resistance impairs insulin transport across the BBB[93].

Insulin acts in the brain playing a direct effect on dopaminergic signalling, hippocampal synaptic plasticity, expression and regulation of some proteins, BBB function, among others. Deregulation of those functions could lead to neurodegenerative and psychiatric disorders [91].

In fact, changes in insulin signalling at the hippocampal level affect the molecular mechanisms underlying neuronal plasticity. These events have a negative impact on the maintenance of mental abilities, such as memory storage, which can be a risk factor for dementia [94]. In the brain upon desensitization of insulin receptors, insulin lose its ability to improve neuronal plasticity [92]. Insulin signalling impaired by the consumption of high fat diet leads to a decrease in the expression of PSD-95, and alterations in functional and structural plasticity [95], [96]. Moreover, studies performed in Zucker rats, a genetic insulin resistant animal model, showed alterations in hippocampal insulin signalling, which have a negative impact in synaptic activity, as these rats exhibit impaired LTP at CA3-CA1 synapses [97]. Studies performed in rodent models of metabolic syndrome, that were fed with high fat diet presented vascular dysfunction, learning impairment and a decreased capacity to memorize tasks [92]. Moreover, there are reports showing that alterations in glucose correlating with cognitive dysfunction in T2D patients [98].

Another consequence of brain insulin resistance is neuronal loss in certain brain regions, leading to neurodegenerative disorders [52], [92].

1.7. T2D and Neurodegeneration

Neurodegeneration is the result of the progressive loss of structure and function of neurons, which ultimately results in cell death. It is a hallmark of multiple brain disorders and a process that is accelerated by the pathological misfolding and aggregation of given proteins [99].

Most cases of neurodegenerative diseases are of sporadic origin, being aging the major risk factor. In last decades, several studies revealed a relevant association between metabolic and neurodegenerative diseases such as AD and PD [100], [101], [102].

1.7.1. Alzheimer's Disease (AD) and other Dementias

Currently, it is estimated that around 50 million people worldwide are living with Alzheimer's Disease, the most prevalent neurodegenerative disorder worldwide, and the most common form of dementia which accounting from 60-70% of cases [103].

AD is characterized by progressive loss of memory and other associated cognitive functions [104]. It can be classified as late-onset sporadic AD and early-onset familial AD, which constitutes only around 5% of all AD cases. Early-onset familial form is caused by mutations in genes of the amyloid-β precursor protein (APP), and/or presenilin-1, or presenilin-2 [105]. In fact, mutations in presenilin are the main cause of Early-onset familial AD [104]. These mutations are the result of a partial loss of function in γ-secretase complex, leading to a wide range of several alterations in downstream effectors. A consequence of this phenomenon is the decrease in the generation of APP, showing that presenilin mutation are associated with its loss of function [106]. Therefore, it affects some function leading to an incomplete digestion of the Aβ [104], [106], [105].

Since most AD cases are sporadic, it is highly relevant to determine the aetiology and mechanisms by which AD occurs. The pathological hallmark of AD is the extra-neuronal formation of senile plaques composed by accumulated amyloid-β (Aβ) peptides, and the intraneuronal accumulation of neurofibrillary tangles (NFTs) of hyperphosphorylated tau protein [107], [108].

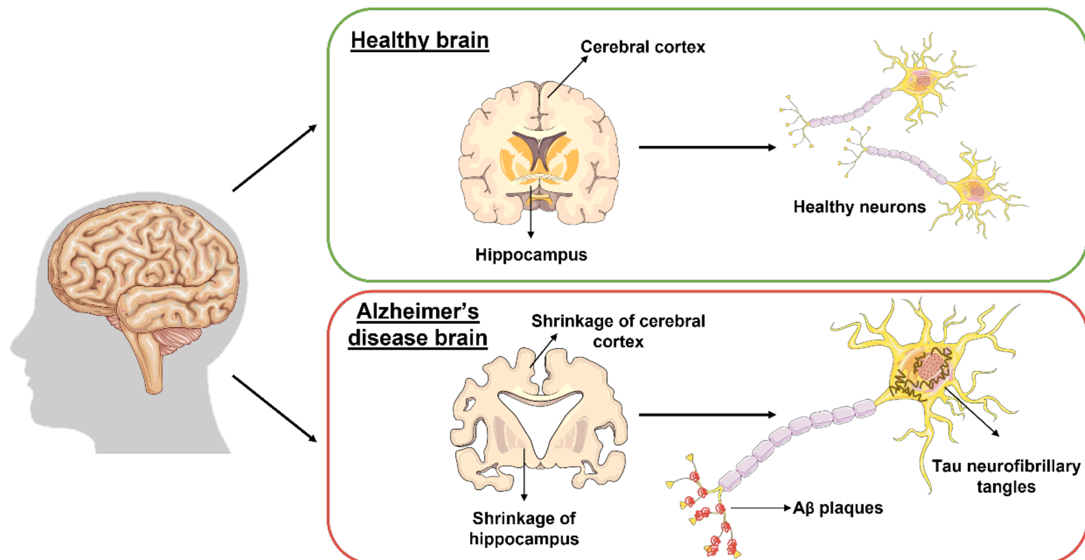


***Figure 5**. Physiopathology of Alzheimer's disease.*

The physiological structure of a healthy brain and Alzheimer's disease brain, presenting extracellular accumulation of Aβ plaques and intraneuronal accumulation of neurofibrillary tangles of hyperphosphorylated tau protein.

Interestingly, AD and other forms of dementia have been linked to metabolic disorders as obesity and/or T2D. Epidemiologic studies demonstrate a close association between obesity and T2D with an increased risk for AD. In fact, T2D patients have 50 to 75% increased risk of developing AD compared to age and gender matched control groups [109], [110], [111]. Furthermore, a cohort study revealed that 81% of AD patients have T2D or exhibit impaired fasting blood glucose [112]. Several studies support the hypothesis that AD is a metabolic degenerative disorder which exhibit impaired brain glucose uptake. Impaired brain glucose metabolism occurs at the early stage of the disease and aggravates with AD progression, suggesting that insulin resistance may contribute to amyloid-β aggregation and accumulation, and to the hyperphosphorylation of tau leading to impaired synaptic plasticity and deficits in memory [104], [113].

The hippocampus and cerebral cortex are the brain regions most vulnerable to aggregation and accumulation of Tau, a feature of AD. These brain regions express high levels of IRs, however, under AD conditions it has been reported that there are decrease in levels of insulin mRNA [114]. In fact, impaired insulin signalling leads to decreased PI3K/AKT signalling, leading to the over-activation of GSK-3β [115]. Consequently, the over-activation of GSK-3β results in the hyperphosphorylation of tau and to a higher production of Aβ peptides, contributing to cognitive impairment [115], [116], [117]. Additionally, insulin deficiency leads to a decrease in GLUT levels, leading to an impairment in glucose uptake/metabolism in the brain [12]. Decreased intraneuronal glucose uptake causes a reduction of intraneuronal generation of ATP,

impairing synaptic activity and cognitive function. It also leads to a decrease in the levels of uridine diphosphate (UDP)- β-N-acetylglucosamine (GlcNAc) via the hexosamine biosynthetic pathway and, consequently, decreasing tau O-GlcNAcylation, a process that inversely regulates tau phosphorylation. An increase of tau phosphorylation induces the formation of tau oligomers, that are neurotoxic, contributing to neuronal loss and degeneration [104].

This unequivocal link between AD and T2DM/impaired insulin signalling prompted the concept of type 3 diabetes to refer to AD [118]. Importantly, some studies referred long-term hyperinsulinemia as a risk factor for dementia. In contrast, insulin administration to AD patients is able to improve memory formation, by keeping glucose levels constant [119]. These pathways require further clarifications.

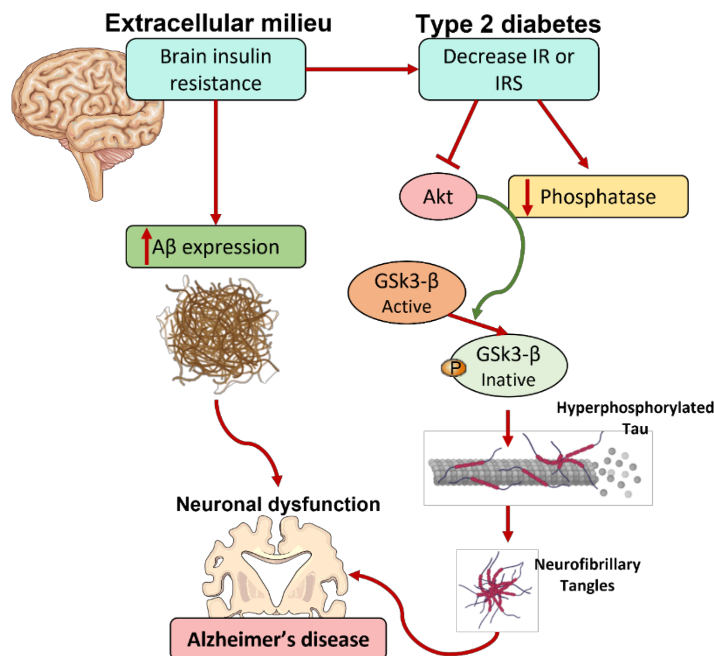


Figure 6. Type 2 diabetes accelerates Alzheimer's disease pathology.
Brain insulin resistance leads to alterations in insulin signalling pathway that inactivate AKT triggering the inactivation of GSK3-β leading to tau hyperphosphorylation and higher production of Aβ peptides.

1.7.2. Parkinson's Disease (PD)

Parkinson's Disease is the second most common neurodegenerative disorder worldwide, currently affecting more than 6 million people [120], [121]. A major hallmark of PD is the loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc), and the presence of intracellular Lewy Bodies, mainly composed of alpha-synuclein (aSyn) protein aggregates [122], [123] [124]. PD is well known for its motor impairments including resting tremor, bradykinesia and postural instability, major consequences from the loss of DA neurons in the SNpc and

striatum. However, PD patients also exhibit non-motor features such as hyposmia, sleep disorders, constipation, cognitive impairment and depression, anxiety and even [125]. Some of these alterations precede the manifestation of motor impairments by a few years.

Only 3-5% of PD cases are genetic, thus as occurs in AD, most PD cases are sporadic, suggesting that nongenetic factors contribute to the onset progression of these pathologies [126]. In fact, a wide range of environmental factors could contribute to the development of PD, as pesticides and heavy metals. The major risk factor for PD is ageing. Moreover, dysmetabolic conditions such as T2D and obesity are also PD risk factors [127], [128], [129], [130]. Recent epidemiological studies reported that 80% of PD patients exhibit impaired glucose metabolism. Additionally, T2D not only increases up to 50% the risk of developing PD, but also accelerates the progression of motor and cognitive deficits [131], [132]. Even more impressive, is the fact that this risk increases up to 380% for individuals who develop T2D at an early age, between 25 to 45 years (Pagano et al., 2018; Yang et al., 2017).

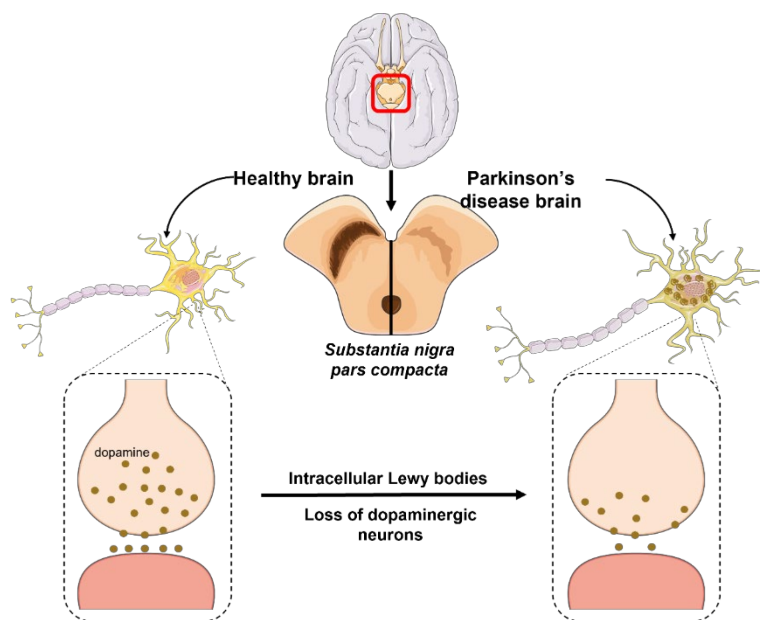


Figure 7. Physiopathology of Parkinson's disease (PD).
The physiological structure of a healthy substantia nigra versus a substantia nigra of PD patient. In PD there is the aggregation and accumulation of aSyn in Lewy bodies which are toxics leading to dopaminergic neuronal loss.

Several reports suggest a molecular association between dysmetabolic diseases and PD. *Ex vivo* findings reveal a close association between insulin resistance and DA neurons survival. In fact, it was observed in the SNpc of PD patients, the death of DA neurons is anticipated by the loss of IR mRNA, and increased levels of IRS phosphorylation in serine residues promoting the inhibition of insulin signalling leading to an insulin resistance state [133]. Other studies showed that the levels of IRS-1 pSer616 in hippocampus and pSer312 in the putamen were increased in the brain of PD patients [134]. More recently, some studies performed in rodents have shown that the

consumption of high fat diets which leads to insulin resistance states produces several effects on the SNpc neurons as impaired iron homeostasis, leading to an increase in iron deposition, which promoted decreased potassium-evoked DA release, which correlated with the degree of insulin resistance. Thus, these alterations in DA neuronal function could precede DA depletion and neurodegeneration [135].

1.7.3. Glycation in Alzheimer and Parkinson' Diseases

Another common mechanism underlying T2D, AD and PD is protein glycation as an age-dependent posttranslational modification, with the ability to change the structure and function of several proteins. Glycation is a reaction that has been vastly studied in the context of DM, due to the deregulation of glucose metabolism. Glucose can react with phospholipids, nucleotides, and proteins in a nonenzymatic process, called Maillard reaction [136], [137]. During this reaction, stronger reducing sugars may be formed and can covalently react with proteins forming advanced-glycated end-products (AGEs), [125], [138]. In fact, glucose has a low capacity to induce glycation. In contrast, several carbonyls which are by-products of glucose metabolism, display an increased glycation reactivity, being methylglyoxal (MGO) one of the most reactive agents unavoidably formed in the cells [139].

MGO arises mainly due to the decomposition of the phosphate group of glyceraldehyde 3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP). It can be also formed by the oxidation of aminoacetone, from the oxidation of ketone bodies, and from the oxidation of acetone. MGO is mainly catabolized by the glyoxalase (GLO) system, comprising GLO-1 and GLO-2 enzymes, and by aldose reductase [125].

Interestingly, MGO levels are increased 2-to 5-fold in diabetic patients, resulting in increased glucose levels. Moreover, hyperglycaemia induces an increase in the formation of AGEs in the SNpc [140], [141],[125]. To investigate the hypothesis that glycation may contribute for the neurodegenerative process in AD and PD, some studies have been recently conducted.

The first studies report that AGEs were detected at the periphery of Lewy bodies [142]. Moreover, PD patients exhibit increased levels of AGEs in brain regions such as cerebral cortex, amygdala and SNpc. Interestingly, glycation was reported to accelerate aSyn oligomerization *in vitro*. MGO is also able to react with dopamine, generating 1-acetyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (ADTIQ), presenting PD patients increased levels of this metabolite in the brain including in the SNpc. Considering that ADTIQ is a neurotoxin, these finding suggests that MGO reacts with dopamine and may trigger dopaminergic neurons degeneration [125], [143].

Glycation was also suggested to be involved in AD pathology, since amyloid plaques from AD patient's brains exhibit more AGEs when compared to healthy age matched controls [125]. Interestingly, glycation promotes the formation of Aβ and tau aggregates, by upregulating the expression of APP and leading to the hyperphosphorylation of tau [125]. All these findings highlight the impact of glycation in neurodegenerative disorders and suggest that inhibition of protein glycation could be used as therapeutic strategy to AD and PD.

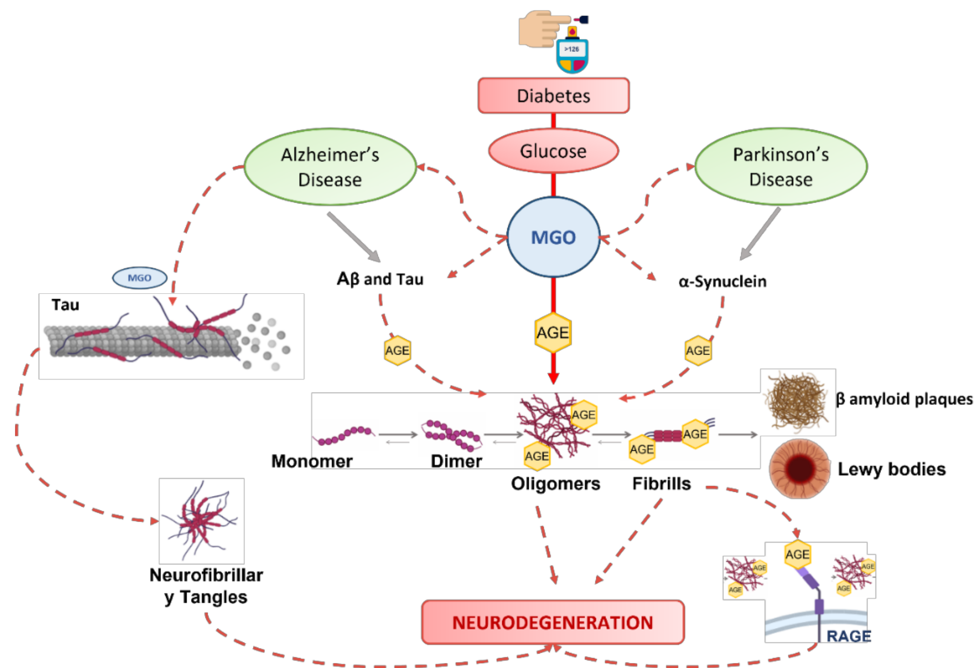


Figure 8. Glycation in metabolic and neurodegenerative disorders.
Diabetes associated with hyperglycaemic states is a known risk factor for the development of AD and PD. Increased sugar levels lead to increased levels of MGO, promoting the formation and aggregation of aSyn and increasing the phosphorylation of tau. Glycated species by the advanced glycation end products (AGEs) active the AGE receptors (RAGE) inducing and inflammatory response. Thus, this process leads to neurodegeneration in both AD and PD, being a missing link between metabolic and neurodegenerative disorders. Adapt [125].

1.8. Therapeutic strategies to neurodegenerative disorders

Neurodegenerative disorders affect millions of people worldwide, and the currently available therapeutics are limited. Moreover, the existing therapies are not disease-modifying. For the case of PD, they are only able to alleviate the symptoms. They are mostly DA-replacing therapies (levodopa-carbidopa, dopamine agonists), able to improve PD-motor features in the initial stages of the disease. Non-motor symptoms require non-dopaminergic approaches as for example, the selective serotonin reuptake inhibitors [121].

For the case of AD, current treatments also aim to attenuate symptoms (e.g. NMDA receptor antagonist) as cognitive impairment, aggression, and seizures, and do not target disease pathology [103].

In the last years, the utility of drugs used for T2D management have also been investigated in PD and AD patients, including Metformin, GLP-1 analogues, dipeptidyl peptidase 4 (DPP-4) inhibitors or gliptins, among others. For example, a study performed in a Taiwanese cohort evaluated the usage of metformin, known to reduce blood glucose levels and to decrease insulin resistance, in PD patients and reported that the combination of metformin with sulfonylurea therapy was able to reduce the risk for PD [125], [144]. Also, Metformin when administered to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) -induced Parkinson's disease mice showed to improve locomotor activity in these animals [145]. Metformin was also demonstrated to be able to cross the BBB and to act as a neuroprotective agent by reducing tau phosphorylation in primary cortical neurons of a transgenic mouse with the lack of microtubule-associated protein tau (MAPT), a known mechanism of AD development [146].

As previously mentioned, these therapies only can attenuate symptoms, and some may present important adverse effects. Therefore, it is important to develop novel disease modifying treatments to stop disease progression and to improve the quality of life of patients [121], [121].

1.9. The Carotid Bodies (CBs)

The carotid bodies (CBs) are small, paired organs located in the bifurcation of the common carotid artery in the neck [147], [148]. They were recently shown to be involved in the regulation of peripheral insulin action and glucose homeostasis. Their dysfunction is implicated in the genesis of metabolic diseases [149], [150], [151].

CBs were first described in the 18th century by von Haller [147]. However, it was with the seminal histological studies of De Castro, in the 1920s, that raised the hypothesis that the CBs were organs with the capacity of sensing and detecting chemicals in the arterial blood. Additionally, Corneille Heymans and De Castro demonstrated that CBs are important in the response to alterations in blood levels of oxygen (O_2), carbon dioxide (CO_2) and H^+ [152], [153] . The CBs are formed by clusters of cells, surrounded by a dense net of capillaries, and embedded by the sensory nerve ending of the carotid sinus nerve (CSN), a thin branch of the glossopharyngeal nerve that integrates the information coming from the CBs to the brain [148], [154], [155]. They are constituted by two different types of cells, in a ratio approximately of 4:1-

being the main component the type I cells, also known as chemoreceptor cells or glomus cells; they are also composed by the type II cells similar to glial cells of the nervous system [156], [157].

Type I cells communicate by both electrical and chemical synapses, being innervated by the afferent terminals of the CSN. Type 1 cells are responsible for the synthesis of neurotransmitters and neuromodulators - ATP, DA, acetylcholine, adenosine, substance P among others. These molecules act postsynaptically in the CSN to inhibit or activate it, or presynaptically in type 1 cells to modulate its function (for a review see [158]). This type of cells also expresses a range of metabotropic and ionotropic receptors, suggesting that there are autocrine and paracrine mechanisms involved in the afferent discharge during chemoexcitation [156]. The information from the CBs is integrated in the brainstem via the CSN to induce an hyperventilatory response to normalize blood gases and the activation of the sympathetic nervous system [148], [153]

In the last years it has been shown that the CBs apart from being a blood gases sensor also play a new important role in the control of energy homeostasis and metabolism [159], [158]. Also, it was shown that CBs play a major role in the development of metabolic diseases. Conde's group hypothesise that the overactivation of the CBs, produced by hypercaloric diets and/or chronic intermittent hypoxia leads to the deregulation of the sympathetic nervous system with consequent metabolic deregulation [160], [161]. In agreement, they found that the CBs from prediabetic and T2D animals [160], [162] and from prediabetic patients were overactivated [163]. Moreover, they showed that the increased CBs chemosensitity in patients evaluated by the Dejour test, where ventilation, -respiratory frequency (RR) and tidal volume (TV) - was measured, while subjects breathed in normoxic conditions (21% O_2- room air) followed by two breaths of hyperoxia (100% O_2) correlated with insulin resistance and increased fasting insulin [163]. In concordance with a role for the CBs in the development of metabolic diseases, the authors showed that the abolishment of CBs activity, via CSN resection or CSN neuromodulation was able to prevent [160] and restore [161], [164] T2D hallmarks, as insulin resistance and glucose intolerance, in rats. Confirming this pre-clinical data, the authors also showed that the functional block of CBs activity by the use of hyperbaric oxygen therapy, commonly used to treat diabetic foot wounds, was able to improve glucose homeostasis in T2D patients [165]

Altogether, it is clear that the CBs plays a major role in glucoregulation and that the modulation of its activity could be used as a therapeutic strategy to treat metabolic disorders in humans.

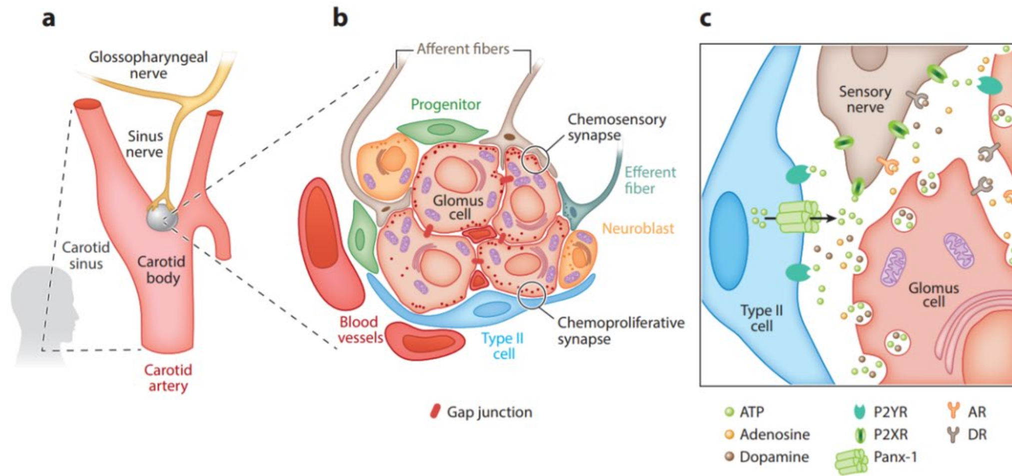


Figure 9. Carotid Bodies (CBs) anatomy.

Carotid artery bifurcation (A), and cellular cluster of the CBs, chemosensory and chemoproliferative synapses are indicated (B); Synaptic activity between CSN, type II cells and glomus cells. Abbreviations: AR, adenosine receptor; DR, dopamine receptor; Panx-1, pannexin-1 channel; P2XR, purinergic 2X ionotropic receptor; P2YR: purinergic 2Y metabotropic receptor [166].

Hypothesis and Objectives

II. Hypothesis and Objectives

It is well stablished that metabolic disorders, characterized by a state of peripheral insulin resistance, are a crucial risk factor for the development neurodegenerative disorders. Additionally, recent studies showed that patients that develop neurodegenerative diseases as dementia, Alzheimer's Disease and Parkinson's Disease have brain hyperinsulinemia, suggesting a state brain insulin resistance.

It is well established that in dysmetabolic states the CBs, peripheral chemoreceptors involved in the regulation of peripheral insulin action and glucose homeostasis, are overactivated. Additionally, it is known that the abolishment of their activity can prevent and reverse dysmetabolic features [161], [160]. Therefore, the main goal of this project was to investigate if the abolishment of CBs activity via bilateral resection of the CSN could prevent/ameliorate the neurodegenerative process, and cognitive impairment associated with brain insulin resistance.

The specific aims of the present work are:

1. To study the impact of hypercaloric diet consumption in brain insulin resistance and neurodegeneration;
2. To investigate the impact of abolishing CBs activity, via the resection of the CSN, on the prevention of neurodegeneration and cognitive impairment associated with brain dysmetabolism.

Material and Methods

III. Materials and Methods

3.1. Animals and experiments

Experiments were performed in male Wistar rats (200–320 g), aged 3 months, obtained from Charles River Laboratory (France) with 6 weeks of age and maintained at the animal house of the NOVA Medical School/Faculdade de Ciências Médicas. Animals were kept under temperature and humidity control (21 ± 1 °C; 55 ± 10% humidity) and a regular light (08.00–20.00 h) and dark (20.00–08.00 h) cycle, with food and water ad libitum. After randomization, animals were included in one of the two groups of animals used: the normal chow diet group (NC) and the high-fat high-sucrose (HFHSu) group, a model which combines IR and glucose intolerance [164], [167]. HFHSu diet was composed by a 60% lipid-rich diet (61.6% fat + 20.3% carbohydrate + 19.1% protein, Test Diets, Missouri, USA) and by the administration of 35% wt./vol. sucrose (Enzymatic, SA, Portugal) in drinking water [167]. The normal chow group fed a standard diet (7.4% fat+75% carbohydrate (4% sugar) +17% protein, SDS diets RM1, Probiológica, Portugal).

Caloric and liquid intake were monitored daily, before and after the surgical procedures in all groups of animals. Body weight and animal behavioural changes were assessed twice per week. Insulin sensitivity through an insulin tolerance test (ITT), glucose tolerance through an oral glucose tolerance test (OGTT) and behaviour tests - locomotor activity, olfactory acuity and spatial memory and cognition were evaluated by open field test (OF), block test and y-maze test, respectively – were performed before CSN denervation (at 14weeks of diet) and at 17, and 20 weeks of diet (2-, and 5-weeks post-surgery)

At 15 weeks of diet, after randomization, half of the HFHSu and the CTL animals were submitted to bilateral CSN resection under ketamine (30mg/kg)/ medetomidine (4mg/kg) anaesthesia and brupenorphine (10µg/kg) analgesia. The control groups (NC sham and HFHSu sham) were submitted to a sham procedure, leaving their CSN intact. After the surgical procedure the animals were kept under the respective diets for more 7 weeks.

At week 22 of diet/protocol, the animals were anaesthetised with pentobarbital (60mg/kg i.p.) and transferred to a heating pad to maintain body temperature at 37, 5 ± 0, 5ºC throughout the experiment. To record blood pressure, the femoral artery was catheterized, and blood pressure recorded using a catheter, and signals were fed to a computer for visualization and storage for later analysis with EMKA software (Emka Technologies, Paris, France) during 15 minutes.

Blood was collected and treated for quantification of plasma insulin. Afterwards, the animals were perfused with phosphate-buffered saline (PBS) to clean the tissues. Half of the animals were perfused with 4% of paraformaldehyde (PFA), for histological analysis. The tissues from the other half of animals were collected after PBS perfusion and the brains dissected and frozen at -80ºC, to protein analysis.

Principles of laboratory care were followed in accordance with the European Union Directive for Protection of Vertebrates Used for Experimental and Other Scientific Ends (2010/63/EU). Experimental protocols were approved by the Ethics Committee of the NMS- NOVA Medical School.

3.2. Metabolic profiling

3.2.1. Insulin tolerance test (ITT)

Insulin sensitivity was assessed using the ITT, that provides an estimate of overall insulin sensitivity, correlating with the hyperinsulinaemic–euglycaemic clamp [168]. For that the animals were fasted for approximately 15 hours with free access to water. An insulin solution of 0.1 U/kg body weight was prepared. Basal blood glucose was measured and then 100 µl of the insulin solution was administered into the tail vein. Glucose was measured from blood collected from the tip of the tail vein over 15 minutes at 1-minute intervals with a glucometer (Precision Xtra Meter, Abbott Diabetes Care, Portugal) and test strips (Abbott Diabetes Care, Portugal) [168].

The constant rate of glucose disappearance (K_{ITT}) was calculated using the formula $0.693/t^{1/2}$ and glucose half-time ($t^{1/2}$) was calculated from the slope of the least-square analysis of plasma glucose concentrations during the linear decay phase [160], [169].

3.2.2. Oral glucose tolerance test (OGTT)

Glucose tolerance was accessed by the oral glucose tolerance test. Overnight fasted animals for approximately 15 hours were administrated with a glucose solution (2g/kg in a 10 ul/g body weight volume) by gavage after the measurement of basal glycemia. Glucose levels were measured at 15, 30, 60 and 120 minutes after the oral gavage by tail tipping using a glucometer (Precision Xtra Meter, Abbott Diabetes Care, Portugal) and test strips (Abbott Diabetes Care, Portugal).

The evaluation of the glycaemic response was performed by calculating the total area under the serum glucose curve using the minimum squares method or the trapezoidal method [170], [171], [172].

3.3. Behavioural Studies

3.3.1. Open field test (OF)

The OF test is used to assess gross motor activity, anxiety, and willingness to explore [173]. In this test, the rat is placed for 5 minutes in a square arena (70 cm x 70 cm x 75 cm). The behaviour of the animals was recorded with a camera placed on the ceiling above the equipment, and the videos were analysed using Bonsai software (version 7.0). For the analysis, an inner and central zone in the maze was defined (40 cm x 40 cm) and it was measured the: total distance covered, distance covered in the inner zone, total immobility time, immobility time in the inner zone, average velocity, and average velocity in the inner zone. It was considered that the animal was in the inner zone when more than half of its body was in the 40 cm area. Taking into account the size of the animal and the number of pixels it represents in the software, a minimum number of pixels is defined so that more than half of the animal are detected in the inner zone. Immobility was considered when the animal was stationary (the animal was not walking), meaning that its coordinates X and Y was the same. The maze was cleaned with diluted 10% ethanol between tests to eliminate odours and residues. Data presented result from a single trial for each rat.

3.3.2. Y-maze test

The Y-maze is used to assess spatial learning and memory capacity, core features affected in some neurodegenerative diseases [174]. A Y-shaped maze with 120° between each arm with 10 cm wide and 30 cm high was used. The A arm or starting arm is 50 cm long, and both arms B and C are 46 cm long. During the training, the rat was placed in the start point (A arm) of the Y-maze with a closed arm and allowed to freely explore it for 5 minutes. After one hour, the "novel" arm was open, and the animal allowed to freely explore the maze for 5 minutes. The behaviour of the animals was recorded with a camera placed on the ceiling above the equipment, and the videos were analysed using Bonsai software (version 7.0). The exploratory capacity of the animal was evaluated as well as the time spent both in the unlocked arm and in the arm that is always open. The first arm choice when both arms are open was also evaluated, as well as the number of triads (i.e., ABC, CAB, or BCA) and entries. The alternative behaviour score (%) for each mouse

was calculated as the ratio of the number of alternations to the possible number (total number of arm entries minus two) multiplied by 100. It was defined a threshold, that is, the minimum number of pixels necessary by the software to detect when more than half of the rat was in the arm. The animal was inside the arm when more than half of its body is detected by the software. The maze was cleaned with diluted 10% ethanol between tests to eliminate odours and residues. Data presented result from a single trial for each rat.

3.3.3. Block test

The block test is used to assess olfactory function. This test evaluates sensitivity to social smells, an ethologically essential ability in rats, thus measuring the olfactory acuity and discrimination [175]. Housed animals were exposed to five wood blocks (10cm x1.5cm x1.5cm) (Ultragene, Portugal) placed inside each cage for 1 week. During this period, the cage bedding was not replaced. The rat was placed on the novel cage and videotaped for 30 seconds. Each rat underwent two training sessions. On the trial test, one block was replaced by a block that was originally in a cage with a different set of animals. The mouse was videotaped for 1 minute. Parameters as the time that animals take to recognize the novel block and the time spent to smell the novel block were evaluated. The animal was only considered to be sniffing the block when its nose was touching/very closely to the block. The cages were cleaned with diluted 10% ethanol between tests to eliminate odours and residues. Data presented result from a single trial for each rat.

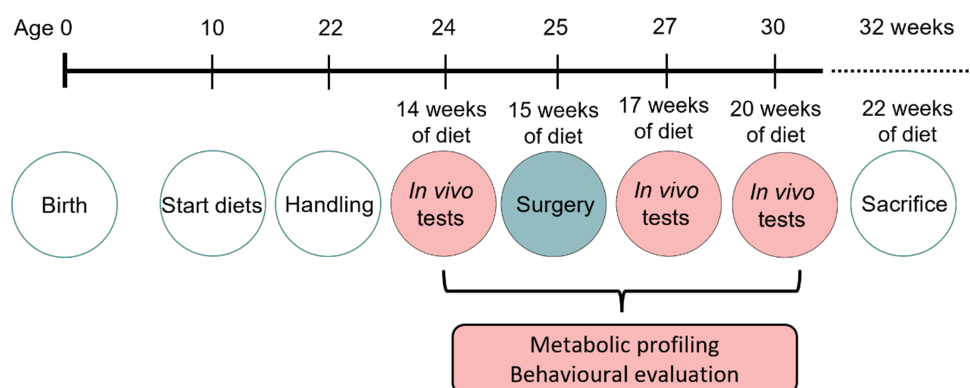


Figure 10. Schematic representation of the in vivo studies performed in Wistar rats submitted either to a NC diet and or to a HFHSu diet.

3.4. *Ex vivo* analysis

After 22 weeks of diet (both NC and HFHSu diets), animals were sacrificed, and brains dissected and harvested for protein analysis. The brain areas dissected were: 1) olfactory bulb, 2) frontal cortex, 3) hypothalamus, 4) hippocampus, 5) midbrain, 6) striatum 7) brainstem 8) cerebellum. Protein analysis was only performed in extracts of the frontal cortex and the hippocampus.

3.4.1. Tissue lysate preparation

For tissue lysate preparation, 200 µl of RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.1 % SDS, 0.25 % sodium deoxycholate) were added per 0,02 g of cortex or hippocampus tissues. Samples were macerated with an automatic pestle. Using a sonicator, samples underwent three cycles of sonication in pulses (1 second on, 45 milliseconds off, for 30 seconds, with 15% of intensity), with 1 minute incubation on ice between them. Then, protein extracts were centrifuged for 10 minutes at 13.000 rpm at 4º C, and supernatant was collected, and protein was quantified using Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific; Waltham, MA, USA).

3.4.2. Immunoblot analysis

Western Blot (WB) technique is used to study the levels of specific proteins extracted from cells or tissues. The separation of proteins is based on their molecular weight, which occurs through polyacrylamide gel electrophoresis. Subsequently, the gel is transferred to a membrane, followed by its incubation with primary and secondary antibodies, specific for the protein of interest. Finally, the results are visualized using a chemiluminescence method.

3.4.2.1. Electrophoresis and Dot-blot system

For the Western blot analysis, 15 mg of total protein from frontal cortex or hippocampus lysates were separated by SDS-PAGE electrophoresis using a Tetra cell (Bio-Rad; Hercules, CA, USA), in 12% polyacrylamide separation gel and a 4% polyacrylamide stacking gel, applying a constant voltage of 120 V.

For the dot-blot analysis, 15 mg of total protein from tissue lysates were also loaded onto nitrocellulose membranes, using a dot- blot vacuum system. Wells were washed twice with PBS before removing the membrane from the apparatus.

3.4.2.2. Western- blotting procedures

Gel separated proteins were transferred to nitrocellulose membranes, using standard procedures with a Mini Trans-Blot system (Bio-Rad; Hercules, CA, USA). To avoid nonspecific

bonds, membranes were incubated with blocking solution (5% bovine serum albumin) in 1x TBS-T (20 mM Tris, 136 mM NaCl, 10% Tween 20, pH 7.6) at room temperature for 1hour. Primary antibody incubations were carried out overnight at 4ºC, using concentrations described in table 2.

Then, membranes were washed and incubated with secondary antibody (see concentrations in table 2) for 1.5 hours. Detection procedures were carried on according to ECL system (GE Healthcare, Life Sciences; Little Chalfont, UK), and the signal detected using a ChemiDoc™ Imaging Systems (Bio-Rad, Hercules, CA, USA). Membranes were reprobed for the β-actin.

Table 2. Primary and secondary antibodies used for the proteins of interest in the present study, and its respective brand, specie, and concentration used.

Proteins	Primary Antibody	Secondary Antibody
aSyn (14 kDa)	Anti- aSyn (1:1000), mouse (BD Transduction Laboratories, code: 610787)	ECL Anti-mouse IgG Horseradish Peroxidase Linked Species (GE Healthcare,code: NA931) (1:5000)
APP (100-140 kDa)	Anti-APP (1:1000), rabbit (Abcam, code: ab32136)	ECL Anti-rabbit IgG Horseradish Peroxidase Linked Species (GE Healthcare,code: NA934) (1:5000)
AGEs (CEL- Anti N (carboxyethyl) lysine)	Anti- CEL (1:1000), mouse (Cosmo Bio Co., LTD, code: AGE-M02)	ECL Anti-mouse IgG Horseradish Peroxidase Linked Species (GE Healthcare,code: NA931) (1:5000)
AKT (60 kDa)	Anti-AKT (1:1000), rabbit (Cell Signalling, code: 9272)	ECL Anti-rabbit IgG Horseradish Peroxidase Linked Species (GE Healthcare,code: NA934) (1:5000)
AMPK (60 kDa)	Anti-AMPK (1:1000), rabbit (Cell Signalling, code: 5831)	ECL Anti-rabbit IgG Horseradish Peroxidase Linked Species (GE Healthcare,code: NA934) (1:5000)
IR (95-100 kDa)	Anti- IR (1:1000), mouse (Santa Cruz, code: sc-57342)	ECL Anti- mouse IgG Horseradish Peroxidase Linked Species (GE Healthcare,code: NA931) (1:5000)

AMPK-p (60 kDa)	Anti-AMPK-p (1:500), rabbit (Santa Cruz, code: sc-101630)	ECL Anti-rabbit IgG Horseradish Peroxidase Linked Species (GE Healthcare,code: NA934) (1:2000)
Synapsin-I (80-90 kDa)	Anti-Synapsin (1:1000), rabbit (Abcam, code: ab64581)	ECL Anti-rabbit IgG Horseradish Peroxidase Linked Species (GE Healthcare,code: NA934) (1:5000)
PSD-95 (95 kDa)	Anti-PSD-95 (1:1000), mouse (Invitrogen, code: 7E3-1B8)	ECL Anti- mouse IgG Horseradish Peroxidase Linked Species (GE Healthcare,code: NA931) (1:5000)
SNAP-25 (25 kDa)	Anti-SNAP-25 (1:1000), mouse (Santa Cruz, code: sc-20038)	ECL Anti- mouse IgG Horseradish Peroxidase Linked Species (GE Healthcare,code: NA931) (1:5000)
Glo-1 (20 kDa)	Anti-Glo-1 (1:1000), mouse (Santa Cruz, code: sc-133144)	ECL Anti-rabbit IgG Horseradish Peroxidase Linked Species (GE Healthcare,code: NA934) (1:5000)
B-actin (42 kDa)	Anti- B-actin (1:5000), mouse (Life technologies, code: AM4302)	ECL Anti- mouse IgG Horseradish Peroxidase Linked Species (GE Healthcare,code: NA931) (1:10000)

Protein densitometry was performed using ImageLab System, BioRad (version 6.0.1). When necessary, membranes were incubated with stripping solution (250 mM Glycine, 0.1 % of 10 % SDS, pH 2.0) for 20 minutes at room temperature with agitation. Then, they were 4 times washed, twice with 1x TBS and twice with 1x TBS-T solutions. Membranes were then incubated in blocking solution for 30 minutes before a re-incubation with the required antibodies. The results were visualized using a chemiluminescence method.

3.5. Data analysis

Data were analysed using GraphPad Prism Software, version 8.0.2 (GraphPad Software Inc., San Diego, CA, USA) and presented as mean values with the standard error of the mean (SEM). The significance of the differences between the groups was calculated by one- and two-way ANOVA with Dunnett’s and Bonferroni multiple comparison tests. Differences were considered

significant as $p \leq 0.05$. Experimental groups are constituted by 6-7 animals in *In vivo* experiments, and by 3-4 animals in *Ex vivo* experiments

Experimental Results

IV. Experimental results

4.1. *In vivo* results

4.1.1. Metabolic profiling: Effect of HFHSu diet and CSN resection on weight gain and caloric intake

Intake of HFHSu diet promotes changes in caloric intake and body weight of the Wistar rats. In table 3, the values of weight gain and caloric intake of NC and HFHSu animals before and after surgery are depicted. HFHSu animals increase their weight by 40.77 % when compared to the NC animals before surgery. This effect was expectable since the HFHSu animals consumed 86.74% more calories than the NC animals. After CSN resection weight gain was attenuated by 23.27% in HFHSu denervated animals as compared with HFHSu sham animals. In agreement, HFHSu denervated animals decreased their caloric intake by 14.36%, when compared to the HFHSu sham animals.

We also observe a non-significant tendency of caloric intake reduction in NC denervated animals (table 3).

Table 3. Effect of HFHSu diet and CSN resection on weight gain and caloric intake.
Values refer to the differences in parameters before CSN resection – intake of diet during 14 weeks in animals with 24 weeks age, and after CSN resection – intake of diet during 5 weeks of diet from animals aged 30 weeks. Data are means ± SEM of 6–7 animals. One-way ANOVA with Bonferroni multicomparison tests: *p < 0.05, **p < 0.01, ***p < 0.001 vs NC group; # p <0,05, ## p <0,01, ### p < 0,001 before vs after surgery; $ p <0,05, $$ p <0,01, $$$ p < 0,001 HFHSu before vs HFHSu after surgery.

Weight Gain (g/day)		Before surgery (24 weeks old)	After surgery (30 weeks old)
NC	Sham	0.6536 ± 0.05209	0.793 ± 0.1399
	Den	0.7638 ± 0.1064	0.6181 ± 0.05663
HFHSu	Sham	0.9201 ± 0.09196 *	1.534 ± 0.2516 **, $
	Den	1.033 ± 0.1147	1.177 ± 0.1872 #
Caloric Intake (Kcal/day/Kg)		**Before surgery**	**After surgery**
NC	Sham	102.8 ± 2.525	103.2 ± 4.451
	Den	112.1 ± 2.406	90.22 ± 7.545
HFHSu	Sham	191.9 ± 7.785 ****	199.1 ± 6.659 ****
	Den	195.3 ± 8.330	170.5 ± 5.43 ##

4.1.2. Metabolic profiling: Impact of HFHSu diet and CSN resection on glycaemia, insulin sensitivity and glucose tolerance

HFHSu diet impacted on basal glycaemia, insulin sensitivity and glucose tolerance, effects altered with the CSN resection. Figure 11A represents the effect of chronic CSN denervation on fasting glycaemia in NC and in HFHSu animals. Before CSN denervation basal glycaemia of HFHSu animals was 20.86% higher than in the NC animals (NC= 69.86 ± 3.84 mg/dL; HFHSu= 84.4286 ± 1.771 mg/dL), effect that was previously observed in other animal models of dysmetabolism [160], [169]. Additionally, and as expected 20 weeks of HFHSu diet led to an aggravation of basal glycaemia by 39.42% in comparison to 14 weeks HFHSu fed animals (HFHSu before surgery= 84.4286 ± 1.771 mg/dL; HFHSu 5 weeks after surgery= 117.71 ± 3.80 mg/dL). This effect was attenuated by 22.55% 5 weeks after CSN denervation (HFHSu= 117.71± 3.80 mg/dL; HFHSu den= 91.16 ± 1.515 mg/dL) (fig. 11A).

Insulin sensitivity was evaluated using the ITT before and 5 weeks after CSN resection. As expected, animals treated for 14 weeks with HFHSu diet exhibit a 54.27% decrease in insulin sensitivity (NC= 4.38 ± 0.2165 glucose/min; HFHSu= 2.00 ± 0.3822 glucose/min), an effect totally reversed 5 weeks post CSN resection, increasing their insulin sensitivity by 220,98% when compared to the HFHSu animals (HFHSu= 1.89 ± 0.2816 glucose/min; HFHSu den= 6.06 ± 0.4174 glucose/min (fig. 11B).

Glucose tolerance was evaluated using the OGTT before and 5 weeks post-surgery. In accordance with a dysmetabolic state, 14 weeks HFHSu treated rats develop glucose intolerance [164], exhibiting an increase of 13.78% in the area under the curve of the glucose excursion curves when compared to the NC animals before surgery (NC= 15212.4 ± 1060 mg/dL/min; HFHSu= 17309.3 ± 1291 mg/dL/min) (fig. 11C). Glucose intolerance aggravates with the increase in the number of weeks under hypercaloric diet with the AUC increasing by 27.27%, upon 20 weeks of diet (HFHSu before surgery= 17309.3 ± 1291 mg/dL/min; HFHSu 5 weeks after surgery= 22028.9 ± 333.2 mg/dL/min). Five weeks post CSN denervation, HFHSu animals show a decrease in the AUC of the glucose excursion curves when compared to the HFHSu sham animals. The CSN resection attenuates the glucose intolerance of these animals by 18.95% (HFHSu= 22028.9 ± 333.2 mg/dL/min; HFHSu den= 17854.2 ± 797.1 mg/dL/min) (fig. 11C).

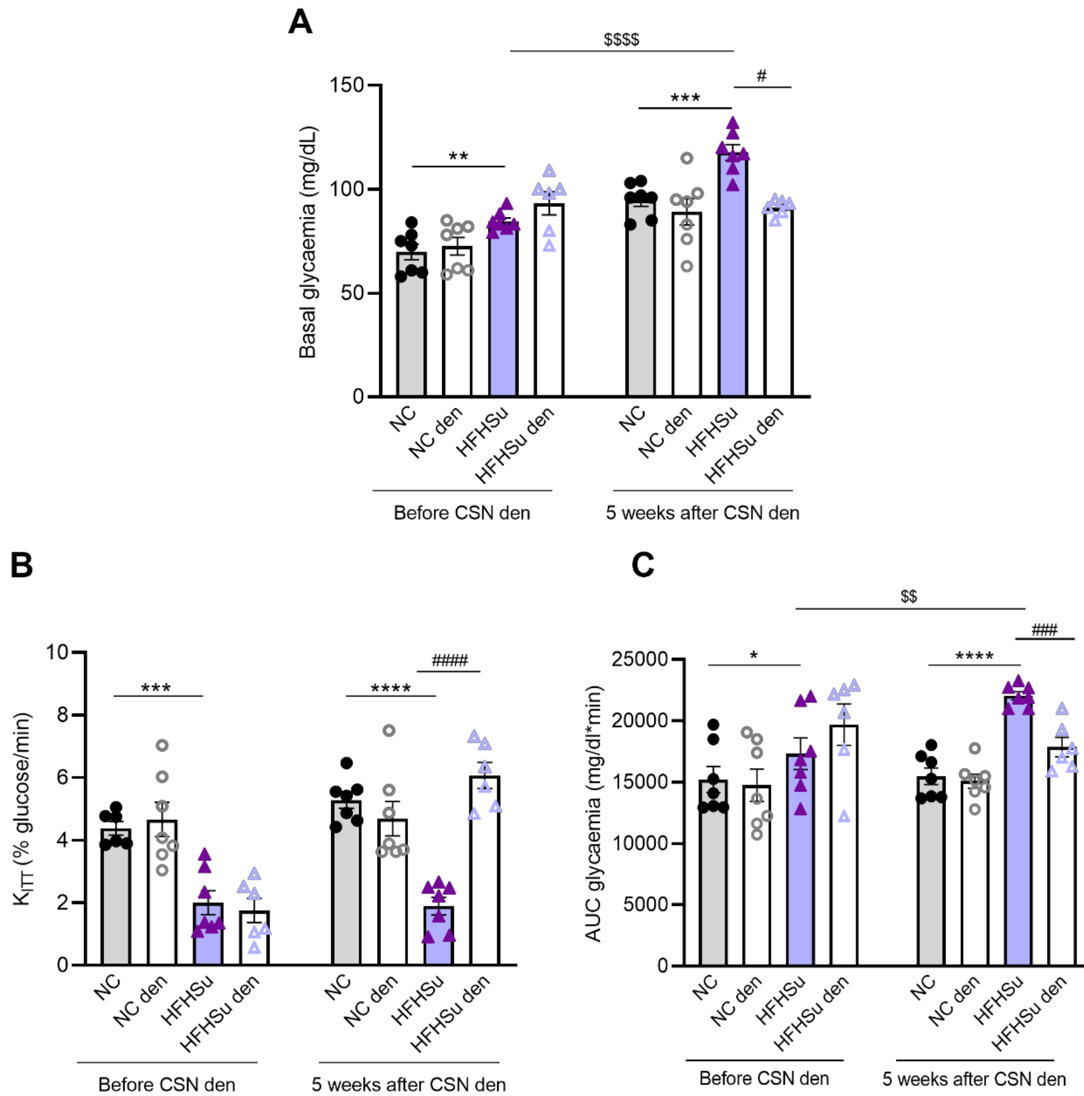


Figure 11. Metabolic profiling of NC and HFHSu animals before and 5 weeks after CSN resection.
Effect of HFHSu diet and CSN resection on fasting plasma glucose **(A)**; insulin sensitivity **(B)**, determined by the insulin tolerance test and expressed as the constant rate of glucose disappearance (KITT); glucose tolerance **(C)**, evaluated by the oral glucose tolerant test (OGTT). It is represented the area under the curve (AUC) of the glucose excursion curves of the OGTTs of the NC and HFHSu animals before CSN resection, and 5 weeks after surgery, respectively. Black circles represent NC animals; open grey circles represent N Data are means ± SEM of 6–7 animals. One-way ANOVA with Bonferroni multicomparison tests: *p < 0.05, **p < 0.01, ***p < 0.001 vs NC group; # p <0,05, ## p <0,01, ### p < 0,001 before vs after surgery; $ p <0,05, $$ p <0,01, $$$ p < 0,001 HFHSu before vs HFHSu after surgery. Black circles represent NC animals; open grey circles represent NC denervated animals; purple triangles represent HFHSu animals; open lilac triangles represent HFHSu denervated animals. Data are means ± SEM of 6–7 animals.

4.1.3. Behaviour phenotyping: Impact of HFHSu diet and CSN resection on locomotor activity

Locomotor activity was evaluated by the open-field test. No significant differences between the groups and the different times of diet in all 6 parameters evaluated were observed - total distance covered (A); distance covered in the inner zone (B); total immobility time (C); immobility time in the inner zone (D); total average speed (E); average speed in the inner zone (F)) (fig.12). Prior to surgery, HFHSu treated animals do not display altered locomotor activity (fig 12A). The same phenotype is observed at 2-, and 5- weeks post-surgery. Also, before surgery, no differences were observed in the distance travelled in the inner zone between animals submitted to the HFHSu diet and the NC group (fig. 12B). In contrast, 2 weeks post-surgery it seems that the sham HFHSu animals walk higher distances in the inner zone than the NC and the HFHSu denervated animals. This effect seems to be maintained until 5 weeks post-surgery.

Two weeks post-surgery, HFHSu sham animals seem to be immobile for less time when compared to HFHSu denervated animals. However, this is not observed until 5 weeks after denervation, since both HFHSu and HFHSu denervated animals are the same time immobile (fig. 12C). Also, before CSN resection it is observed a tendency in HFHSu animals to stay more time immobile in the inner zone when compared to the other groups. Two weeks after CSN denervation it is observable that the animals did not stop in the inner zone. However, this tendency seems to be different 5 weeks post-surgery, since the HFHSu animals seem to stop more time in the inner zone when compared to the other groups (fig. 12D).

The total average speed and the average speed in the inner zone (cm/s) does not change between the experimental groups (NC vs HFHSu) for any of the feeding durations (fig. 12E and F). The average speed in the inner zone is also not altered upon CSN denervation (fig. 12F).

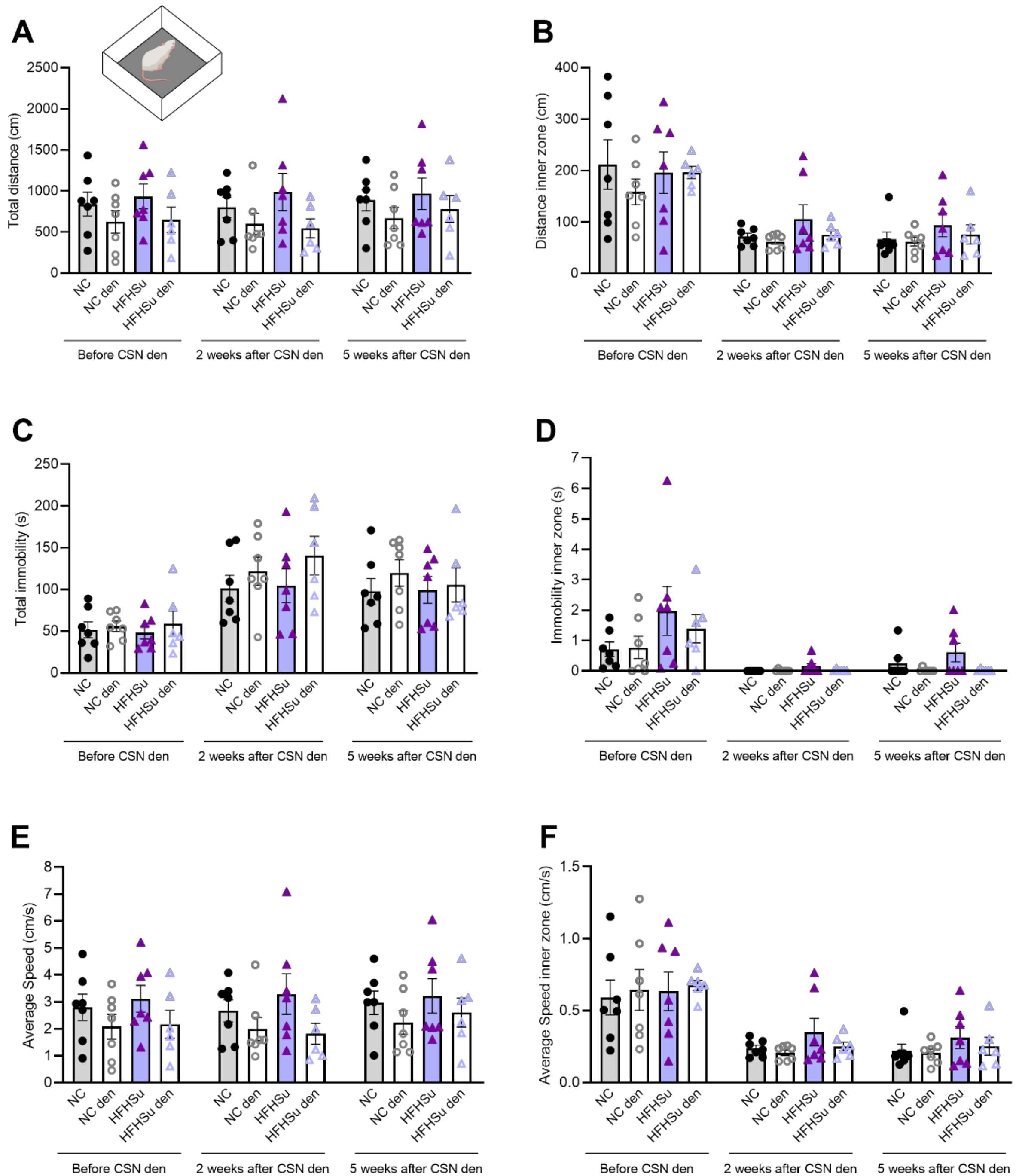


Figure 12. Effect of HFHSu diet and CSN resection on locomotor activity evaluated by the OF test, of NC and HFHSu animals before, 2- and 5-weeks post-surgery.

(A) and **(B)** represent the distances travelled (cm) by both groups of animals in the whole arena (70x 70 cm) and in the inner zone (40x 40 cm), respectively; **(C)** and **(D)** the total immobility (s) and the immobility in the inner zone of both groups of animals are respectively represented; **(E)** and **(F)** represent the average speed (cm/s), and the average speed in the inner zone, respectively. Black circles represent NC animals; open grey circles represent NC denervated animals; purple triangles represent HFHSu animals; open lilac triangles represent HFHSu denervated animals. Data are means ± SEM of 6–7 animals.

4.1.4. Behaviour phenotyping: Effect of HFHSu diet and CSN resection on spatial learning and memory capacity

Spatial learning and memory capacity of NC and HFHSu animals were evaluated by the Y-maze test. No significant differences were observed in the time that the animals spend in the novel arm before CSN resection and 2 weeks after CSN resection (fig.13A). However, HFHSu sham animals at 5 weeks post-surgery spend 62.1% less time in the novel arm in comparison to the NC animals (NC = 109.24 ± 5.62 s; HFHSu = 41.41 ± 10.30 s). The effect of HFHSu diet on the time spent in the novel arm was prevented by CSN denervation, that increases this parameter by 128.9% (HFHSU= 41.4± 10.30%; HFHSu den= 94.78 ± 19.97%) (fig.13A).

Prior to denervation, 65% of the NC animals (mean between the 2 groups of NC animals before surgery) and 62% of HFHSu animals (mean between the 2 groups of HFHSu animals before surgery) choose the novel arm (NC= 71.4%; NC den= 57.1%), (HFHSu= 57.1%; HFHSu den=66.6%) (fig 13B). At 2 weeks post- surgery, all the NC sham animals choose the novel arm (NC= 100 %), in contrast with the 57.1% from the NC denervated animals. Interestingly, 85.7% of HFHSu sham animals and 83.3% of HFHSu denervated animals choose the novel arm. At 5 weeks post CSN denervation the NC sham, NC denervated, and HFHSu animals choose approximately 86% the novel arm. Surprisingly, all HFHSu denervated animals choose the novel arm (100%), improving their behaviour comparing to 2 weeks post-surgery animals by 20% (HFHSu den 2 weeks post-surgery= 83.3%; HFHSu den 5 weeks post-surgery= 100%) (fig.13B).

The percentage of novel arm entries in the Y-maze was also evaluated, and the results are presented in figure 13C. No differences were observed before surgery and 2 weeks post-surgery. However, HFHSu sham animals at 5 weeks post-surgery exhibit a decrease of 22.1% in novel arm entries in comparison with the NC group (NC= 40.64 ± 2.12%; HFHSu= 31.65 ± 1.69%). CSN resection in HFHSu animals did not modify this phenotype (HFHSu den= 35.08 ± 1.69%).

The spontaneous alternative behaviour, assessed as the ratio of the number of alternations to the possible number (total number of arm entries minus two) multiplied by 100, is presented in figure 13D. Both, HFHSu diet and CSN resection, do not modify the spontaneous alternative behaviour.

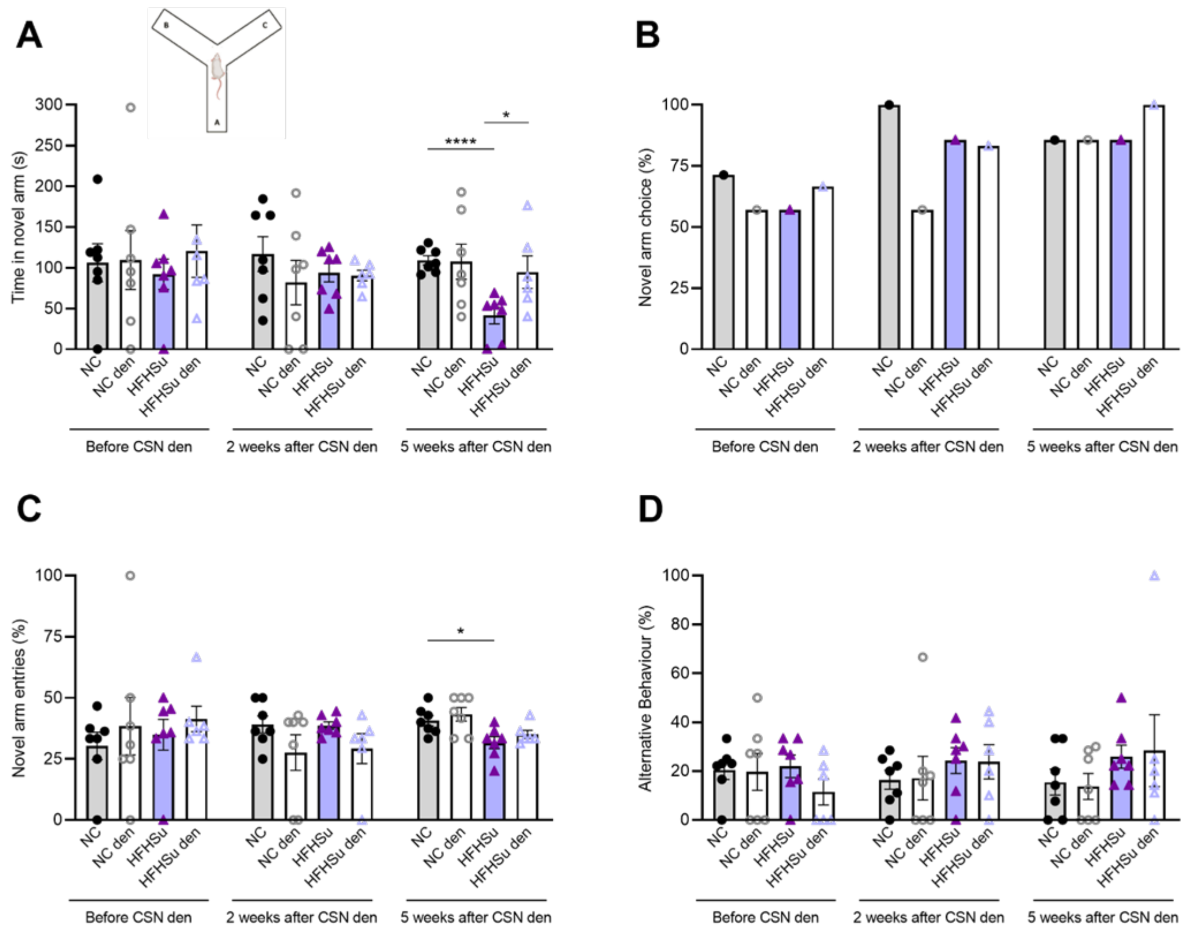


Figure 13. Effect of HFHSu diet and CSN resection on special memory and learning capacity evaluated by the y-maze test of both NC and HFHSu animals before, 2-, and 5- weeks post-surgery.

(A) shows the time that animals spend in the novel arm (s); **(B)** percentage of animals in each group that chose the novel arm; **(C)** percentage of novel arm entries; **(D)** shows the alternative behaviour score calculated by the ratio of the number of alternations to the possible total number (total number of arm entries minus two). Black circles represent NC animals; open grey circles represent NC denervated animals; purple triangles represent HFHSu animals; open lilac triangles represent HFHSu denervated animals. Data are means ± SEM of 6–7 animals. One-way ANOVA with Bonferroni multicomparison tests: *p < 0.05, **p < 0.01, ***p < 0.001.

4.1.5. Behaviour phenotyping: Effect of HFHSu diet and CSN resection on olfactory acuity

The olfactory acuity of the animals was evaluated using the Block test, and the results are depicted in figure 14. No significant differences between experimental groups were observed for the time that the animals need to identify the novel block (fig. 14A). However, a trend is observed in HFHSu animals at 5 weeks post-surgery that require more time to identify the novel block than NC, or HFHSu denervated animals (fig. 14A).

The time sniffing the novel block (s) is presented in the graph B of figure 14. After fourteen weeks of HFHSu diet, these animals spent a similar amount of time sniffing the novel block as

NC-fed animals (NC before surgery = 3.92 ± 0.48 s; HFHSu before surgery = 4.64 ± 1.16 s). However, upon longer exposures, HFHSu animals 2 weeks post-surgery seem to spend less time sniffing the object, comparing to sham group (NC 2 weeks post-surgery = 12.09 ± 2.21 s; HFHSu 2 weeks post-surgery = 7.11± 1.96 s). This phenotype was significant at 5 weeks post-surgery (NC 5 weeks post-surgery = 10.95 ± 2.03 s; HFHSu 5 weeks post-surgery = 5.22 ± 1.16 s). CSN resection seems to prevent the decrease in olfactory acuity in the HFHSu animals, since HFHSu denervated animals and NC animals spent similar time sniffing the novel block (NC 5 weeks post-surgery =10.95 ± 2.03 s HFHSu den= 7.14 ± 0.47 s) (fig. 14B). Additionally, it was performed the ratio between the time that the animals spent sniffing the novel block and the time that they spent sniffing the objects with their own scent, and the results are presented in the graph C of figure 14. While before surgery, no differences are observed, both at 2- and 5-weeks post-surgery, the HFHSu animals spent less time sniffing the novel block comparing to NC animals (2 weeks: NC= 0.36 ± 0.08 s; HFHSu= 0.18 ± 0.03 s; 5 weeks: NC= 0.34 ± 0.08 s; HFHSu= 0.13 ± 0.02 s). CSN resection seems to improve the olfactory acuity of HFHSu denervated animals by 33.33% when compared to the HFHSu animals (HFHSu= 0.13 ± 0.02 s; HFHSu den= 0.17 ± 0.01 s).

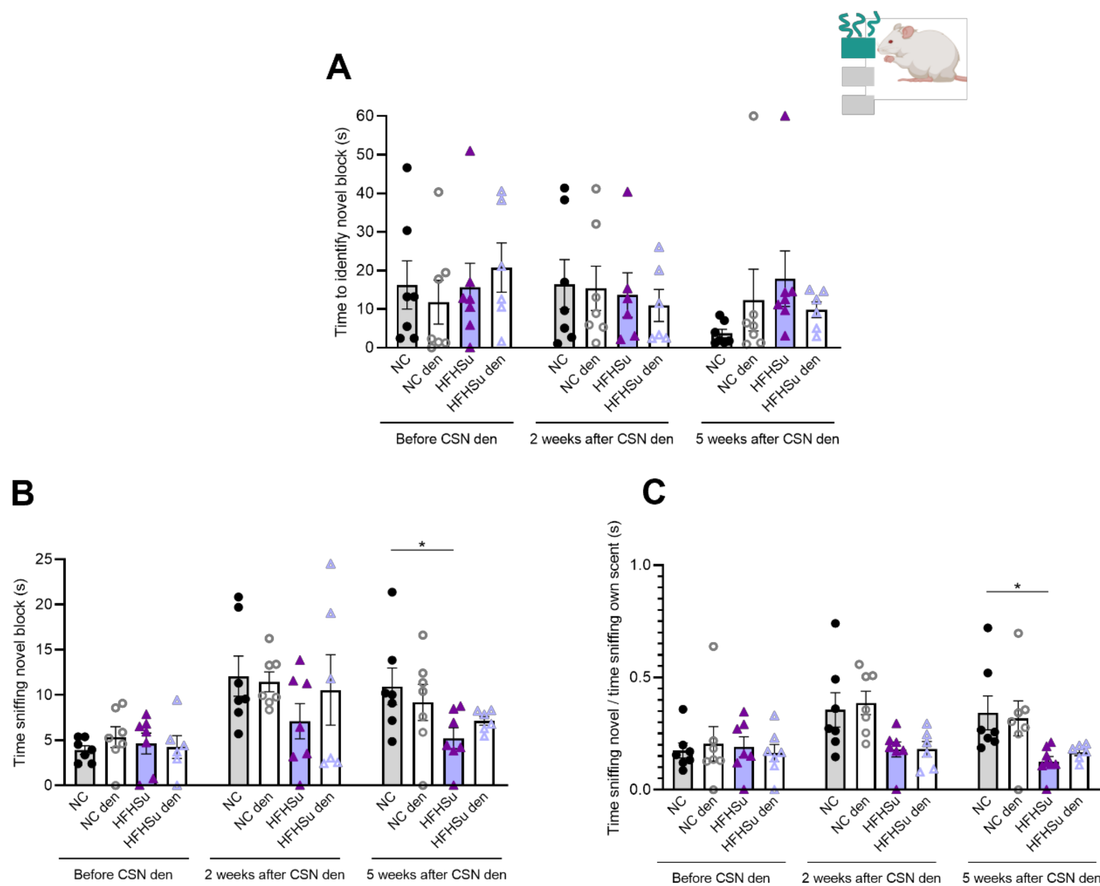


***Figure 14. Effect of HFHSu diet and CSN resection on olfactory acuity evaluated by the bock test before surgery and, 2- and 5- weeks post-surgery*.**

(A) represents the time spent to identify the novel block (s) (importantly, the values which correspond to the 60 seconds, meaning that the animals did not find the novel block); **(B)** shows the time spent sniffing the novel block (s);

(C) represents the ratio between the time that animals spent sniffing the novel block divided by the time that they spent sniffing the own scent (s). Black circles represent NC animals; open grey circles represent NC denervated animals; purple triangles represent HFHSu animals; open lilac triangles represent HFHSu denervated animals. Data are means ± SEM of 6–7 animals. One-way ANOVA with Bonferroni multicomparison tests: *$p < 0.05$.

4.2. *Ex vivo* results: Biochemical Analysis

The *in vivo* results showed that the most pronounced alterations, caused by HFHSu diet and by CSN resection, were at the level of their cognitive function and spatial memory (results from Y-maze test see figure 13). Therefore, we proceed to the analysis of brain areas mostly related to these functions - the prefrontal cortex and the hippocampus [71], [89].

4.2.1. Impact of HFHSu diet and CSN resection on brain insulin signalling

Insulin signalling in the brain was evaluated by the levels of IR, AKT, and phosphorylated AMPK/total AMPK (p-AMPK/t-AMPK) ratio in the prefrontal cortex and in the hippocampus, respectively. No significant differences were observed in the levels of both IR, AKT and p-AMPK/t-AMPK in prefrontal cortex, as reflected in figure 15. However, HFHSu diet seems to promote a non-significant slight decrease of 18.9% and 18.82%, respectively in the levels of IR ($p = 0.0824$) and p-AMPK/total AMPK ($p = 0.3652$) (fig. 15 A1 and A3 respectively). CSN resection in HFHsu animals did not alter this result.

As in the prefrontal cortex, HFHSu diet and CSN resection did not impact the levels of IR or of AKT (fig. 15 B1 and B2, respectively). Nevertheless, IR levels seems to decrease non-significantly by 22.13% in HFHSu animals in comparison to the NC animals. CSN resection did not change this result. Additionally, the HFHSu diet promoted a non-significant decrease of 46.65% in the ratio p-AMPK/t-AMPK ($p = 0.0503$) (fig. 15 B3), an effect attenuated by 40.90% in HFHSu CSN resection (HFHSu= 53.35 ± 5.71%; HFHSu den= 75.16 ± 12.37%)

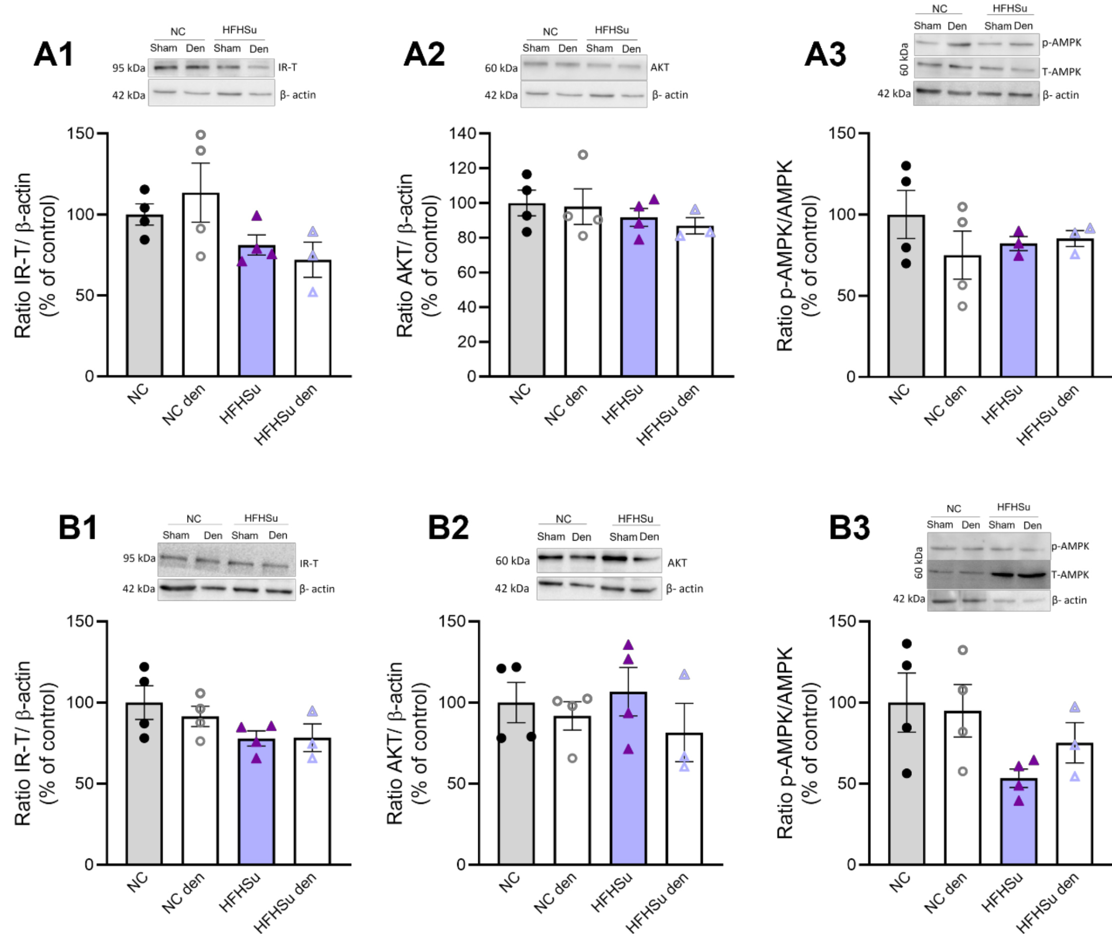


Figure 15. Effect of HFHSu diet and CSN resection on the levels of proteins involved in insulin signalling in prefrontal cortex (A), and in the hippocampus (B), evaluated by Western Blot.

(A1) and **(B1)** represents the levels of Insulin Receptor (IR- 95 kDa), in prefrontal cortex and in hippocampus, respectively. Panels **(A2)** and **(B2)** depict the levels of Protein Kinase B (AKT-60 kDa) in prefrontal cortex and hippocampus; and panels **(A3)** and **(B3)** depict the ratio between phosphorylated AMPK/total AMPK (AMPK- 60 kDa) in prefrontal cortex and hippocampus, respectively. Proteins were normalized to the loading control β-actin (42 kDa). Top of the graphs show representative Western Blots for each protein studied. Black circles represent NC animals; open grey circles represent NC denervated animals; purple triangles represent HFHSu animals; open lilac triangles represent HFHSu denervated animals. Data are means ± SEM of 3-4 animals.

4.2.2. Impact of HFHSu diet and CSN resection on brain glycation

Glycation is a reaction deeply associated with alterations in proteins structure and function. The effect of HFHSu diet and of CSN resection on the levels of 2 different glycation markers: the enzyme GLO-1 and Advanced Glycation End Products (AGEs) in the prefrontal cortex and in the hippocampus is depicted in figure 16.

Both the diet and CSN resection did not altered the levels of GLO-1 (fig. 16 A1) or AGEs (fig. 16A2) in the prefrontal cortex.

At the hippocampus, HFHSu diet or CSN resection did not modify the levels of GLO-1 (fig. 16 B1). In contrast, HFHSu diet intake increased the levels of AGEs (fig. 16 B2) by 32.70%. CSN resection in HFHSu animals seems to promote a slightly non-significant attenuation of these effects (HFHSu= 132.70 ± 12.22 %; HFHSu den= 120.53 ± 7.98 %).

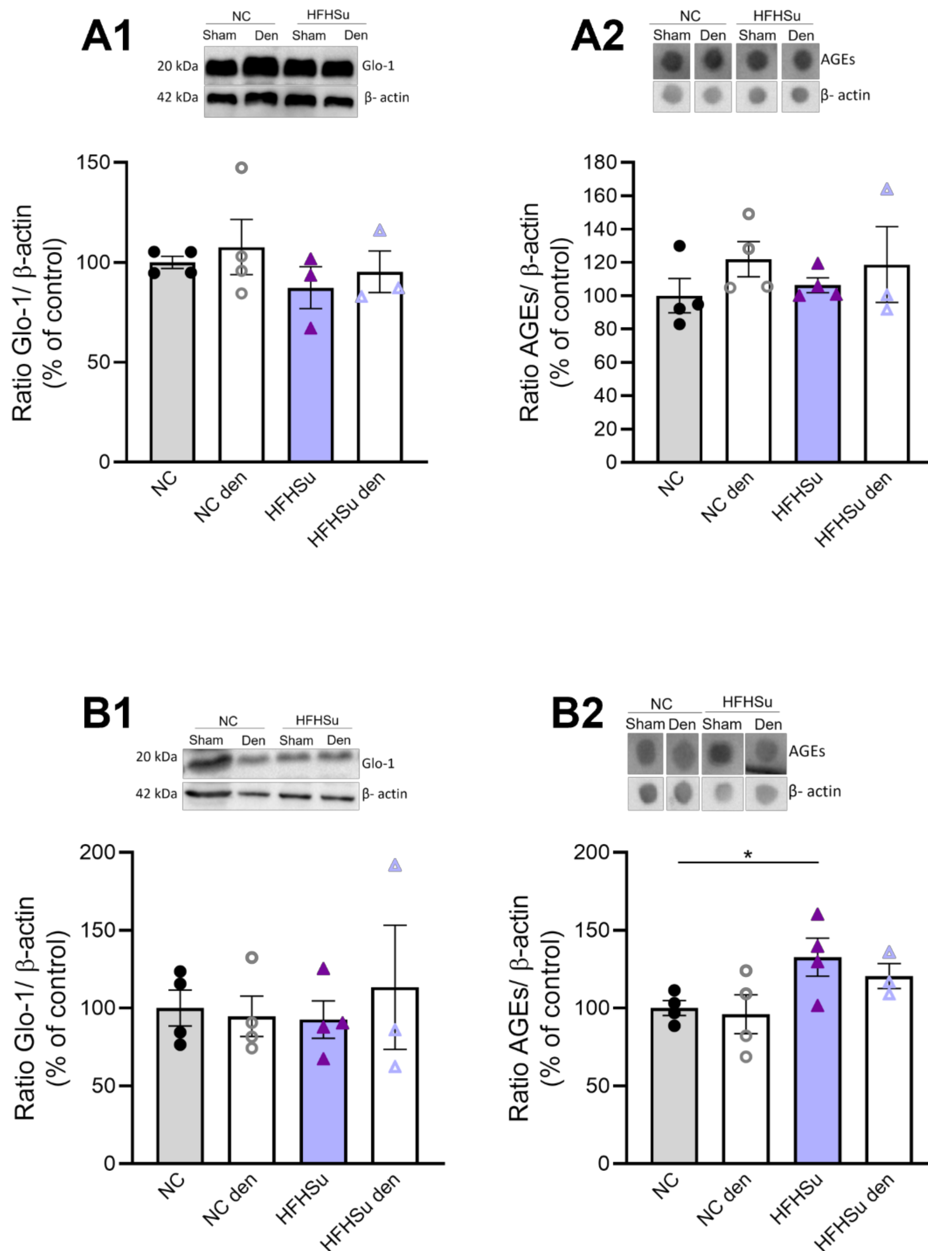


Figure 16. Effect of HFHSu diet and CSN resection on the levels of Glioxalase-1 (GLO-1) and Advanced Glycation End Products (AGEs) in prefrontal cortex (A) and in hippocampus (B), evaluated by Western Blot and DotBlot, respectively.

(A1) and **(B1)** show, respectively, the levels of GLO-1 (20 kDa), in prefrontal cortex and in hippocampus. **(A2)** and (**B2**) show, respectively, the levels of AGEs, in prefrontal cortex and in hippocampus. Proteins were normalized to the loading control β-actin (42 kDa). Top of the graphs show representative Western Bots and Dotblots for each protein studied. Black circles represent NC animals; open grey circles represent NC denervated animals; purple triangles represent HFHSu animals; open lilac triangles represent HFHSu denervated animals. Data are means ± SEM of 3-4 animals. One-way ANOVA with Bonferroni multicomparison tests: *p < 0.05 vs NC group.

4.2.3. Impact of HFHSu diet and CSN resection on synaptic markers

The levels of 3 different synaptic markers, synapsin I and SNAP25 - presynaptic markers and a post synaptic marker - PSD-95, were evaluated in prefrontal cortex (fig. 17A) and in hippocampus (fig. 17B) since these proteins can be deregulated in neurodegenerative conditions.

In the prefrontal cortex, no alterations in the levels of synapsin I were observed upon HFHSu diet or CSN resection (fig. 17 A1). However, HFHSu diet promoted a non-significant decrease of 32.9% in the levels of SNAP-25 in comparison with NC animals (p= 0.1983). Interestingly, HFHSu-CSN resected animals exhibited SNAP-25 levels similar to NC animals (fig. 17 A2). Additionally, neither the HFHSu diet nor the CSN resection change PSD-95 levels in prefrontal cortex (fig. 17 A3).

Interestingly, at the hippocampus both synapsin I and SNAP-25 decreased non-significantly by 35.13% (p = 0.1730) and 19.11% (p = 0.1461), respectively, in response to HFHSu diet. CSN resection in HFHSu animals did not alter these results (fig. 17 B1 and B2). HFHSu diet did not change PSD-95 levels, however, CSN resection in HFHSu animals promoted a non-significant increase of 53.36% (p = 0.0731) in the levels of this post synaptic marker (fig. 17 B3).

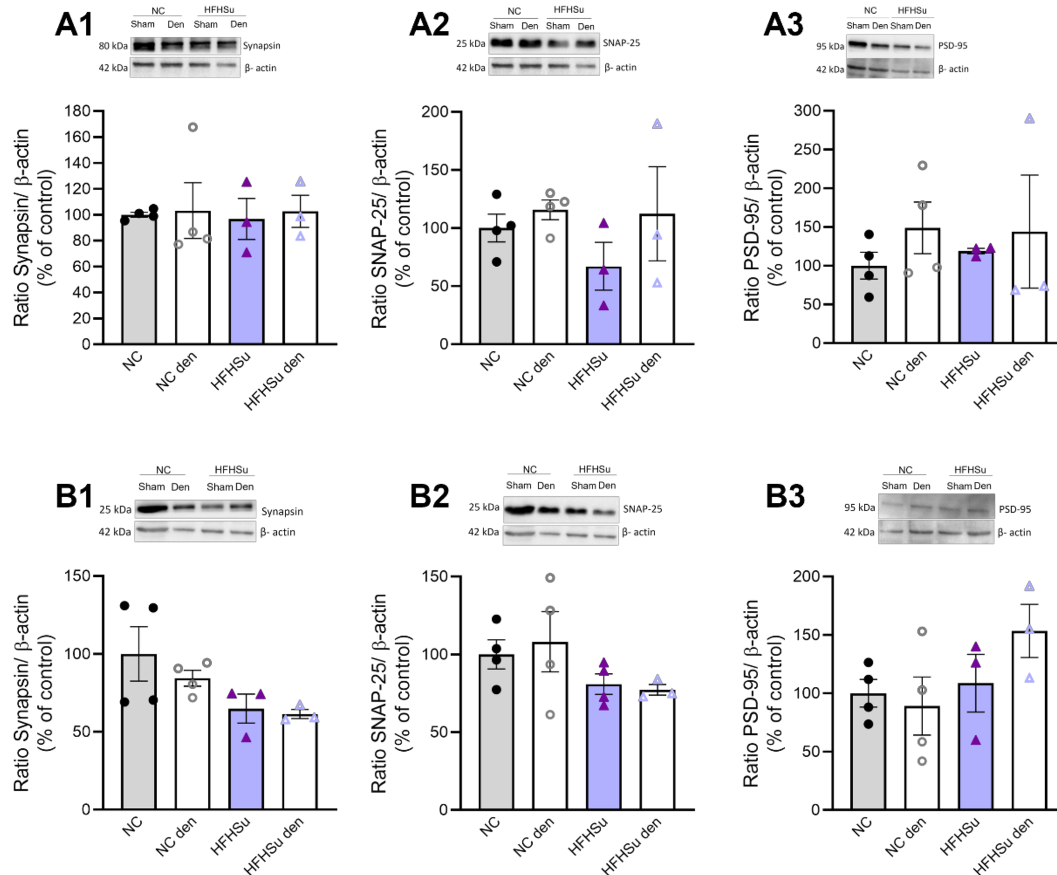


Figure 17. Effect of HFHSu diet and CSN resection on the levels of 3 different synaptic markers in prefrontal cortex (A), and in the hippocampus (B), evaluated by Western Blot.
(A1) and **(B1)** represent respectively, the levels of synapsin I (80 kDa) in prefrontal cortex and in hippocampus, respectively. **(A2)** and **(B2)** show the levels of SNAP-25 (25 kDa); and **(A3)** and **(B3)**. shows the levels of PSD-95 (95 kDa) a post-synaptic marker. The levels of levels of proteins were normalized to the loading control β-actin (42 kDa). Top of the graphs show representative Western Blots for each protein studied. Black circles represent NC animals; open grey circles represent NC denervated animals; purple triangles represent HFHSu animals; open lilac triangles represent HFHSu denervated animals. Data are means ± SEM of 3-4 animals.

4.2.4. Impact of HFHSu diet and CSN resection on aSyn and APP

The increase and accumulation of alpha-synuclein (aSyn) and Aβ levels, are reported to occur in the context of neurodegenerative processes in prefrontal cortex and in hippocampus. The levels of aSyn and APP proteins were evaluated and presented in figure 18.

In the prefrontal cortex (fig. 18 A), HFHSu diet promoted an increase in the levels of aSyn of 20.1% in comparison to NC animals. Interestingly, HFHSu-CSN resected animals did not exhibited this increase. Moreover, the levels of aSyn in these animals were even lower than the basal levels in NC animals (HFHSu= 120.09 ± 4.60 %; HFHSu den= 64.69 ± 6.97 %) (fig. 18 A1).

HFHSu diet also promoted an increase of 29.8% in the levels of APP when compared to NC animals (fig. 18 A2). Again, CSN resection in HFHSu animals prevent this phenotype (HFHSu= 129.81 ± 8.54 %; HFHSu den= 77.12 ± 4.75 %) (fig. 18 A2).

The levels of aSyn and APP were also evaluated in the hippocampus (fig. 18 B). HFHsu diet seems to promote a non-significant increase in the levels of both aSyn (p=0.1839) and APP (p=0.2420). In contrast, HFHSu CSN-resected animals present lower levels of aSyn (similar to NC animals) and of APP (46.96% and 30.89%, respectively) in comparison with HFHSu and NC animals (fig. 18 B1 and B2, respectively).

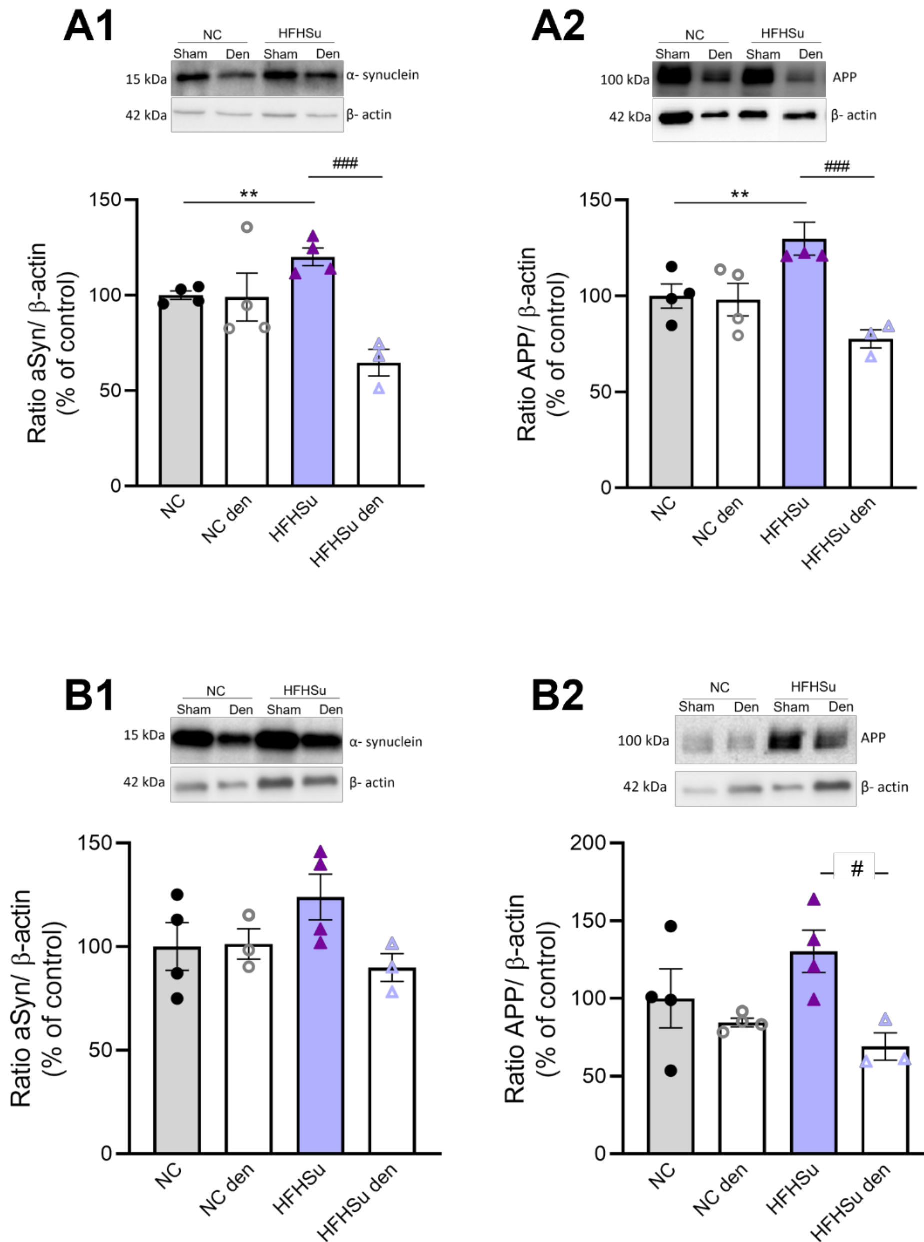
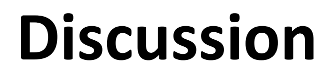


Figure 18. Effect of HFHSu diet and CSN resection on the levels of alpha-synuclein (aSyn) (A), and Amyloid β (Aβ) precursor protein (APP) (B) in prefrontal cortex (A) and in hippocampus (B).

(A1) and **(B1)** represent, respectively, the levels of aSyn (15 kDa) in prefrontal cortex and in hippocampus. The levels of APP (100 kDa) is represented both in prefrontal cortex and in hippocampus (**A2** and **B2**, respectively). Proteins were normalized to the loading control β-actin (42 kDa). Top of the graphs show representative Western Blots for each protein studied. Black circles represent NC animals; Black circles represent NC animals; open grey circles represent NC denervated animals; purple triangles represent HFHSu animals; open lilac triangles represent HFHSu denervated animals. Data are means ± SEM of 3-4 animals. One-way ANOVA with Bonferroni multicomparison tests: $**p < 0.01$, $***p < 0.001$ vs NC group; # p <0,05, ## p <0,01, ### p < 0,001 before vs after surgery.


Discussion

V. Discussion

In this study, we showed for the first time that the modulation of CBs activity impact brain function, namely the cognitive processes as well as the levels of proteins associated with neurodegenerative processes. Herein, it was showed that the abolishment of CBs activity via bilateral resection of CSN prevents cognitive alterations in rats fed with a hypercaloric diet enriched in 60% of lipids and 35% of sucrose in the drinking water. Additionally, CSN resection prevents the HFHSu-derived increase of the protein levels of APP and aSyn not only in the prefrontal cortex but also in the hippocampus.

5.1. CSN resection improves peripheral metabolic function

In the present work we have observed that the HFHSu diet intake, as expected and previously reported by our group, promoted a significant increase in the weight gain of the animals. It was previously showed that the intake of HFHSu diet for 14 or 25 weeks led to an increased weight gain when compared to their corresponding age-matched controls [167], [164]. In agreement, we obtained the same effects after 14 and 20 weeks of diet. This increase in weight gain is probably correlated with the increased caloric intake in the animals under the HFHSu diet since they consumed more calories than the control group fed with a standard diet. Comparing the consequences in the weight gain of the dysmetabolic experimental model tested herein with other procedures that induce dysmetabolism such as HF diet exposure (without sucrose in the drinking water), the HFHSu diet -animals exhibit a lower weight gain than the HF animals (results from the present work and[164],[167]). These differences might be explained by the presence of sucrose in the diet composition allowing the animals to consume more sugar than the HF diet, that is known to promote an increase in fat accumulation and expansion, leading to an increase in the number, and size of the adipocytes [176], [177].

As also expected, the basal glycaemia levels of the HFHSu animals were higher than the NC animals and progressively increased throughout time. It is important to note that although the animals exhibited a 24% higher basal glycaemia levels (117 mg/dl) than NC animals, these levels were below the threshold value for diabetes diagnosis (126 mg/dL, [40]) meaning that these animals are in a prediabetes stage. These results agree with previous findings from Conde's lab, where they observed that HFHSu-treatment for 25 weeks increase the basal glycaemia levels by 17% [167]. In agreement with this dysmetabolic state observed already in fasting glucose levels,

HFHSu diet led to a decrease in insulin sensitivity and glucose tolerance, results already reported for animals under HFHSu diet for 14 and 25 weeks [167], [178] , [164]. Overall, we can state that in this study we were able to reproduce the dysmetabolic model previously reported in our lab, since HFHSu diet for 20 weeks promotes an increase in the weight gain, fasting glycaemia, insulin resistance and glucose intolerance.

In the present study and in concordance with the previous publications from our laboratory we also show that CSN resection was able to attenuate the increase in weight gain, the increase in fasting glycaemic levels, being also able to restore insulin sensitivity and ameliorate glucose intolerance, 5 weeks after surgery. These beneficial effects of CSN resection on metabolic homeostasis have been attributed to the positive impact on visceral AT and the liver [161]. In fact, in those models, it was shown that CSN resection restores glucohomeostasis and lipohomeostasis. The mechanisms that underlying these beneficial effects involves an improvement in glucose uptake by the liver and a re-establishment of glucose uptake by perienteric AT, concomitantly with an improvement in autonomic function. More recently, our laboratory has showed that the positive impact of CSN resection on dysmetabolism involves the beiging of the visceral AT and the activation of brown adipose tissue through the modulation of sympathetic nervous system activity [179]

Altogether it can be concluded that CSN resection positively impacts the dysfunctions promoted by hypercaloric diets, leading to restoration of peripheral metabolism.

5.2. CSN resection prevents cognitive impairment induced by HFHSu diet, evaluated *in vivo* by the y-maze test

In our study we demonstrate that hypercaloric diets, as described by several authors [133], [102], [79] could have a negative impact in several brain functions, namely those associated with cognitive processes and olfactory acuity. Moreover, we show that CSN denervation prevents the alterations on brain features associated with cognition.

Herein it was showed that animals treated for 20 weeks with HFHSu develop behavioural function alterations, measured in the y-maze and block test. Although we did not observe significant differences in the OF test, some trends are interesting to pursue and suggest that HFHSu diet consumption can impact the behaviour performance of these animals.

It was clear that the amount of time that the animals were under the HFHSu diet, in the present work, was not sufficient to promote locomotor function alterations. In fact, the distance covered

by the 4 experimental groups tested herein was not significantly different from each other, which agrees with some studies performed with other animal models of metabolic, or neurodegenerative disorders. For example, in the study by Skye Hsin-Hsien Yeh et al.,(2020) using C57BL/6 as control animals and APP/PS1 transgenic mice, both fed a NC diet or an high sucrose diet (67.3% of carbohydrate, 4.3% of fat, and sucrose at 35% by weight) for 10 weeks showed, in the OF test, that the total distance covered, and the time spent in the inner zone was not significantly different between groups [180]. These results suggest that even a model of neurodegenerative disease consuming high sucrose diet was not able to cause changes in the locomotor activity, what may indicate that induce this phenotype is difficult even in neurodegenerative models. It is also important to refer that the OF test is used to access general and gross locomotor activity and thus, to better evaluate the locomotor activity of these animals it will be necessary to perform more sensitive behavioural tests that analyse fine motor activity such as the mouse walker or the rotarod [173], [181]. Also, the OF test is used to access animals exploration habits and anxiety states being reported that the more anxious the animals are, the more time they spend near the walls of the maze, staying consequently, less time in the inner zone [182]. Our results show a tendency to decrease the total distance covered, to decrease the distance covered in the inner zone, and consequently to decrease the time spent in the inner zone throughout the time, independently of diet consumption or surgery intervention, since the trend is the same in all experimental groups. Since the OF test assesses exploration habits and animals were tested in the arena three times, they might decrease the interest in the task as this has no longer novelty. Moreover, these effects were not altered with the CSN resection. It is interesting to note that 5 weeks after surgery, HFHSu sham animals show a tendency to increase the time they spend in the inner zone when compared to other groups, and even compared to their own behaviour at the previous timepoint. This result may be due to the consumption of the sucrose in the drinking water, that has been shown to decrease anxiety-related behaviour. In agreement, Avena et al. (2008) showed that rats under 3 months of sucrose intake, decreased anxiety-related behaviour in the OF and elevated plus maze tests [183], [184] Additionally, as explained before, protein glycation, which is known to occur in T2D as well as in AD and PD, is driven by the reaction between proteins and reduction sugars forming AGEs, being MGO one of the most reactive agents unavoidably formed in the cells. Considering that our animal model consumed a hypercaloric diet with sucrose in the drinking water, we can speculate that the levels of MGO could be increased, therefore impacting anxiety states. In fact, Hansen and colleagues (2016), showed that Wistar rats injected intracerebroventricularly with MGO and evaluated 3 weeks post-surgery exhibited decreased anxiety-like behaviour in the OF test, since they increased in the distance covered [185]. Interestingly, the same result is not

observed in the HFHSu animals submitted to the CSN resection. As observed in table 3, CSN resection promoted a significant decrease in the caloric intake of HFHSu denervated animals, suggesting that they decrease not only the diet consumption but also the liquid consumption. Probably, due to this, the amount of sucrose consumed was not sufficient to promote an increase in MGO levels in the brain and consequently promote this anxiolytic effect [185]. However, to make further conclusions, the measurement of brain MGO levels in our animals, should be assess.

Several reports have shown the negative impact of dysmetabolism in brain function[52], [92]. What is truly novel in this project is the beneficial effect of the CSN modulation in some brain processes. In particular, the analysis of the animals spatial learning and memory capacity by the Y-maze test, shows promising protective effects. At 20 weeks of diet, HFHSu animals show lower number of entries and lower time spent in the novel arm, compared to NC animals. Our results are in line with previous reports. For example, Abbott and co-workers (2019) conducted a meta-analysis of the impact of different diets (HF, High sucrose (Hsu), or HFHSu), and reported that each type of diet adversely affects cognitive performance, with the largest effect produced by exposure to a combined HFHSu diet for 8 wees [186]. Fu and colleagues (2017) also demonstrated that long-term HF diet induces hippocampal microvascular insulin resistance and cognitive dysfunction. In particular, the intake of a HF diet for 6 months significantly decreased the cognitive function of 8 months animals assessed by the two-trial spontaneous alternation behaviour and the novel object recognition tests [187]. Thériault and colleagues (2016) showed that a HF diet exacerbated AD-related pathology in APPswe/PS1 mice, a mouse model of AD that overexpress the Swedish mutation of APP together with PS1 deleted in exon 9. In this study, 3 and 12 months old APPswe/PS1 mice were fed for 4 months with a HF diet and demonstrated that this diet accelerated age-associated cognitive decline without affecting parenchymal Aβ [188]. What is interesting is that we show herein that HFHSu CSN-denervated animals spend a similar time in the novel arm when compared to the sham group (5 weeks post-surgery), suggesting that CSN resection impacts cognitive function. As previously mentioned, CSN resection decreased the caloric intake, and improved metabolic function by increasing insulin sensitivity and glucose tolerance. We hypothesize that this improvement in metabolic function is contributing for the prevention of cognitive impairment. Thus, dysmetabolism is a putative mechanism underlying the development of cognitive deficits with the intervention of CBs overactivation.

Another parameter evaluated in the Y-maze test was the % of animals that choose the novel arm in the test. Before CSN surgery, the NC group showed increased % of novel arm choice in

comparison to the other groups. However, over time, the animals seem to become familiar to the maze and the test, and it seems that almost all the animals learned the task since the % of novel arm choice is similar between the groups being almost 100%. In fact, 2 weeks post-surgery, only one animal from the NC, HFHSu and HFHSu CSN-denervated groups failed the task and did not choose the novel arm. Interestingly, 5 weeks after surgery, the HFHSu CSN-denervated group was the only where all animals chose the novel arm (100% novel arm choice). This result further supports that CSN denervation may have a role in preventing cognitive decline.

Regarding the spontaneous alternative behaviour evaluation, no differences were observed between the experimental groups. This result suggests that although the HFHSu animals spent less time in the novel arm, they are still performing the correct sequence in the alternative behaviour evaluation, leading us to hypothesize that the time under HFHSu diet only promoted slight alterations in these animals' cognitive functions.

Knowing that hyposmia is frequently associated with PD, we evaluated the olfactory acuity of the animals using the block test. This test is frequently used in mice to access their olfactory acuity and discrimination, sensitivity to social smells, an ethologically essential ability in rodents. Herein we shown, that HFHSu animals exhibit reduced olfactory performance. To discard that the decreased amount of time sniffing the novel block is not due to loss of interest in performing the task caused habituation and disinterest, we analysed the ratio between the time the animals spend sniffing the novel block and the total time sniffing the other blocks. This analysis showed that in fact the time sniffing the novel object is decreased, suggesting no habituation or disinterest for the task. These findings suggest that hypercaloric diets may promote olfactory alterations. In the present work CSN resection did not prevent olfactory acuity changes, suggesting that the mechanism involved in the HFHSu-driven decrease in olfactory acuity probably does not involve peripheral mechanisms or circuits mediated by the CBs.

5.3. CSN resection decreased the levels of aSyn and APP proteins in the cortex and hippocampus of the HFHSu animals

Our findings suggest that HFHSu may induce an impairment in some brain functions such as cognition and olfactory performance, effects attenuated at the level of cognition in the CSN-denervated animals. Thus, considering the key role of prefrontal cortex and hippocampus in the cognitive processes, we evaluated the levels of different proteins associated with insulin

signalling, protein glycation, synaptic and proteinopathy markers, which are parameters associated with neurodegenerative processes [52].

The important role of insulin in brain function, led us to evaluate the levels of IR, AKT and the ratio between p-AMPK/ t-AMPK. Of interest, cortex and hippocampus are the brain regions that express higher concentrations of IR [67]. No differences were observed in these proteins in both prefrontal cortex and hippocampus, neither due to the HFHSu diet consumption, nor the CSN resection, although the levels of IR seem to be decreased in both regions of HFHSu animals. Nevertheless, until the moment we only have assessed the levels of the total isoforms of the proteins involved in insulin signalling, as the IR and the AKT but not the phosphorylated forms. Since it is well known that hypercaloric diets promote the downregulation of IRS-1 and phospho-AKT levels [189] the assessment of phosphorylated forms of proteins involved in insulin signalling cascade and/or of proteins only activated with the binding of insulin to its receptor will be key to take further conclusions. Another possibility for the absence of effects of HFHSu diet on insulin cascades could be the time under diet treatment, as in this case it would be important to analyse animals under a longer treatment with HFHSu diet. Importantly, other animal models of dysmetabolism, using different feeding protocols such as a HF diet for 6 months, present an abolishment of insulin-mediated microvascular responses and AKT phosphorylation. The authors claim that hippocampal microvascular IR may play a critical role in the development of cognitive impairment, since they present cognitive impairment in Y-maze test [187].

We further observed a non-significant decrease in the ration of p-AMPK/t-AMPK in the hippocampus with the HFHSu diet that seems to slightly increase with the CSN resection, effects that must be confirmed with the increase in the number of samples evaluated. AMPK is a metabolic sensor both in the periphery and in the brain and its deregulation has been shown to be involved in T2D and in several neurodegenerative diseases as AD and PD [190]. AMPK can influence several processes as autophagy, cell growth, and mitochondrial quality control. It is reported that in mice, the activation of AMPK protected neurons against metabolic and excitotoxic insults [191]. Thus, activating or enhancing the AMPK activity, will trigger the increase in the autophagic process, and reducing the aggregates or misfolded proteins [192]. These findings are in line with our results showing that HFHSu animals show a tendency to decrease AMPK levels and that CSN resection seems to improve it, what may indicate that the surgery intervention is contributing to the activation of AMPK improving its role in brain functions.

This effect, that should be confirmed in further studies, could be due to the possible decrease of IR levels. It is possible that a reduction of IR levels, due to a state of insulin resistance, may lead to the downregulation of its downstream proteins.

Considering that glycation is a cardinal feature of T2D as a consequence of hyperglycaemia, and that also has been shown to potentiate the toxicity and aggregation of aSyn and Aβ in neurodegenerative disorders, we evaluated the levels of GLO-1 and the AGEs in the prefrontal cortex and in the hippocampus. While in the prefrontal cortex we found no alterations, in the hippocampus the levels of AGEs were significantly higher in the HFHSu animals. In agreement with the results herein obtained, Wang and colleagues (2009) showed that adult male diabetic Sprague–Dawley rats exhibited elevated levels of AGEs in the hippocampus, an effect that led to impaired hippocampal neurogenesis and behavioural alterations [193]. This result may suggest that the increase in AGEs levels due to HFHSu treatment, may induce those behavioural alterations by affecting neurogenesis and impairing normal cognitive processes. These events may also allow to understand our results showing that HFHSu animals spent less time in the novel arm. We also found that animals with CSN resection did not present these alterations, suggesting that CSN modulation may be protective by preventing brain dysmetabolism.

It is reported that alterations in synaptic function are associated with neurodegenerative processes [194],[195]. Therefore, we evaluated the levels of synapsin I, SNAP-25 and PSD-95 in both brain regions. No significant differences were observed. However, the levels of SNAP-25 in the prefrontal cortex and in the hippocampus, shows a clear tendency to decrease with diet, that will probably become significant if we increase the number of samples analysed. SNAP-25 is a presynaptic protein that belongs to the SNARE protein complex, with important functions in the process of exocytosis and neurotransmitter release, by regulating the docking/or fusion of synaptic vesicles to the plasmatic membrane [196]. The decrease in the levels of SNAP-25 in those brain regions may indicate that exocytosis and neurotransmission are affected, meaning that less neurotransmitters are being released. It was previously shown in human brain homogenates that the membrane-bound levels of SNAP-25 were significantly decreased in samples of AD patients [197]. In agreement we also found a non-significant decrease in the protein levels of synapsin I in the hippocampus of HFHSu animals. As SNAP-25, synapsin I is a presynaptic protein which is important to modulate the release of neurotransmitters from the presynaptic- terminal, controlling the number of vesicles that fusion in the active zone. In fact, decreased levels of synapsin I are associated with cognitive impairment and AD [194]. It was reported that AD patients present a significant decrease in synapsin I immunoreactivity in the striatum of the CA1 neurons and in the molecular layer of the dentate gyrus [194]. Our results

show the same tendencies, what may indicate that the HFHSu animals are developing features associated with AD pathology. Interestingly we did not find any alterations in the levels of PSD-95 a post-synaptic marker, this contrasting with previous data from Bhat and Thirumangalakudi (2013) where they showed that mice submitted to high fat/high cholesterol diet exhibit a down-regulation of PSD-95 in the hippocampus and this indicating altered synaptic plasticity [189]. Interestingly and in contrast with other proteins evaluated herein, CSN resection was incapable of produce visible effects on these synaptic markers. However, as the number of samples evaluated was small further studies are needed to confirm this data.

The most interesting and surprising results on the brain analysis performed in the present work are related to the role of HFHSu diet and CSN resection on proteinopathies. Herein we showed that the intake of HFHSu diet was capable of promoting a significant increase in the protein levels of aSyn and APP in the prefrontal cortex, and of APP in the hippocampus, where a tendency of aSyn increase is also observed. These results are expectable since several reports showed that hypercaloric diet consumption are involved in the development of neurodegenerative disorders [198]. For example, Kothari and collaborators (2017) demonstrated that HFHSu diet induced brain insulin resistance and cognitive impairment in mice. Notably, C57BL/6NHsd mice under a HFHSu diet (40% energy from fat, 42 g/L liquid sugar) present increased Aβ deposition and neurofibrillary tangle formation and decreased synaptic plasticity [199]. In our work we did not quantify the levels of Aβ and therefore we cannot ascertain if there is an increase in these levels. Yet, we assessed the levels of APP, a protein that has a central role in the development of AD, since its misprocessing lead to the generation of Aβ peptides, major components of the amyloid plaques [200]. Thus, it remains to be measured the ratio between the APP and its fragments such as C99, which results from the APP processing [201]. If this ratio is increased, it would be possible to conclude that there is an increase in APP processing that contributes for higher formation of Aβ. Of note, usually Aβ increases is associated with neuronal death, this deserving further assessment in the future by measuring β3 tubulin. Additionally, in our results we observe that both in prefrontal cortex and in the hippocampus, CSN resection was able to restore the increased levels of APP, what may indicate that by improving peripheral metabolism we are impacting the levels of total APP, by decreasing it to the values similar to the NC animals.

We also found that the levels of aSyn were increased in the prefrontal cortex and the hippocampus in HFHSu animals, however being only significant in the cortex. Importantly, in PD, the levels of aSyn are reported to be increased in the SNpc, but also in other brain regions [202]. Therefore, our results may indicate that the HFHSu diet can contribute for the appearance of

biochemical features associated with the onset of PD, suggesting that the diet can trigger aSyn pathology in the brain. Although no major changes were observed at a motor level in the OF test, we observed a decrease in olfactory acuity in HFHSu animals (an early feature of PD). Interestingly, we observe that in CSN resected animals the levels of aSyn are below the NC animals in the prefrontal cortex, and similar to the NC animals in the hippocampus, suggesting that CBs modulation may prevent aSyn accumulation.

In fact, the in vivo results related to the evaluation of PD like features, only demonstrated that there is an impairment of olfactory acuity, a non-motor symptom of PD, which precedes the motor symptoms. Considering that gut dysmetabolism is also a non- motor symptom of PD, we can speculate that our results are in same line of a recent theory by Rietdijk and colleagues (2017). The authors showed that PD derives from the migration of aSyn precursors and aggregates from the gut to the central nervous system through the autonomic nervous system, in particular by the vagus nerve [203] . This idea was supported by the fact that PD patients that were submitted to the truncal vagotomy exhibited a decreased risk to develop PD about 40-50% after 10–20 years of surgery. Furthermore, truncal vagotomy in mice prevented the spread of aSyn fibrils injected in the gut through the gut-to brain axis, thus preventing synucleinopathies and associated neurodegeneration, and behavioural deficits [204]. Additionally, more recently Van Den Berge and colleagues (2021) showed that robust spread of aSyn from the gut to the brain and from the brain to the gut involves not only the parasympathetic nervous system, namely the vagus, but also the sympathetic nervous system [205]. Knowing that the CBs are deeply connected with both the sympathetic and parasympathetic nervous system, we can postulate that the CBs and the CSN might be involved in the spread of aSyn from the periphery to the brain. All these speculations deserve future clarifications.

Concluding remarks

VI. Concluding remarks

The prevalence of aging-associated chronic pathologies, as metabolic and neurodegenerative disorders, has dramatically increased along with the increase in life expectancy. These metabolic disorders are often associated with a modern lifestyle, characterized by physical inactivity, sedentarism and with increased consumption of hypercaloric diets. Despite the efforts that have been done to change bad habits of life, the number of people suffering from, for example T2D is still increasing. Moreover, there are several comorbidities developed with T2D as neurodegenerative disorders. Therefore, it is important to search novel therapeutic approaches to prevent the development of metabolic disorders and consequently their comorbidities as neurodegenerative states.

In the last years Conde's group have been dedicated to the study of the modulation of CBs activity to prevent the development of insulin resistance, glucose intolerance and obesity. In this project we investigated if the modulation of CBs activity via bilateral resection of CSN could prevent the neurodegenerative process and cognitive deficits by preventing and/or reversing brain dysmetabolism.

We showed that animals fed a HFHSu diet, submitted to the CSN resection, present an improvement in peripheral metabolism, by increasing insulin sensitivity, glucose tolerance and decrease weight gain. Moreover, we describe for the first time that CSN bilateral resection has a beneficial impact in brain functions related to cognitive processes, since the HFHSu denervated animals spent less time in the novel arm in the y-maze test, and CSN resection prevented this phenotype.

These results suggest that the modulation of CBs activity could be a therapeutic strategy to the prevention of neurodegenerative disorders.

Future work

VII. Future work

Despite the novelty of the results here presented, and after one year working on this project, there are questions that need to be further explored, and experiments that must be concluded.

The results presented in this work are very preliminary. Regarding the levels of insulin signalling proteins, it will be important to evaluate the phosphorylated form of the IR, and AKT. In this project we only access the total levels of these proteins, and we did not observe differences, probably because the alterations occur at a post-translational level. In fact, probing only the phosphorylated form of those proteins, will give us more detailed information to assess the ones that are being activated in the insulin signalling pathway after insulin binding.

Considering also that we only evaluated the APP levels we cannot conclude if more Aβ is being produced in HFHSu animals, being therefore crucial to evaluate APP fragments as C99, C191 and the ratio between APP and its fragments to understand if there is more APP processing, and consequently, more Aβ being formed. Considering that due to the fact that APP is a protein widely expressed, and that alterations in the total levels of APP are usually accompanied by neuronal death, it will be important to assess the level of β3 tubulin, a measurement of general neuronal mass. It will be also important to assess the levels of Tau and phosphorylated Tau protein to understand if this diet and the CSN resection are having an impact on this protein deeply involved in the development of AD features.

Additionally, it will be important to clarify if the increased levels of aSyn and APP are in the aggregated form. To address this question, we can perform immunohistochemistry assays in brain slices of the prefrontal cortex and hippocampus. Thus, we will evaluate not only the levels of aggregated aSyn but also, we will be able to observe if aSyn is colocalizing with other proteins and in which type of neurons these are present at a higher density. In immunohistochemistry assays we will also evaluate the levels of Aβ and understand if it is increased in the HFHSu animals, as APP is, and understand if CSN resection might have an impact on this.

Considering that in the block test we observe a decreased olfactory function in the HFHSu animals, and that it is a non-motor symptom of PD that precedes the motor symptoms, it will be important to evaluate the olfactory bulb brain extracts and conclude if this brain region has some proteins being altered, as the levels of aSyn.

As explained in the discussion, the aggregates of aSyn in the gut could have an impact on aSyn pathology in the brain. Thus, it will be important to perform protein analysis in the gut of these

animals to evaluate if there are alterations in the levels of aSyn levels, not only under the HFHSu diet but also with the CSN resection.

As this is an exploratory project without any preliminary data regarding the role of CBs in brain insulin resistance, we based it on some results that we obtained from some metabolic studies involving CBs activity. Thus, the model used in the present work was the HFHSu rats submitted to 14 weeks until the CSN recession. As observed by the behavioural tests, the alterations observed were slight. Thus, in a new cohort of animals, we will test more time on the HFHSu diet, for example 24 weeks and at 25 weeks we will proceed with surgery and the animals will be followed for more 5 weeks. It will be also important to add some behavioural test, specifically, more motor tests, since the OF evaluates gross motor activity, and we did not observe differences between the groups. Thus, performing the mouse walker and the rotarod tests will give us more detailed information regarding this function. We could also include other cognitive tests such as novel object recognition test and to evaluate anxiety more precisely, we could perform the elevated plus maze test. Considering that rats are not so sensitive to social smells as mice, we should change the olfactory test to the recognition of new smells.

After all the *in vivo* tests, all the *ex vivo* evaluations will be the same that we performed in this cohort of animals, and the new cohort might give us new clarifications of the mechanisms behind the role of CBs in brain insulin resistance and neurodegeneration.

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VIII. References

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