MODULATION OF CAROTID BODY ACTIVITY AS A THERAPEUTIC INTERVENTION IN METABOLIC DISEASES

JOANA FILIPA CANAIS DA COSTA SACRAMENTO

Tese para obtenção do grau de Doutor em Mecanismos de Doença e Medicina Regenerativa

Doutoramento em associação entre:

Universidade NOVA de Lisboa (Faculdade de Ciências Médicas | NOVA Medical School - FCM|NMS/UNL)

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Projects approved by the Ethics Committee of NMS|FCM-UNL:

- Corpo carotídeo: um potencial alvo terapêutico para as doenças metabólicas.
- Estudo das vias neuronais envolvidas nas doenças metabólicas associadas sobreativação dos corpos carotídeos: definindo o caminho para o uso de dispositivos bioeletrónicos.
- Carotid chemoreceptor neuromodulation for the treatment of type ii diabetes mellitus.

Project approved by DGAV:

- In-Vivo study to investigate the electrical blocking of the carotid sinus nerve activity in early stage type 2 diabetic rat models: Impact on insulin sensitivity and glucose tolerance.

The present work:

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- **Sacramento JF**, Chew DJ, Melo BF, Donegá M, Dopson W, Guarino MP, Robison A, Prieto-Lloret J, Patel S, Holinski BJ, Ramnarain N, Famm K, Conde SV. (2018) Bioelectronic modulation of carotid sinus nerve activity in the rat: a potential therapeutic approach for type 2 diabetes. *Diabetologia*. 61(3):700-710.

- Conde SV, Monteiro EC, **Sacramento JF.** (2017) Purines and carotid body: new roles in pathological conditions. *Front Pharmacol.* 8:913.

- **Sacramento JF**, Ribeiro MJ, Rodrigues T, Olea E, Melo BF, Guarino MP, Fonseca-Pinto R, Ferreira CR, Coelho J, Obeso A, Seiça R, Matafome P, Conde SV. (2017) Functional abolishment of carotid body activity restores insulin action and glucose homeostasis: key roles for visceral adipose tissue and the liver. *Diabetologia*, 60(1):158-168.

Won the following awards:

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- **Sacramento JF**, Prego CS, Conde SV. (2018) Can the modulation of adenosine and/or ATP signaling in the carotid body be used to treat type 2 diabetes? Early Career Physiologists' Symposium (ECPS 2018), London, UK.

- **Sacramento JF***, Chew DJ*, Melo BF, Donegá M, Dopson W, Guarino MP, Robinson A, Prieto-Lloret J, Patel S, Holinski BJ, Ramnarain N, Pikov V, Famm K, Conde SV. (2018) Bioelectronic neuromodulation of the carotid sinus nerve as a potential therapeutic for type 2 diabetes. I NoTeS Congress - Novel Therapeutic Strategies for Noncommunicable Diseases, Porto, Portugal.

- Sacramento JF, Prieto-Lloret J, Conde SV. (2017) Overactivation of carotid sinus nerve activity in high-fat animals is mediated by A_2 adenosine receptors. 20th International Society for Arterial Chemoreception (ISAC) Congress, Baltimore, USA.

Oral communications in national meetings:

- **Sacramento JF**, Chew DJ, Melo BF, Donegá M, Dopson W, Guarino MP, Robison A, Prieto-Lloret J, Patel S, Holinski BJ, Ramnarain N, Famm K, Conde SV. (2018) Modulação bioeletrónica da actividade do nervo do seio carotídeo: potencial terapêutica para diabetes tipo 2. 14th Portuguese Congress of Diabetes, Albufeira, Portugal.

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- **Sacramento JF**, Prieto-Lloret J, Melo BF, Conde SV. (2017) Effect of high-fat diet on carotid sinus nerve activity in basal conditions and in response to hypoxia. XLVII Annual Meeting of the Portuguese Society of Pharmacology, Coimbra, Portugal.

- **Sacramento JF**, Melo BF, Rodrigues T, Coelho JC, Olea E, Ribeiro MJ, Obeso A, Seiça RM, Guarino MP, Matafome P, Conde SV. (2016) Efeito da ressecção do nervo do seio carotídeo no transporte da glucose em modelos animais de resistência à insulina. 12nd Portuguese Congress of Diabetes, Vilamoura, Portugal.

- **Sacramento JF**, Melo BF, Rodrigues T, Coelho JC, Olea E, Ribeiro MJ, Obeso A, Seiça RM, Guarino MP, Matafome P, Conde SV. (2016) Is the effect of carotid sinus nerve resection on insulin sensitivity mediated by an increase in glucose uptake? XLVI Annual Meeting of the Portuguese Society of Pharmacology, Porto, Portugal.

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glucose tolerance in rodent type 2 diabetes models. 53rd Annual Meeting of the European Association for the Study of Diabetes, Lisbon, Portugal.

- **Sacramento JF**, Melo BF, Rodrigues T. Coelho JC, Olea E, Ribeiro MJ, Guarino MP, Obeso A, Seiça RM, Matafome P, Conde SV. (2016) Carotid body transection restores glucose and lipid homeostasis in prediabetes animal models. 52nd Annual Meeting of the European Association for the Study of Diabetes, Munich, Germany.

- **Sacramento JF**, Prieto-Lloret J, Melo BF, Conde SV. (2016) High fat diet increases basal carotid sinus nerve activity and the responsiveness to hypoxia. Physiology 2016, Joint Meeting of the American Physiological Society and The Physiological Society, Dublin, Ireland.

- Conde SV, **Sacramento JF**, Melo BF, Patel S, Chew D, Dopson W, Pickov V, Robinson A, Holinski B, Ramnarain N, Guarino MP. (2016) Electrical Neuromodulation of the Carotid Sinus Nerve as a Novel Therapeutic Approach for the Treatment of Type 2 Diabetes. 76th American Diabetes Association Scientific Sessions, New Orleans, United States of America.

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Abbreviations

AACE	American Association of Clinical Endocrinology
AC	Adenylyl cyclase
ADP	Adenosine diphosphate
AHA/NHLBI	American Heart Association/National Heart, Lung, and Blood Institute
AK	Adenosine kinase
AMP	Adenosine monophosphate
AMPK	5′AMP-activated protein kinase
ATP	Adenosine-5’-triphosphate
AUC	Area under the curve
BP	Blood pressure
Bpm	Breaths per min
CA	Carotid artery
cAMP	Cyclic adenosine monophosphate
CB	Carotid Body
CCA	Common carotid artery
CGI	Combined glucose intolerance
CNT	Concentrative nucleoside transporter
CSN	Carotid Sinus Nerve
CTL	Control
DC	Direct current
DPP4-i	Dipeptidyl peptidase-4 inhibitor
ECA	External carotid artery
ECG	Electrocardiogram
EGIR	European Group for the study of Insulin Resistance
EMG	Electromyography
ENT	Equilibrative nucleoside transporter
FPG	Fasting plasma glucose
fxs	Fractures

GI	Gastrointestinal
GLP1	Glucagon-like peptide 1
GLUT2	Glucose transporter 2
GLUT4	Glucose transporter 4
GPN	Glossopharyngeal nerve
HbA_{1c}	Haemoglobin A_{1c}
HDL	High-density lipoprotein
HF	High-fat
Hf	High frequency
HFHSu	High-fat/High-sucrose
HR	Heart rate
HSu	High-sucrose
Hypo	Hypoglycemia
ICA	Internal carotid artery
IDF	International Diabetes Federation
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
IP3	Inositol 1,4,5-trisphosphate
ITT	Insulin Tolerance Test
IRS	Insulin receptor substrate
IVGTT	Intravenous glucose tolerance test
LDL	Low-density lipoprotein
Lf	Low frequency
KHFAC	Kilohertz frequency alternating curreny
K_{ITT}	Constant of the Insulin Tolerance Test
MAP	Mean arterial pressure
MAPK	Mitogen-activated protein kinase
mRNA	messenger ribonucleic acid
microCT	Microcomputed tomography
NBTI	Nitrobenzylthioinosine

NCEP:ATPIII	National Cholesterol Education Program Adult Treatment Panel III
NGT	Normal glucose tolerance
OGTT	Oral glucose tolerance test
$PaCO_2$	Partial arterial pressure of carbon dioxide
PaO_2	Partial arterial pressure of oxygen
PDE	Phosphodiesterase
PI3K	Phosphatidylinositol 3-kinase
PKB/AKT	Protein kinase B
PO_2	Partial oxygen pressure
PSD	Power spectral density
RA	Receptor agonist
Rg′	Glucose metabolic index
SAHase	S-adenosyl homocysteine hydrolase
SaO_2	Oxygen saturation
SGLT2-i	Sodium-glucose co-transporter-2 inhibitor
SU	Sulfonylurea
TBST	Tris-buffered saline with Tween
TZD	Thiazolidinediones
T2D	Type 2 Diabetes
VE	Minute ventilation
VG	Vagus nerve
VT	Tidal volume
WHO	World Health Organization

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Abstract

Type 2 diabetes (T2D) is one of the most common chronic diseases, whose prevalence continues to increase, being expected to affect 629 million people in the world in 2045. The principal defects in T2D are peripheral insulin resistance, abnormal hepatic glucose metabolism and progressive pancreatic beta cell failure. Despite the several different drugs available for T2D treatment, over time, glucose control deteriorates progressively and even with the rearrange of medication, a sizeable proportion of individuals remain poorly control. Therefore, is crucial the development of new therapeutic strategies to control this epidemic.

In the last years, the carotid body (CB), a peripheral chemoreceptor that sense changes in blood O_2, CO_2 and pH, have also been described as a metabolic sensor implicated in the control of energy homeostasis. In fact, it was described that CB overactivity is involved in the genesis of insulin resistance and hypertension induced by the hypercaloric diets.

The aims of the present work were to investigate the role of CB in the control of glucose homeostasis and to search a method/approach to modulate CB activity aiming to treat T2D.

Chapter I introduces general concepts in T2D field, as the insulin signaling, glucose homeostasis and the therapeutic options for T2D treatment. Additionally, fundamental concepts of CB function and the role of ATP and adenosine in the CB neurotransmission, as well as, the role of CB as a metabolic sensor are also addressed.

In **chapter II** are described the general and specific aims of the present work.

In **chapter III** it was demonstrated that the carotid sinus nerve (CSN), the CB sensitive nerve, resection restores the insulin sensitivity in two prediabetes animal models, an effect that was maintained even when the animals were continuously fed hypercaloric diets. Moreover, it was also demonstrated that CSN resection normalized systemic sympathetic nervous system activity, blood pressure, endothelial function, lipid profile and plasma glucose and insulin levels. Additionally, the mechanism behind the repair of glucohomeostasis involves an improvement in glucose uptake in the liver and perienteric adipose tissue and a restored insulin signaling pathways in skeletal muscle and adipose tissue.

In **chapter IV** it was demonstrated that the bioelectronic modulation of the CSN by using the kilohertz frequency alternating current (KHFAC) is capable to restore the

insulin sensitivity and the glucose tolerance in a diet-induced early stage T2D animal model. Furthermore, it was also described that these effects were reversed after discontinuation of the electrical stimulus. This work support a potential role for bioelectronic medicines in the treatment of T2D.

Another approach that could be used to modulate the CSN activity is a pharmacological approach. In **chapter V**, it was explored the role of ATP and adenosine on the basal and CSN chemosensory activity evoked by hypoxia. It was shown that the CSN frequency of discharge is overactivated in a prediabetes animal model, being this effect modulated by ATP and adenosine. Since adenosine contributes more than ATP to generate CSN activity in moderate hypoxia, while ATP shows a more preponderant role during intense hypoxia and knowing that intense hypoxias are less prone to occur, it is suggested that the modulation of ATP signaling in the CB could be a therapeutic target to treat T2D.

Finally, in **chapter VI**, it is presented a general and integrated discussion of this PhD thesis. In conclusion, the data present in this work contribute to strengthen that the modulation of CB/CSN activity represents a novel therapeutic approach for T2D.

Resumo

A diabetes tipo 2 é uma das doenças crónicas mais comuns no mundo, cuja prevalência continua a aumentar. Em 2045 estima-se que esta doença afete cerca de 629 milhões de pessoas no mundo. A diabetes tipo 2 é caracterizada pela resistência periférica à insulina, o anormal metabolismo hepático de glucose e a falha progressiva das células beta do pâncreas. Existem vários fármacos disponíveis para o tratamento da diabetes tipo 2 contudo, com o tempo, o controlo da glucose deteriora-se progressivamente e, mesmo com ajustes na medicação, uma proporção considerável de indivíduos continua pouco controlado. Deste modo, é essencial o desenvolvimento de novas estratégicas terapêuticas para o controlo da diabetes tipo 2.

Nos últimos anos, o corpo carotídeo (CB), um quimoreceptor periférico que deteta alterações de O_2, CO_2 e pH no sangue, tem também sido descrito como um sensor metabólico envolvido no controlo da homeostasia energética. De facto, foi descrito que o CB está envolvido na génesis da resistência à insulina e hipertensão induzidos pelas dietas hipercalóricas.

Este trabalho teve como objetivo avaliar o papel do CB no controlo da homeostasia da glucose e investigar uma possível metodologia para modular a atividade do CB com o intuito de encontrar um novo tratamento para a diabetes tipo 2.

O capítulo I introduz conceitos gerais na diabetes tipo 2, tais como, a sinalização da insulina, a homeostasia da glucose e as opções terapêuticas para o tratamento da diabetes tipo 2. Para além disso, conceitos relacionados com a função do CB e o papel do ATP e da adenosina na neurotransmissão do CB, bem como, o seu papel como sensor metabólico serão também abordados.

No capítulo II estão descritos os objetivos gerais e específicos do presente trabalho.

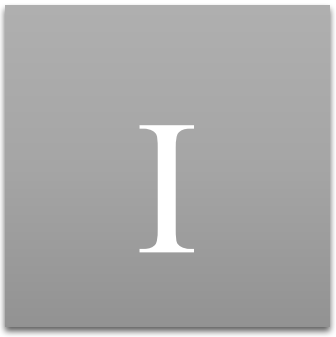
No capítulo III demonstrou-se que a ressecção do nervo do seio carotídeo (CSN), o nervo sensitivo do CB, restaura a sensibilidade à insulina em dois modelos animais de prediabetes, um efeito que se mantém com a continuação da administração das dietas hipercalóricas. Para além disso, foi observado que a ressecção do CSN normaliza a atividade do sistema nervo simpático, a pressão arterial, a função endotelial, o perfil lipídico e os níveis plasmáticos de glucose e insulina. Foi também descrito que o mecanismo pelo qual é restaurada a homeostasia da glucose envolve uma melhoria na

captação de glucose no fígado e no tecido adiposo perientérico bem como, o restauro das vias de sinalização da insulina no músculo esquelético e no tecido adiposo.

No capítulo IV foi descrito que a modulação bioelectrónica do CSN através da aplicação de uma corrente alternada de alta frequência (KHFAC) restaura a sensibilidade à insulina e a tolerância à glucose num modelo animal de diabetes tipo 2. Para além disso, observou-se que estes efeitos são reversíveis após o término do estímulo elétrico de alta frequência. Assim, este trabalho suporta o potencial terapêutico da medicina bioelectrónica na diabetes tipo 2.

Uma abordagem farmacológica poderá também ser utilizada para modular a atividade do CSN. Desta forma, no capítulo V foi estudado o papel do ATP e da adenosina na atividade do CSN no basal e em resposta à hipóxia. Observou-se que a frequência de descarga do CSN está aumentada num modelo animal de prediabetes, sendo este efeito modulado pelo ATP e pela adenosina. Tendo em conta que a adenosina contribui mais do que o ATP para gerar atividade do CSN na hipóxia moderada, enquanto que o ATP tem um papel preponderante durante a hipóxia intensa e, sabendo que hipóxias intensas são menos frequentes em situações fisiológicas, é sugerido que a modulação da sinalização do ATP no CB poderá ser um alvo terapêutico para o tratamento da diabetes tipo 2.

Finalmente, no capítulo VI, é apresentada uma discussão geral e integrada da presente tese de Doutoramento. Em conclusão, os resultados apresentados neste trabalho contribuem para reforçar que a modulação do CB/CSN representa uma nova estratégia terapêutica na diabetes tipo 2.



Introduction

1. Introduction

1.1. Metabolic syndrome

The metabolic syndrome is a complex disorder that is considered a worldwide epidemic, with high socioeconomic impact. It is defined by a cluster of metabolic abnormalities that increase the risk of coronary heart disease, other forms of cardiovascular diseases and type 2 diabetes (T2D) (Kassi *et al.*, 2011). The main risk factors for metabolic syndrome are: obesity, insulin resistance, raised fasting plasma glucose, hypertension and atherogenic dyslipidemia (elevated serum triacylglycerols and apolipoprotein B, increased small low-density lipoprotein (LDL) particles, and a reduced level of high-density lipoprotein (HDL)) (Grundy *et al.*, 2005; Kassi *et al.*, 2011). There are other metabolic abnormalities, such as chronic proinflammatory and prothrombotic states, non-alcoholic fatty liver disease and obstructive sleep apnea, that have been associated with metabolic syndrome (Kassi *et al.*, 2011).

It is estimated that around 20-25% of the adults worldwide have metabolic syndrome (Nolan *et al.*, 2017), a number that continue to increase. However, it is difficult to determine the prevalence of this epidemic disorder due to the multiple definitions that have been suggested for the metabolic syndrome. Moreover, the use of different definitions to identify subjects with metabolic syndrome makes impossible to compare data from different studies (Mancia *et al.*, 2010). Additionally, differences in population age, sex, genetic background, diet and physical activity also influence the prevalence of metabolic syndrome (Pan & Pratt, 2008; Novak *et al.*, 2013; Devers *et al.*, 2016; Krishnadath *et al.*, 2016).

Historically, Reaven (1988) used the term "Syndrome X" (later renamed metabolic syndrome) and suggested that insulin resistance is the common and the key pathological feature of this syndrome, contributing to the development of T2D and cardiovascular diseases. After this definition, several international organizations and expert groups such as the World Health Organization (WHO) (WHO, 1999), the European Group for the study of Insulin Resistance (EGIR) (Balkau & Charles, 1999), the National Cholesterol Education Program Adult Treatment Panel III (NCEP:ATPIII) (Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults, 2001), the American Association of Clinical Endocrinology (AACE) (Bloomgarden, 2003), the International Diabetes Federation (IDF) (Alberti *et al.*, 2006), and the American Heart Association/National Heart, Lung, and Blood Institute

(AHA/NHLBI) (Grundy *et al.*, 2005), have tried to incorporate all the different parameters used to define metabolic syndrome.

The two most widely used definitions for metabolic syndrome are those from the NCEP:ATP III and IDF (Table 1.1), that focus specifically on waist circumference, which is an alternative measure of central obesity. In contrast, the definitions from WHO, EGIR and AACE focused on insulin resistance (Cornier *et al.*, 2008; Kassi *et al.*, 2011).

Table 1.1 - International Diabetes Federation (IDF) definition for metabolic syndrome.

For a person to be defined as having metabolic syndrome they must have: • **Central obesity*** • **Plus any two of the following four factors:**	
Raised triacylglycerols	≥ 150 mg/dl (1.7 mmol/l) or specific treatment for this lipid abnormality
Reduced HDL cholesterol	< 40 mg/dl (1.03 mmol/l) in males < 50 mg/dl (1.29 mmol/l) in females or specific treatment for this lipid abnormality
Raised blood pressure (BP)	Systolic BP ≥ 130 or diastolic BP ≥ 85 mm Hg or treatment of previously diagnosed hypertension
Raised fasting plasma glucose (FPG)	FPG ≥ 100 mg/dl (5.6 mmol/l) or previously diagnosed T2D If above 100 mg/dl or 5.6 mmol/l, OGTT is strongly recommended but is not necessary to define presence of the syndrome.

* Central obesity is defined as waist circumference with ethnicity specific values. If body mass index (BMI) is > 30 kg/m^2, central obesity can be assumed and waist circumference does not need to be measured. In Europe, values for waist circumference are ≥ 94 cm in males and ≥ 80 cm in females. OGTT – Oral Glucose Tolerance Test.

Regarding the problems derived from the multiple definitions suggested for the metabolic syndrome, IDF proposed a new set of criteria with ethnic/racial specific cutoffs for waist circumference. Furthermore, the IDF criteria do not emphasize insulin resistance, focusing on plasma glucose concentrations (Table 1.1) (IDF, 2006a).

1.2. Type 2 Diabetes

T2D is one of the most common chronic diseases in the world, that is increasing in number and significance, as the changes in lifestyles lead to reduced physical activity and increased obesity (Shaw *et al.*, 2010). It is estimated that in 2017, 425 million people had diabetes in the world, a number that continue to increase and in 2045, it is estimated that 629 million people will have diabetes (Fig. 1.1) (IDF, 2017).

Furthermore, approximately 4 million people aged between 20-79 years were estimated to die from diabetes in 2017, being this is equivalent to one death every eight seconds (IDF, 2017).

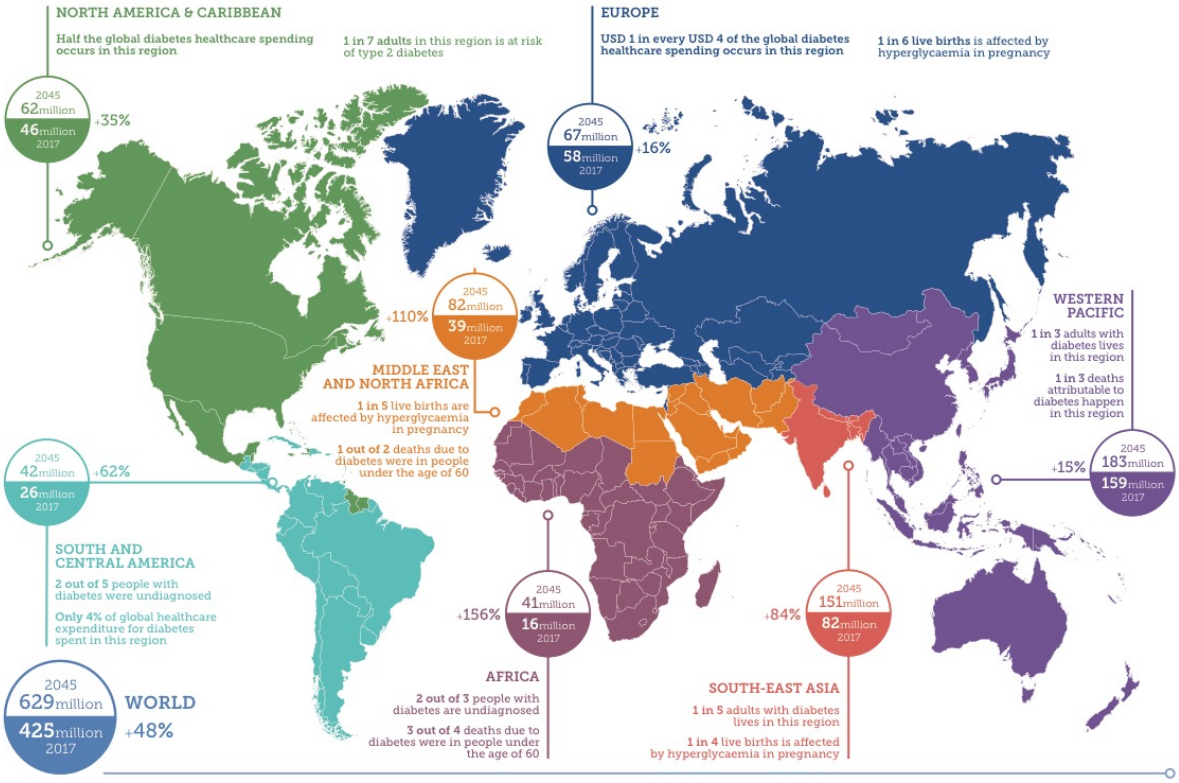


Figure 1.1 - Number of people with diabetes worldwide and per region in 2017 and 2045 (20-79 years) (IDF, 2017).

T2D is the most common type of diabetes, accounting for approximately 90% of all cases of diabetes (Bruno *et al.*, 2005; Holman *et al.*, 2015). In Portugal, if prediabetes is also considered, it is estimated that 34.9% of the population aged 20-79 years have diabetes (Gardete-Correia *et al.*, 2010). Moreover, it was detected a high percentage of people with undiagnosed diabetes (43.6%) (Gardete-Correia *et al.*, 2010).

Prediabetes is typically defined as blood glucose concentrations higher than normal, but bellow the diabetes diagnostic thresholds for impaired glucose tolerance based on a two-hour post 75g OGTT or impaired fasting glucose (Table 1.2) (Tabák *et al.*, 2012). Impaired glucose tolerance is defined as a fasting plasma glucose concentration below 126mg/dl and a glucose concentration of 140-199mg/dl 2h after OGTT and, it is characterized by insulin resistance in muscle and impaired late (second-phase) insulin secretion after a meal. Impaired fasting glucose is defined as a fasting plasma glucose concentration between 110-126mg/dl, without impaired glucose

tolerance and, it is characterized by hepatic insulin resistance and impaired early (first-phase) insulin secretion (Fig. 1.2) (Abdul-Ghani *et al.*, 2006b; IDF, 2006b).

Table 1.2 - Diagnostic reference values for prediabetes and type 2 diabetes.

Parameters	Normal*	Prediabetes	T2D
Haemoglobin A_{1c} (HbA_{1c})	< 5.7% # < 6.0% ϕ	5.7 - 6.4% # 6.0 - 6.4% ϕ	≥ 6.5%
Fasting plasma glucose	< 100 mg/dl # < 110 mg/dl ϕ	100 - 125 mg/dl # 110 - 125 mg/dl ϕ	≥ 126 mg/dl
Two-hour plasma OGTT	< 140 mg/dl	140 -199 mg/dl	≥ 200 mg/dl

*Normal glucose metabolism. #American Diabetes Association. ϕWorld Health Organization. (DeFronzo *et al.*, 2015). OGTT - oral glucose tolerance test; T2D - Type 2 Diabetes.

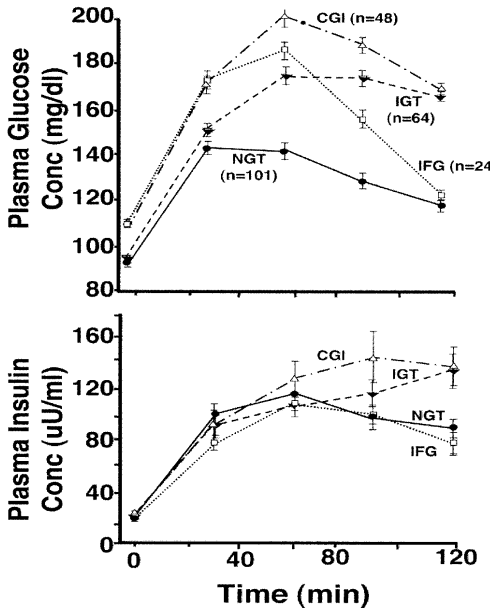


Figure 1.2 - Plasma glucose and insulin concentrations during oral glucose tolerance tests (OGTTs). The tests were performed in subjects with normal glucose tolerance (NGT), impaired glucose tolerance (IGT), impaired fasting glucose (IFG), and combined glucose intolerance (CGI). Subjects with CGI have both IGT and IFG. In a healthy subject, after an induction of hyperglycemia, for example after a meal or an OGTT, a large and rapid insulin secretion occurs that quickly peaks (first phase) and then falls to levels near or above the basal levels for a period of time (second phase). Data represent results from Abdul-Ghani *et al.* (2006a).

People with prediabetes have a high risk of developing T2D. However, not everyone with prediabetes develop T2D. It is estimated that in 2017, 352.1 million people worldwide have impaired glucose tolerance. In 2045, the number of people with age between 20-79 years with impaired glucose tolerance is predicted to increase to 587

million (IDF, 2017). Prediabetes is characterized by decreased insulin sensitivity or insulin resistance and β-cell dysfunction, that start before glucose changes are detectable (Tabák *et al.*, 2012). The risk factors of prediabetes are the same as for T2D: overweight, aging, poor diet and excess of calories, reduced physical activity, smoking and family history (Vazquez *et al.*, 2007; Forouzanfar *et al.*, 2015).

The development of T2D from normal glucose tolerance is a continuous process. The British Whitehall II study (Tabak *et al.*, 2009) observed in individuals who develop T2D higher levels of fasting and post-load glucose and insulin secretion, and lower insulin sensitivity as early as 13 years before the diagnosis of T2D. Moreover, 3-6 years before the diagnosis, it was observed a rapid increase in fasting and post-load glucose values (Tabak *et al.*, 2009). Other studies have also showed this rapid increase in fasting glucose levels before the diagnosis of T2D (Mason *et al.*, 2007; Sattar *et al.*, 2007). Additionally, insulin sensitivity decreased abruptly during the last 5 years before diagnosis and insulin secretion showed a significant compensatory increase 3-4years before diagnosis, and then decreased until diagnosis of T2D (Tabak *et al.*, 2009). These results demonstrated that insulin resistance begins years before the development of T2D and that diminished β-cell function is already present in prediabetes (Abdul-Ghani *et al.*, 2006b). Therefore, T2D is characterized by dysregulation of carbohydrate, lipid and protein metabolism, and results from defects on insulin secretion (β-cell dysfunction) and insulin action (insulin resistance), that originates a decrease in whole-body glucose disposal (DeFronzo *et al.*, 2015). Several factors contribute to β-cell dysfunction: aging, genetic abnormalities in incretin hormones (glucagon-like peptide 1 (GLP1) and gastric inhibitory polypeptide) resistance and/or deficiency, lipotoxicity, glucotoxicity, reactive oxygen stress, hypersecretion of islet amyloid polypeptide and activation of inflammatory pathways (DeFronzo *et al.*, 2015). Additionally, post-mortem analysis of pancreas from T2D patients showed a reduction of 35-39% in β-cell mass compared with tissues from subjects without T2D (Rahier *et al.*, 2008). The loss of β-cells mass in T2D occurs through apoptosis (Butler *et al.*, 2003) and dysregulated autophagy (Masini *et al.*, 2009).

1.2.1. Insulin signalling pathway

In order to exert its action on glucose metabolism, insulin binds to a specific receptor that is a glycoprotein consisting of two α subunits and two β subunits linked by

disulfide bonds. The α subunits are entirely extracellular and contain the insulin-binding domain. The β subunits presented an extracellular, transcellular and an intracellular domain that expresses insulin-stimulated kinase activity (Pessin & Saltiel, 2000).

The first step in the insulin action on glucose metabolism is the phosphorylation of the insulin receptor β subunit, with subsequent activation of insulin receptor tyrosine kinase (Fig. 1.3) (Bajaj & Defronzo, 2003). This activation leads to the phosphorylation of proximal substrates on tyrosine, which includes members of insulin receptor substrate (IRS) family, namely IRS1 and IRS2, Gap-1 and Shc. The phosphorylated IRS1 and IRS2 proteins bind to the phosphatidylinositol 3-kinase (PI3K), which is a heterodimeric enzyme composed by a p85 regulatory subunit and a p110 catalytic subunit (Bajaj & Defronzo, 2003). Once PI3K is activated, it initiates a signaling pathway that activates protein kinase B (PKB/AKT), leading to the translocation of glucose transporter type 4 (GLUT4) vesicles to the plasma membrane (Hajduch *et al.*, 2001) and to the activation of glycogen synthase (Saltiel & Kahn, 2001).

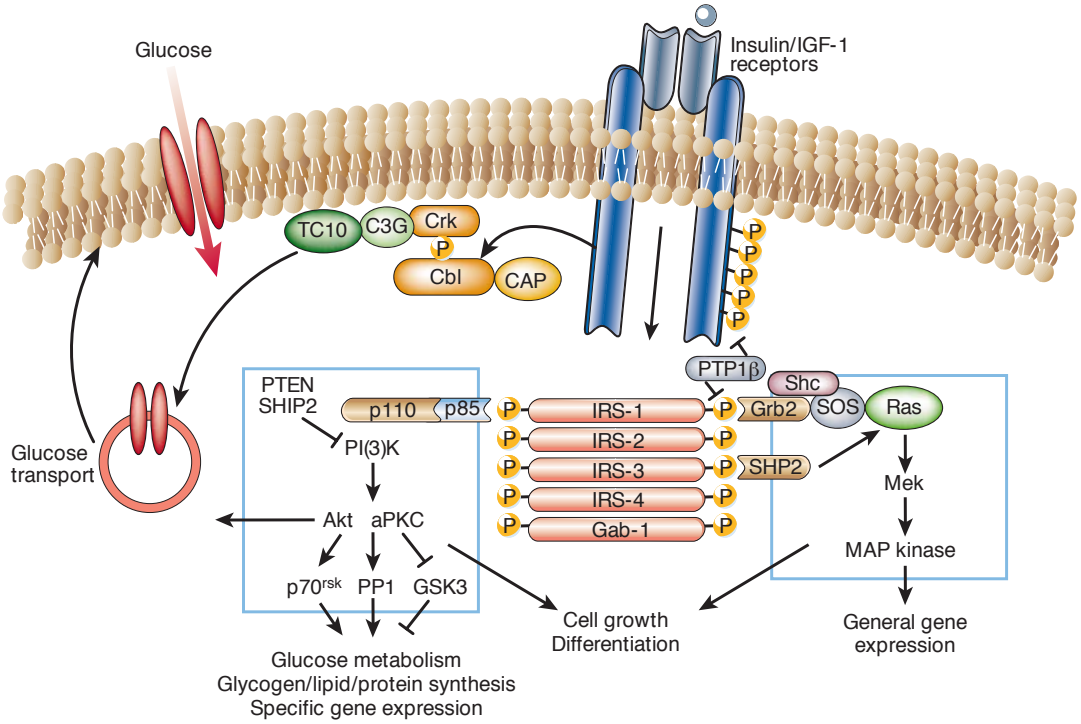


Figure 1.3 - Insulin signalling pathway. Insulin binding to its receptor induces receptor autophosphorylation resulting in the activation of insulin receptor substrates (IRS). This allows the association of IRS with the regulatory subunit (p85) of PI3K, which activates PKB/Akt, leading to the translocation of GLUT4 vesicles to the plasma membrane. Insulin also activates the MAPK signalling pathway (Saltiel & Kahn, 2001).

Insulin also stimulates the mitogen-activated protein kinase (MAPK) signaling pathway through Grb2/Sos and ras, which is involved in gene expression, cell growth and differentiation (Fig. 1.3) (Cusi *et al.*, 2000).

1.2.1.1. Insulin signalling defects in type 2 diabetes

Several problems in the insulin signaling pathway have been detected in T2D, with the most expectable result being a downregulation of insulin receptors due to the generalized hyperinsulinemia. However, the results between studies that aimed to study the insulin binding to the insulin receptor are not consistent. Some studies observed a modest reduction in insulin binding to monocytes and adipocytes from individuals with T2D resulting from the downregulation of insulin receptors (Molina *et al.*, 1989), while others in skeletal muscle and liver have shown that the insulin binding to its receptor was normal in obese and lean diabetic individuals (Caro *et al.*, 1987). In agreement with this last study, Kashiwagi *et al.* (1983)was unable to demonstrate a decrease in insulin receptor number in over half of T2D subjects.

Another possible defect on insulin signaling in T2D is the reduction in insulin-stimulated tyrosine kinase activity (Saltiel & Kahn, 2001). In obese individuals with insulin resistance it was observed that the ability of insulin to activate the insulin receptor and phosphorylate IRS1 in muscle was modestly reduced, an effect that was severely impaired in individuals with T2D (Cusi *et al.*, 2000). Moreover, it has also been observed that the association of the PI3K p85 subunit with IRS1 and the activation of PI3K was significantly reduced in obese and T2D individuals compared with lean healthy controls (Cusi *et al.*, 2000; Krook *et al.*, 2000). In contrast, insulin stimulated-MAPK pathway was intact in individuals with T2D and nondiabetic obese subjects (Cusi *et al.*, 2000).

Glucose transport activity in T2D has also found to be affected, since it was observed a decrease in glucose transport in both adipocytes (Kashiwagi *et al.*, 1983) and muscle (Zierath *et al.*, 1996). In adipocytes, T2D induced a reduction in GLUT4 transporter messenger ribonucleic acid (mRNA) and protein levels and an impairment in GLUT4 translocation to plasma membrane (Kashiwagi *et al.*, 1983). In contrast, in muscle the GLUT4 transporter mRNA expression and protein levels were normal in obese subjects with T2D, however, GLUT4 translocation was impaired in this tissue (Zierath *et al.*, 1996).

1.2.2. Glucose homeostasis

After an overnight fast (10-12h), the majority of total body glucose utilization takes place in insulin-independent tissues. Approximately 50% of all glucose disposal occurs in the brain and 25% of glucose uptake takes place in the splanchnic area (liver plus gastrointestinal tissues) (DeFronzo, 2004). The remaining 25% of glucose disposal occurs in insulin-dependent tissues, mainly the skeletal muscle and with less extend in the adipose tissue. Regarding glucose production, 85% is produced by the liver and the remaining 15% is derived from the kidney, contributing the glycogenolysis and the gluconeogenesis equally to the hepatic glucose production (Magnusson *et al.*, 1992; Gerich *et al.*, 2001).

After glucose ingestion, the rise in plasma glucose concentration induces the insulin release from pancreatic β-cells. The resultant hyperinsulinemia and hyperglycemia suppresses the hepatic glucose production and stimulates glucose uptake by splanchnic (liver and gut) and muscle (DeFronzo *et al.*, 1985; DeFronzo, 2004).

The majority of glucose uptake by peripheral tissues occur in muscle and only a small amount is metabolized in adipose tissue (DeFronzo *et al.*, 1985). There are different glucose transporters with distinctive tissue distributions. In the muscle and adipose tissue, the glucose transporter that is present and responds to insulin is GLUT4. In the liver and pancreatic cells the predominant is GLUT2 (Watson & Pessin, 2001).

Although the adipose tissue is responsible for only a small amount of total body glucose uptake, it plays a very important role in the maintenance of whole body glucose homeostasis (Boden & Shulman, 2002). Insulin is an antilipolytic hormone and therefore, leads to a decreased in plasma free fatty acids levels. To exert its function, insulin binds to insulin receptors in the adipocytes, leading to the activation of IRS and PI3K protein that, in turn, activate the enzyme phosphodiesterase 3A. This enzyme catalyzes the breakdown of cyclic adenosine monophosphate (cAMP) into 5'AMP, which diminished intracellular cAMP levels, leading to the inactivation of protein kinase A and hormone-sensitive lipase (Arner, 2005). Therefore, the hydrolysis of triglycerides to fatty acids and glycerol decrease, leading to inhibition of lipolysis (Arner, 2005). The decrease in plasma free fatty acids levels leads to the increase in muscle glucose uptake and contributes to the inhibition of hepatic glucose production (Bergman, 2000).

Glucagon also plays an important role in the maintenance of glucose homeostasis (Fig. 1.4). After an overnight fast, approximately half of the total hepatic

glucose output is dependent on the maintenance of normal basal glucagon levels (Cherrington, 1999). Whereas, after glucose ingestion, α-cell production of glucagon is inhibited by hyperinsulinemia contributing to the suppression of hepatic glucose production and the maintenance of normal post-prandial glucose tolerance (DeFronzo, 2004).

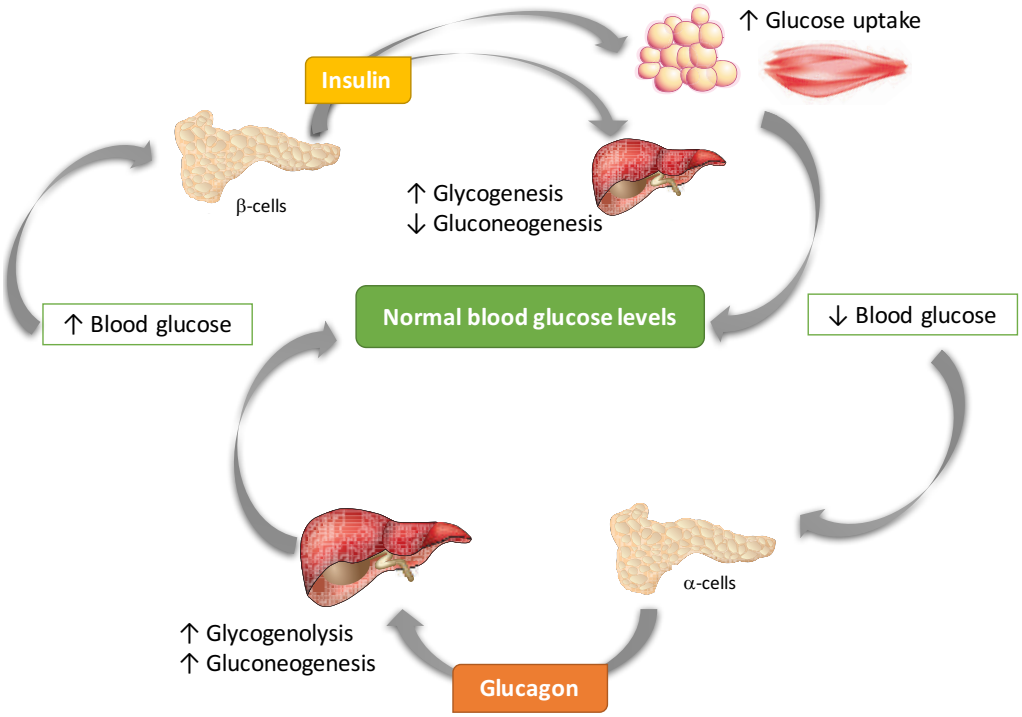


Figure 1.4 - Glucose homeostasis maintained by glucagon and insulin. After an overnight fast, the blood glucose levels are low, promoting the release of glucagon by pancreatic α-cells, which leads to the increase in hepatic glucose production. After a meal, blood glucose levels are high, promoting the release of insulin by pancreatic β-cells, which leads to the decrease in hepatic glucose production and the increase in muscle and adipose tissue glucose uptake.

1.2.2.1. Glucose homeostasis in type 2 diabetes

T2D is characterized by defects on insulin secretion and insulin resistance in the insulin sensitive tissues, such as the muscle, liver and adipose tissue. In subjects with T2D, after a meal, an increase in hepatic glucose production occurs, even in the presence of high levels of plasma insulin, demonstrating an hepatic resistance to the insulin action (Firth *et al.*, 1986). Moreover, after an overnight fast, the increase in whole body glucose production that is observed in T2D is due to an increase in gluconeogenesis (Magnusson *et al.*, 1992).

The muscle is another insulin sensitive tissue that is known to develop insulin resistance in T2D, which contributes to the decreased of glucose uptake. In individuals

with T2D, it has been described through an euglycemic insulin clamp that the skeletal muscle is the first place in which insulin resistance develop (DeFronzo *et al.*, 1985). Together, the decrease in glucose uptake in the muscle with the increase in hepatic glucose production contributes to the hyperglycemia in the postprandial state.

In the adipose tissue, insulin action is also diminished in T2D. Consequently, the ability of insulin to inhibit the lipolysis is compromised, leading to an increase in free fatty acids levels (Bajaj & Defronzo, 2003). The rise in plasma free fatty acids induce insulin resistance in both skeletal muscle and liver (Boden & Chen, 1995) and impaired insulin secretion (Kashyap *et al.*, 2003). Therefore, the increase in plasma free fatty acids levels contribute to the increase in hepatic glucose production (Boden *et al.*, 2001) and to an impaired glucose uptake by the skeletal muscle (Fig. 1.5) (Roden *et al.*, 1996).

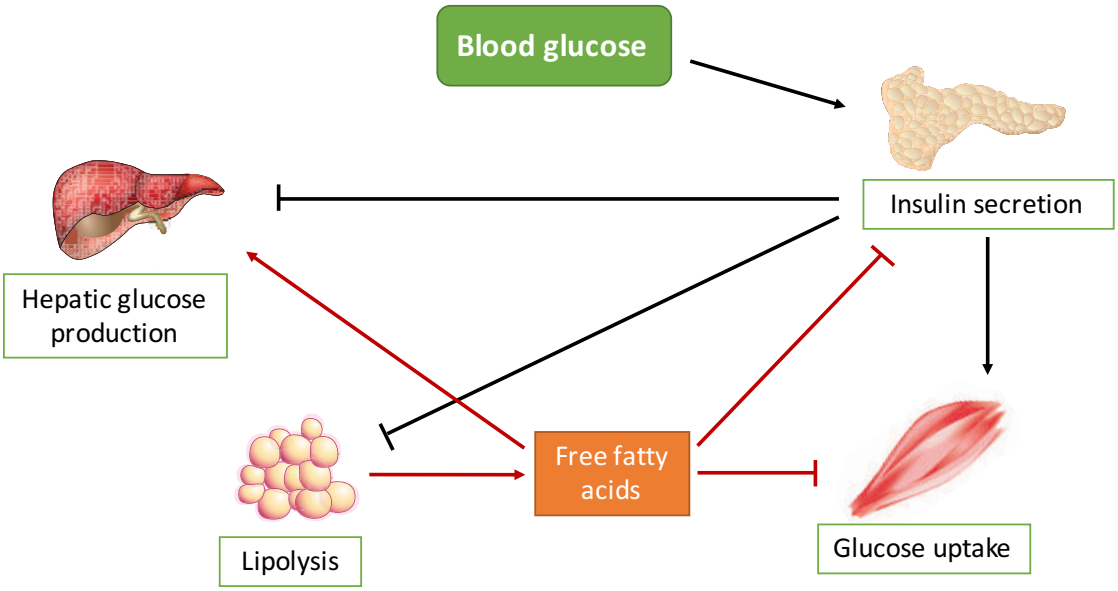


Figure 1.5 - Tissues and processes involved in normal glucose homeostasis and in glucose homeostasis in type 2 diabetes (T2D). The increase in plasma glucose levels stimulates insulin secretion, which leads to the augment in muscle glucose uptake and decreases the hepatic glucose production and the lipolysis in adipose tissue (black arrows). In T2D, the rise in plasma free fatty acids, lead to the increase in hepatic glucose production and to a decrease in insulin secretion and muscle glucose uptake (red arrows).

1.2.3. Type 2 diabetes: role of sympathetic nervous system

Insulin resistance, obesity and hypertension have been associated with an increase in sympathetic nervous system activity (Esler *et al.*, 2001; Tentolouris *et al.*, 2006). The activation of the sympathetic nervous system exerts several physiological responses in target organs, such as, the liver, pancreas, skeletal muscle, adipose tissue, adrenal medulla, arteriole and kidney (Fig. 1.6) (for a review see (Lambert *et al.*,

2010)). This activation is organ specific and is dependent on the adrenergic receptors present in the target organ, the number of neurons recruited and if the individual is in a fasted or postprandial state (Thorp & Schlaich, 2015). In the liver, the acute sympathoexcitation results in the increase in glycogenolysis following a meal and promotes gluconeogenesis in fasting conditions. In the adrenal medulla, sympathetic stimulation promotes the release of catecholamines that also stimulate hepatic glucose production (Thorp & Schlaich, 2015). Activation of the pancreas leads to increased glucagon secretion and to the inhibition of insulin secretion (Lambert *et al.*, 2010).

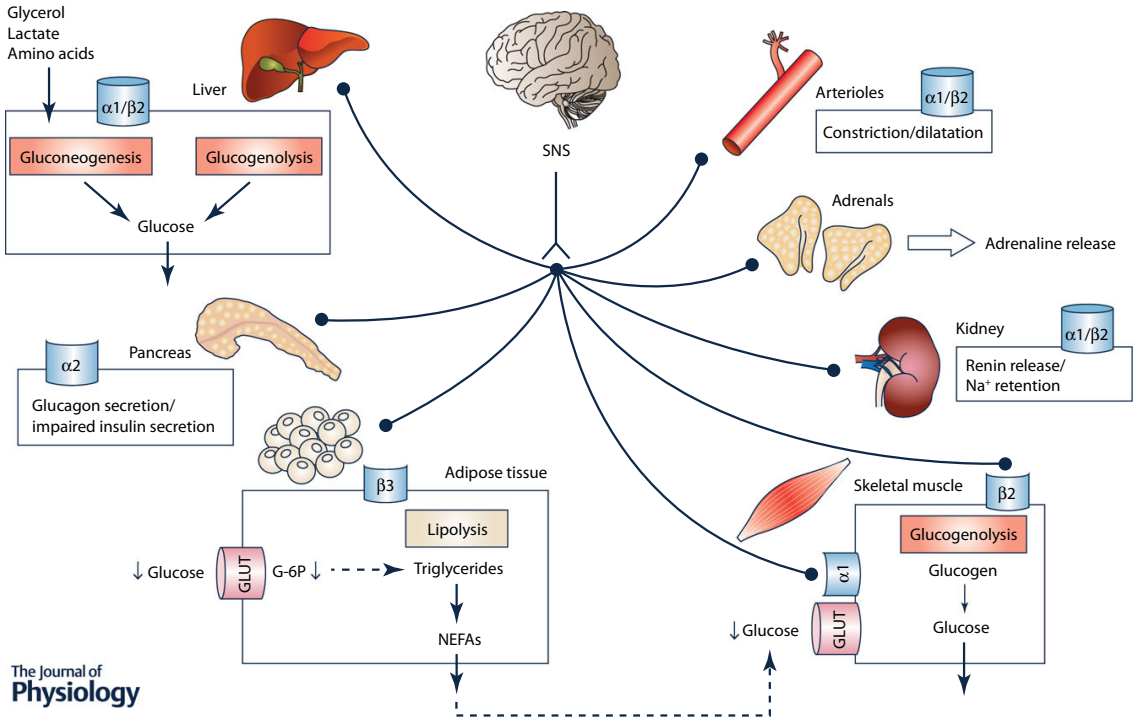


Figure 1.6 - Link between the sympathetic nervous system and the development of metabolic syndrome. The acute activation of the sympathetic nervous system induced the release of norepinephrine and the subsequent stimulation of regionally specific adrenergic receptors. In the liver, gluconeogenesis and glycogenolysis are increase in response to sympathetic overactivity. In pancreas, sympathoexcitation inhibits insulin secretion and promote the release of glucagon. In adipose tissue, it occurs the stimulation of lipolysis which leads to the increase in free fatty acids into to the circulation. In skeletal muscle, glucose uptake is impaired and glycogenolysis is activated. In the kidney, sympathetic activation promotes renin release and sodium (Na^+) retention, that only occurs at higher neuronal firing rates. In adrenal glands, sympathoexcitation leads to the release of adrenaline (epinephrine) into the circulation. (Conde *et al.*, 2017b)

The activation of sympathetic fibers that innervate skeletal muscle modulates glucose uptake. Conversely, the stimulation of α-adrenergic receptors in arterioles, leading to vasoconstriction, impairs glucose uptake in skeletal muscle (Thorp &

Schlaich, 2015). In the adipose tissue, sympathoexcitation leads to lipolysis and to the release of free fatty acids into the bloodstream (Lambert *et al.*, 2010). Activation of the kidney induces the release of renin and, at higher neuronal firing rates, sodium retention and local vasoconstriction also occurs (Thorp & Schlaich, 2015). However, all these mechanisms happen in response to an acute sympathetic nervous system stimulation and when chronic stimulation occurs, it can contribute to the development of insulin resistance, hyperglycaemia, obesity and hypertension, risk factors for metabolic syndrome (Thorp & Schlaich, 2015).

Even with the increase knowledge in this area, the mechanisms linking metabolic diseases and the sympathetic overactivity are not completely understood. The debate if the sympathetic nervous activation is a consequence or a cause of metabolic dysfunction continues, with several hypotheses being postulated. Landsberg (1986) proposed that sympathetic overactivity represents an insulin-mediated adaptive response to overeating, which promotes thermogenesis and acts as a buffer against weight gain. Reaven (1988) suggested that insulin resistance is the primary defect leading to hyperinsulinaemia, hypertension and sympathetic activation. Alternatively, it has been proposed by Grundy (2004) that visceral fat causes metabolic syndrome due to increased release of free fatty acids and adipokines, which cause secondary insulin resistance and sympathetic activation. Another hypothesis postulated is that sympathetic overactivity is the primary defect that leads to insulin resistance and weight gain (Julius *et al.*, 2000). This latter hypothesis is supported by results obtain in prospective trials, demonstrating that elevated norepinephrine levels precedes and predicts the development of insulin resistance, obesity and hypertension (Masuo *et al.*, 1997; Masuo *et al.*, 2003; Flaa *et al.*, 2008).

Insulin resistance states are characterized by increased sympathetic activity in resting/basal state and a diminished sympathetic nervous system responsiveness to physiological sympathetic stimuli. In individuals with insulin resistance associated with obesity, it was observed that the sympathetic nervous system responses to physiological hyperinsulinaemia and glucose consumption are blunted (Grassi *et al.*, 2005). In fact, Straznicky *et al*. (2009) demonstrated that the sympathetic neural response to glucose ingestion is blunted in subjects with insulin resistance, compared with age-matched insulin-sensitive subjects. Moreover, it was also observed that resting muscle sympathetic activity is significantly increased in individuals with T2D, compared with individuals with impaired glucose tolerance (Straznicky *et al.*, 2012). This results

suggest that the progression from prediabetes to T2D is characterized by severe abnormalities in sympathetic nervous system activity.

1.2.4. Therapeutic options for the treatment of type 2 diabetes

T2D is frequently associated with other pathologies such as obesity, hypertension and dyslipidemia. The major objective in the management of patients with T2D is glucose control. The normalization of blood glucose levels reduces the risk of microvascular complications, such as retinopathy, nephropathy and neuropathy (EMA, 2012) as well as the risk of macrovascular complications as coronary artery disease, peripheral arterial disease and stroke.

The first approach recommended by EASD/ADA to control blood glucose levels is the lifestyle intervention that includes weight loss and exercise (Inzucchi *et al.*, 2012). Several clinical trials demonstrated that diet and lifestyle modification contribute to a reduction between 28.5 and 58% in the development of T2D in different ethnic and racial groups (Pan *et al.*, 1997; Tuomilehto *et al.*, 2001; Knowler *et al.*, 2002; Ramachandran *et al.*, 2006; Li *et al.*, 2008). In contrast, other studies showed that despite initial weight loss, several patients regain the lost weight over the subsequent 1-2 years (Dansinger *et al.*, 2007; Purcell *et al.*, 2014). When lifestyle intervention is not enough to normalize blood glucose levels, patients should start a monotherapy with metformin, which is considered the first line agent for T2D treatment (Fig. 1.7) (Inzucchi *et al.*, 2015; Davies *et al.*, 2018).

Metformin is an insulin-sensitizer drug being one of its mechanisms of action the suppression of hepatic glucose production, leading to a decrease in fasting plasma glucose levels (Song, 2016). However, if blood glucose levels or HbA1c levels remain uncontrolled with metformin, a second oral anti-diabetic agent may be added to the therapy. Additionally, when the glucose control remains poor despite the use of three oral antihyperglycemic drugs, insulin therapy is frequently recommended (Fig. 1.7) (Inzucchi *et al.*, 2015). The uncontrolled glucose levels that are observed in these cases could be due to progressive β-cell dysfunction, a characteristic of T2D (Inzucchi *et al.*, 2015; Davies *et al.*, 2018).

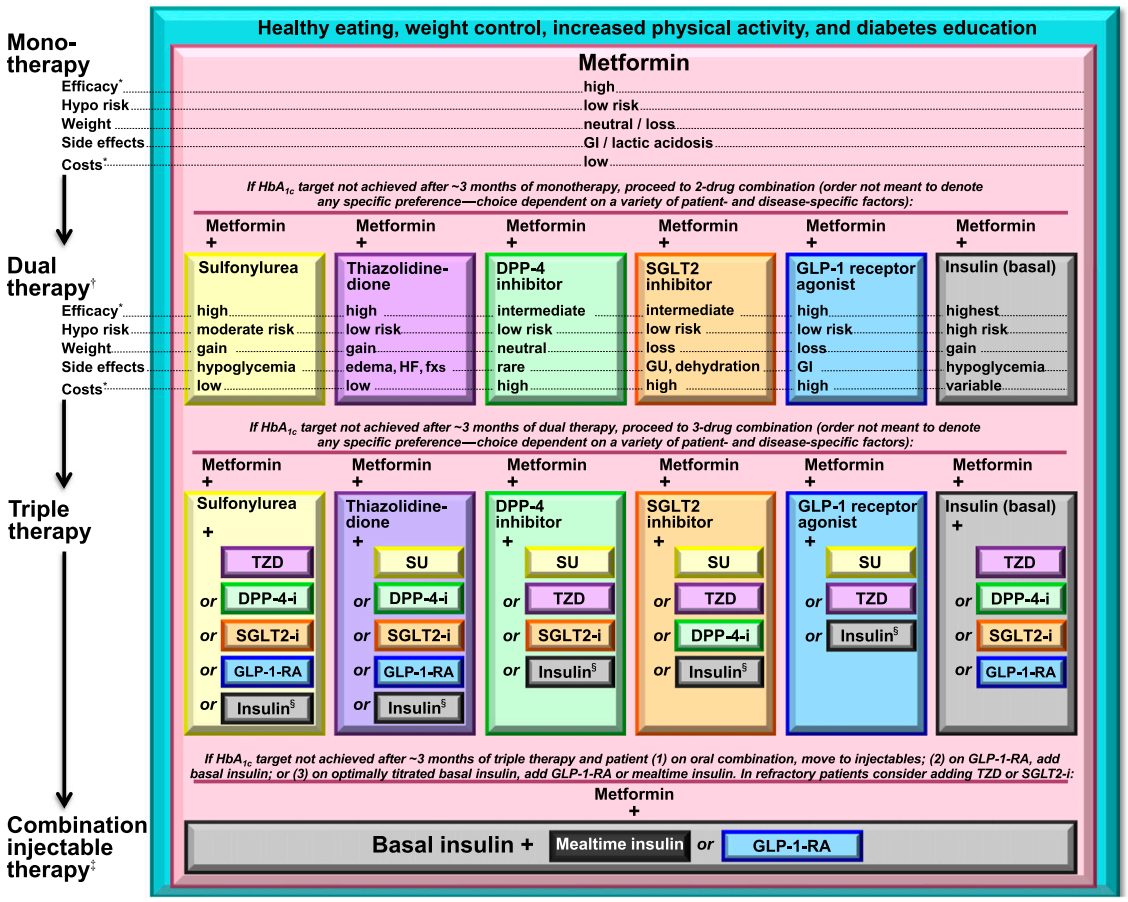


Figure 1.7 - Guidelines approach to drug therapy for the treatment of type 2 diabetes (T2D) recommended by EASD/ADA. Metformin is usually the first therapeutic approach to treat T2D. If the HbA_{1c} target is not achieved after 3 months, it is recommended the use of metformin in combination with: sulfonylurea (SU), thiazolidinediones (TZD), Dipeptidyl peptidase-4 inhibitor (DPP4-i), sodium-glucose co-transporter-2 inhibitor (SGLT2-i), GLP-1 receptor agonist (GLP-1-RA) or basal insulin. fxs, fractures; GI, gastrointestinal; GU, genitourinary; HF, heart failure; Hypo, hypoglycemia. †Consider initial therapy at this stage when HbA1c is ≥9% (≥75 mmol/mol). ‡Consider initial therapy at this stage when blood glucose is ≥300 - 350 mg/dL (≥16.7 - 19.4 mmol/L) and/or HbA1c ≥10 - 12% (≥86 - 108 mmol/mol), especially if patient is symptomatic or if catabolic features (weight loss, ketosis) are present, in which case basal insulin + mealtime insulin is the preferred initial regimen. §Usually a basal insulin. (Inzucchi et al., 2015).

Even with all the therapeutic options available for the treatment of T2D, which show different advantages and disadvantages (Fig. 1.7) and different mechanisms of action (Fig. 1.8), the control of plasma glucose levels deteriorates progressively over time. Therefore, patients have to be continuously evaluated and there is a need for new interventions with oral antihyperglycemic agents every 3-4 years, aiming to obtain a normalization of blood glucose levels (EMA, 2012). Furthermore, despite the combination of therapies and/or insulin treatment that is available, a sizeable proportion of subjects with T2D remain poorly controlled (EMA, 2012), demonstrating the need of

new therapeutic approaches for the treatment of T2D.

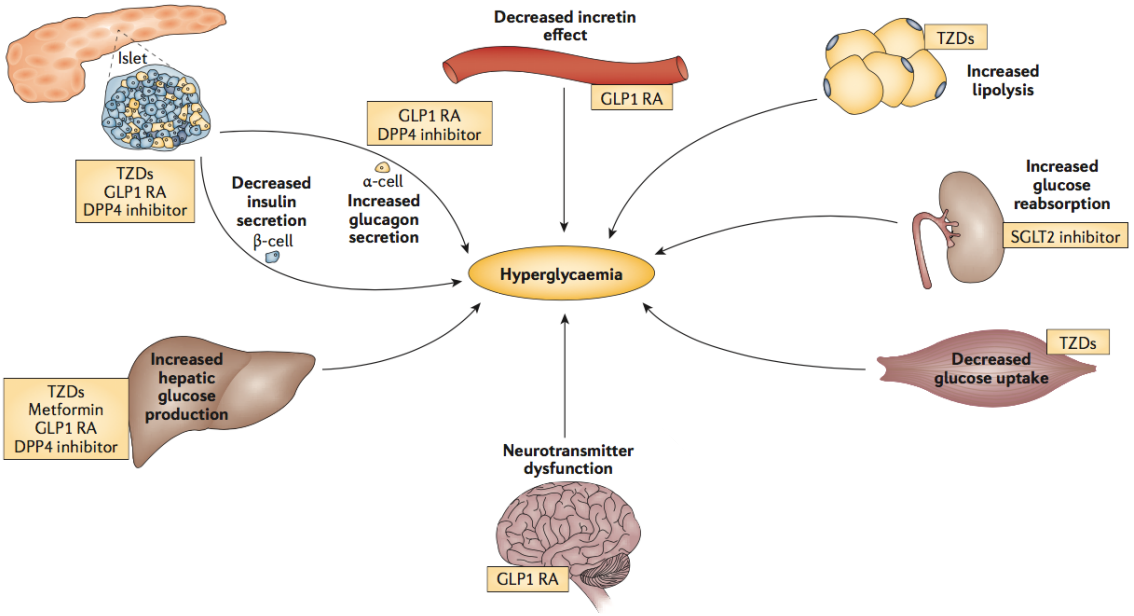


Figure 1.8 - Antihyperglycemic agents that are used for the treatment of type 2 diabetes (T2D) and their mechanism of action. T2D is a complex disorder with multiple pathophysiologic anomalies. Insulin resistance in muscle and liver and β-cell dysfunction represent the core defects of T2D. Moreover, adipocytes (increased lipolysis), gastrointestinal tract (incretin deficiency/resistance), α-cells (hyperglucagonemia), kidney (increased glucose reabsorption) and brain (insulin resistance and neurotransmitter dysregulation) also play important roles in development of hyperglycemia in T2D. DPP4, dipeptidyl peptidase 4; RA, receptor agonist; SGLT2 inhibitor, sodium-glucose co-transporter-2 inhibitor; TZDs, thiazolidinediones. Adapted from Defronzo *et al*. (2015).

1.3. Carotid body

The carotid bodies (CB) are paired chemoreceptor organs, located in the bifurcation of the common carotid artery, involved in the sensing of changes in arterial blood gasses such as hypoxia, hypercapnia, and acidosis (Fig. 1.9). These stimuli activate the CB leading to the release of neurotransmitters that act on the CB sensitive nerve, the carotid sinus nerve (CSN), to generate action potentials or to inhibit its activity (Gonzalez *et al.*, 1994). The action potentials generated postsynaptically are integrated in the brainstem to induce cardiorespiratory responses, to normalize blood gases via hyperventilation (Gonzalez *et al.*, 1994), and to regulate blood pressure and cardiac performance via activation of the sympathetic nervous system (Marshall, 1994).

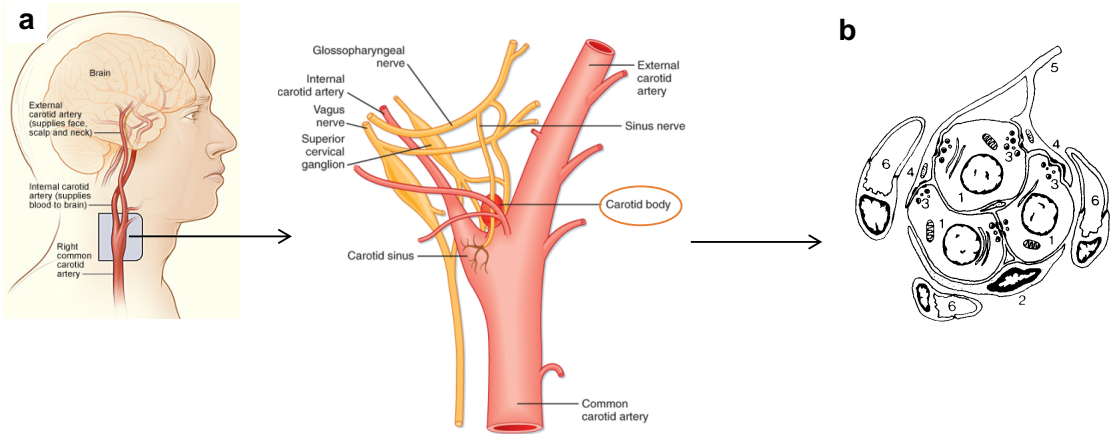


Figure 1.9 - Carotid body (CB) localization and innervation (a) and basic cellular arrangement (b) within the CB. b) The CB is composed by clusters of type I cells (1) that are encapsulated by type II cells (2). Type I cells, exhibit in their cytoplasm a heterogeneous population of synaptic vesicles (3) which are located near the sensory nerve endings (4) of the carotid sinus nerve (5). The clusters are surrounded by a complex net of capillaries (6) (Gonzalez *et al.*, 1992; Koeppen & Stanton, 2008).

The CB is organized into glomeruli, which are clusters of cells in close contact with an abundant network of capillaries and connective tissue (Fig. 1.9). Each glomerulus contains chemoreceptor cells, also known as glomus or type I cells, which are derived of the neural crest and that are synaptically connected with the sensory nerve endings of the CSN (Gonzalez *et al.*, 1994). These cells are enveloped by type II cells or sustentacular cells. It has been proposed that type II cells exhibit properties of stem cells that in response to hypoxia can proliferate and differentiate into new type I cells (Pardal *et al.*, 2007).

Chemoreceptor cells contain several classical neurotransmitters as catecholamines (dopamine and norepinephrine), serotonin, acetylcholine, neuropeptides (substance P and enkephalins), but also contain ATP and adenosine (Gonzalez *et al.*, 1994; Zhang *et al.*, 2000; Rong *et al.*, 2003; Buttigieg & Nurse, 2004; Conde & Monteiro, 2004; Conde *et al.*, 2012a). Moreover, type I cells, express several voltage-ligand-gated ion channels and by using the patch-clamp technique it was shown that these cells contain voltage-dependent Na^+, Ca^{2+} and K^+ currents (Lopez-Barneo *et al.*, 2008).

1.3.1. Chemotransduction mechanisms at the carotid body: oxygen sensing

The classical stimulus for the CB chemoreceptors is hypoxia. However, other physiological stimuli can be also sensed by the CB, such as, hypercapnia/acidosis, hyperthermia, hyperkalemia, hypotension and osmolarity (Kumar & Bin-Jaliah, 2007).

The cellular mechanisms involved in CB response to hypoxia are well characterized, however the molecular mechanisms of oxygen sensing that are translated into the decrease of K^+ conductance that evokes cell depolarization, are still unknown. There are several possible oxygen-sensing mechanisms: 1) the membrane hypothesis that involves a direct inhibition of oxygen-sensitive K^+ channels; 2) the mitochondrial hypothesis that suggests that mitochondria is the site of oxygen sensing in type I cells; 3) the bioenergetic hypothesis that proposed that the oxygen sensor is a change in the energy status of the cell by 5´AMP-activated protein kinase (AMPK) and 4) the biosynthetic hypothesis that suggests that the sensor is an oxygen-dependent enzymatically produced mediator such as reactive oxygen species and carbon monoxide (for a review see (Gonzalez *et al.*, 2010; Kumar & Prabhakar, 2012). Despite the debate regarding the oxygen sensor and the coupling mechanisms, it is generally accepted that the oxygen transduction cascade involves the following steps: 1) decrease in arterial oxygen tension; 2) oxygen sensing by an oxygen sensor; 3) closure of K^+ channels; 4) cell depolarization; 5) activation of voltage dependent Ca^{2+} channels; 6) increase in intracellular Ca^{2+}; 7) release of neurotransmitters; 8) increase in the frequency of action potentials of the CSN that are integrated in the brainstem (Gonzalez *et al.*, 2010) (Fig. 1.10).

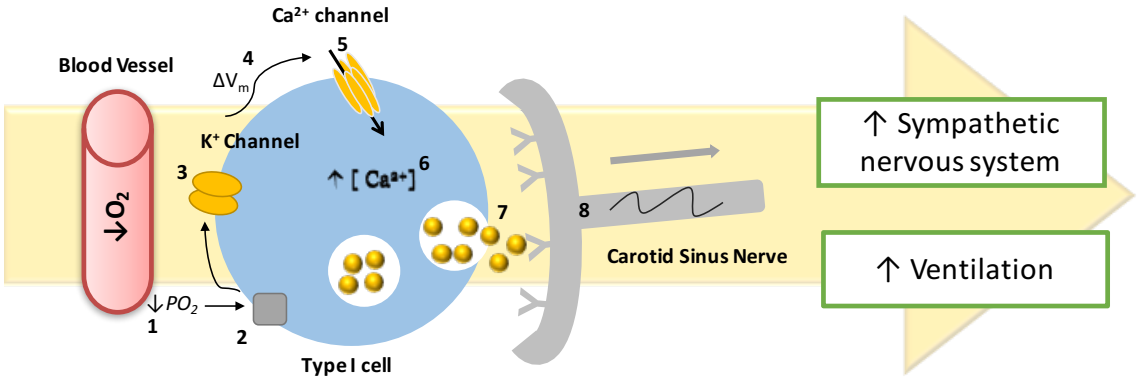


Figure 1.10 - Oxygen sensing by the type I cells of the carotid body. The decrease in blood oxygen (1) is sensed by an oxygen sensor (2) causing the closure of K^+ channels (3) and cell depolarization (4), which induced the opening of Ca^{2+} channels (5). The increase in intracellular Ca^{2+} (6) triggers transmitters release (7) that activate the sensory fibers of the carotid sinus nerve (8). ΔV_m – change in membrane voltage. Adapted from Lopez-Barneo (2003) and Gonzalez *et al.* (1992; 2010).

1.3.2. Chemoreflexes elicited by the carotid body

The CB stimulation by hypoxia or hypercapnia induces ventilatory and cardio-circulatory responses, aiming to normalize blood gases and to reduce the harmful effects of these changes in blood gases in the body (Gonzalez *et al.*, 1994; Marshall, 1994). The role of CB in the ventilatory control has been extensively described. It was observed that hypoxia and hypercapnia, for example, induced an increase in respiratory rate, tidal volume (VT), airway secretions and airway resistance (Fig. 1.11) (Fitzgerald & Shirahata, 1997). The CB is also vital in the ventilatory alterations that are associated with exercise, adaptation to high altitude and pregnancy (Kumar & Prabhakar, 2012). Furthermore, the role of CB in maintaining resting ventilation was also described, since CB denervation induced changes in resting ventilatory parameters, such as a decrease in minute ventilation and an increase in the Partial arterial pressure of CO_2 ($PaCO_2$) (Bisgard *et al.*, 1976; Feustel *et al.*, 1981).

In addition to respiratory reflexes, the CB stimulation can also induce direct cardiovascular responses (Fig. 1.11), that include bradycardia, decreased cardiac output and peripheral vasoconstriction (Marshall, 1994; Kumar & Bin-Jaliah, 2007). However, these effects are often masked by negative feedback responses through baroreceptors and thoracic afferents (Kara *et al.*, 2003).

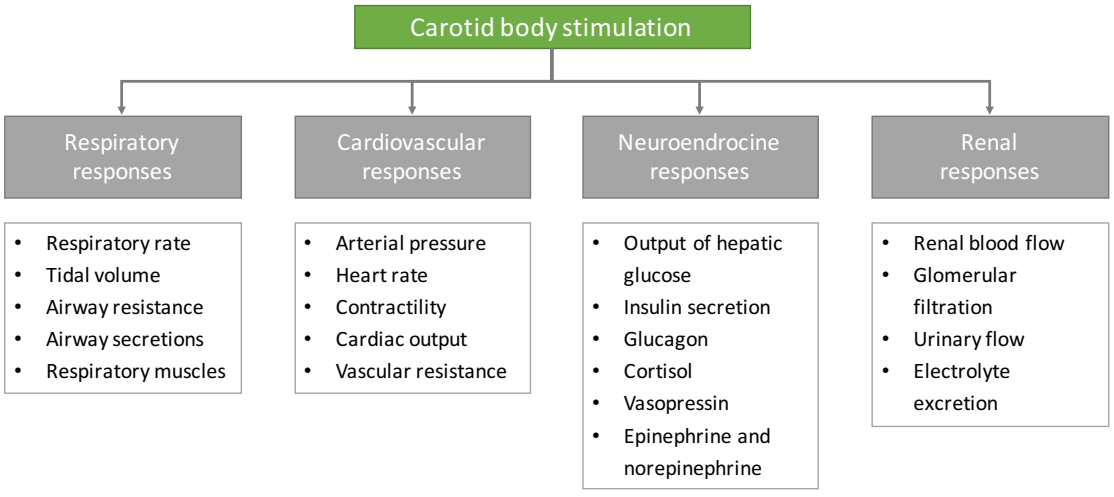


Figure 1.11 - Schematic representation of the chemoreflexes elicited by the carotid body. The stimulation of the carotid body is capable to generate respiratory, cardiovascular, neuroendocrine and renal responses. Adapted from (Fitzgerald, 2000; Conde *et al.*, 2014).

CB stimulation can also promote renal responses (Fig. 1.11). It was observed that CB stimulation results in a decrease in renal blood flow, renal mass, glomerular filtration rate, urine flow, sodium and potassium secretion and osmolar excretion, being

these effects abolished by the CB denervation (Karim *et al.*, 1987; Behm *et al.*, 1993). Moreover, it was also described that the effect of CB stimulation on renal function and haemodynamics is mediated through renal sympathetic nerves (Karim *et al.*, 1987).

In the 60's, Anichkov *et al.* (1960) shown that CB stimulation releases corticotrophin from the anterior lobe of pituitary gland, and consequently corticosteroids from the adrenal cortex. Later, Critchley *et al*. (1982) described that CB stimulation by hypoxia promoted the release of catecholamines from adrenal medulla, being the immediate release of catecholamines in response to hypoxia abolished by adrenal gland denervation.

1.3.3. Neurotransmission in the carotid body: role of adenosine and ATP

Adenosine and adenosine-5'-triphosphate (ATP) act extracellularly to mediate several biological effects via cell-surface receptors, the purine receptors. ATP has a fundamental intracellular role as universal source of energy for all living cells. The demonstration of its release into the extracellular space and the identification and localization of specific receptors on target cells have been essential in establishing its extracellular physiological role.

1.3.3.1. Metabolic pathways of adenosine formation and release

Adenosine is mostly formed by the catabolism of 5'adenosine phosphates (ATP, adenosine diphosphate – ADP and AMP). Intracellular adenosine production is mediated by an intracellular 5'-nucleotidase that dephosphorylates AMP (Schubert *et al.*, 1979; Zimmermann *et al.*, 1998) or by the hydrolysis of *S*-adenosylhomocysteine by *S*-adenosylhomocysteine hydrolase (Broch & Ueland, 1980) (Fig. 1.12). Extracellular adenosine comes from ATP hydrolysis via 5'ectonucleotidases (Fredholm *et al.*, 2001; Yegutkin, 2008) and by its intracellular production and release by nucleoside transport system (Conde *et al.*, 2009). Another source of adenosine that is present extracellularly is cAMP that can be released by secretory cells and converted by extracellular ectophosphodiesterases in AMP and then into adenosine by 5'-ectonucleotidases (Fredholm *et al.*, 2001).

In contrast with other neurotransmitters, adenosine is not stored in synaptic vesicles or acts exclusively on synapses. Its release and uptake occurs through nucleoside transporters, which are constituted by two families: a Na^+ independent

family and another one dependent of the same ion (Griffith & Jarvis, 1996). The Na^+ dependent-nucleoside transport system is concentrative, carrying nucleosides against a concentration gradient. The Na^+ independent-nucleoside transport system (equilibrative nucleoside transport system, ENT) is bi-directional and is formed by two different families (*es* and *ei*), classified based on their sensitivity to nitrobenzylthioinosine (NBTI). The *es* transport is inhibited by low nanomolar concentrations of NBTI, while *ei* transport requires micromolar concentrations to be inhibited (Griffith & Jarvis, 1996; Cass *et al.*, 1998; Podgorska *et al.*, 2005).

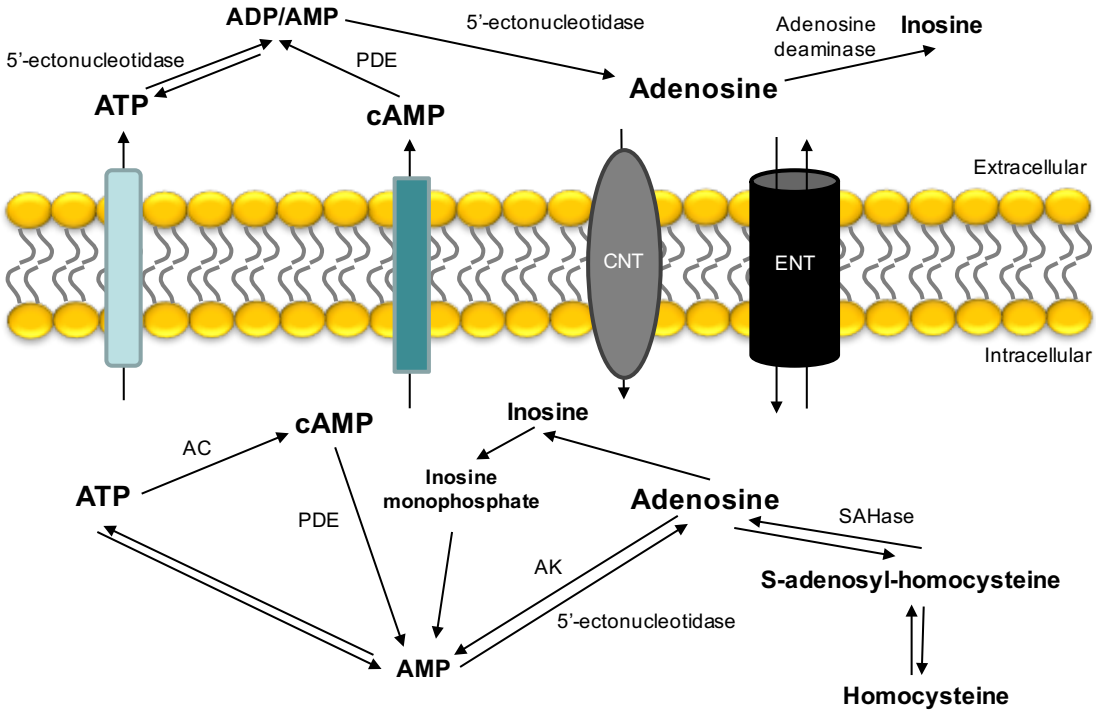


Figure 1.12 - Extra- and intracellular adenosine metabolism and nucleoside transporters that contribute to its release, uptake and production. AC, adenylyl cyclase; AK, adenosine kinase; CNT, concentrative nucleoside transporter; ENT, equilibrative nucleoside transporter; PDE, phosphodiesterase; SAHase, *S*-adenosyl homocysteine hydrolase (Conde *et al.*, 2017a).

The major pathways of adenosine removal or degradation involve reactions catalyzed by two enzymes: adenosine kinase (AK) and adenosine deaminase (Fredholm *et al.*, 1999), which leads to the formation of inosine and AMP, respectively (Conde *et al.*, 2009). Adenosine deaminase is mostly found in the intracellular space, however it is also found in some extracellular compartments. This enzyme has relevance when adenosine concentrations are high (Arch & Newsholme, 1978) and alterations in its activity have been associated with several pathologies, such as *miastenia* gravis and diabetes mellitus (Hoshino *et al.*, 1994; Oliveira *et al.*, 2015).

1.3.3.2. Adenosine receptors in the carotid body

Adenosine exerts is action through four different type of adenosine receptors coupled to G proteins A_1, A_{2A}, A_{2B}, and A_3 (Conde *et al.*, 2009). These receptors are activated by different endogenous adenosine concentrations being the affinity for adenosine: $A_1 > A_{2A} > A_{2B} > A_3$. In order to activate these receptors, the available endogenous adenosine is in equilibrium with the density of adenosine receptors at the site of action, which help to control the different physiological responses of this nucleotide (Conde *et al.*, 2009).

A_1 and A_2 adenosine receptors have been subdivided based on their capacity to inhibit and stimulate adenylyl cyclase and therefore, their ability to decrease and increase the cAMP levels, respectively. In fact, A_1 and A_2 adenosine receptors are G_i and G_S-coupled receptors, respectively. The A_3 adenosine receptors are also coupled to G_i proteins (Fredholm *et al.*, 2001). However, nowadays there are some evidences that adenosine receptors may activate signaling pathways via other G proteins. Apart from the activation of enzymes, the activation of G coupled proteins act on ion channels. It has been shown in hippocampal slices that A_1 adenosine receptors activate N, P, and Q-type Ca^{2+} channels (Wu & Saggau, 1994) and several types of K^+ channels in cultured striatum mouse neurons (Trussell & Jackson, 1985) and also lead to the activation of phospholipase C (Fredholm *et al.*, 2001). The main second messenger involved in the activation of A_{2A} and A_{2B} receptors is cAMP, with the stimulation of these receptors originating an increase in the intracellular levels of this mediator, however, other actions, including mobilization of intracellular calcium, have also been described (for a review see Fredholm *et al*., (2001)).

In the CB the presence of A_1 receptors at the CB is not consensual. Rocher *et al.* (1999) described that A_1 receptors are present in rabbit CB chemoreceptor cells, since A_1 antagonists, DPCPX (10 μM) and 8-cyclopentyl-1,3-dimethylxantine (0.1 μM) prevented the inhibitory action of adenosine on L-type Ca^{2+} currents and on the release of catecholamines. A_1 receptors were also detected in the whole rat CB structure (Bairam *et al.*, 2009). However, other authors described that A_1 receptors are absent in rat CB type I cells (Gauda *et al.*, 2000; Kobayashi *et al.*, 2000) being present in the petrosal ganglion neurons that also express tyrosine hydroxylase (TH) mRNA (Gauda, 2002). The discrepancies described between the existence of A_1 receptors in the CB could be due to different receptor localization in the CB structures and due to the different species studied.

Among the different adenosine receptor subtypes, A_{2A} and A_{2B} receptors were the main receptors localized in the CB type I cells. A_{2A} mRNA expression is developmentally regulated in the CB (Gauda *et al.*, 2000). Immunocytochemical studies have demonstrated the expression of A_{2A} receptors and their colocalization with TH in rat CB type I cells (Gauda *et al.*, 2000; Kobayashi *et al.*, 2000). A_{2B} receptors were also present in rat CB type I cells, as they colocalize with TH (Conde *et al.*, 2006). Moreover, it was demonstrated, through the pharmacological decomposition of the effects of caffeine, a non-selective adenosine receptors antagonist, on the CSN chemosensory activity, that A_{2A} are also present postsynaptically on the CSN (Conde & Monteiro, 2006)).

The expression of A3 adenosine receptors was not detected in CB type I cells (Kobayashi *et al.*, 2000).

1.3.3.3. ATP receptors in the carotid body

ATP exerts its physiological actions via activation of its receptors that have been divided in two families: the P2X ionotropic ligand-gated ion channel receptors and the P2Y metabotropic G-protein-coupled receptors (Abbracchio & Burnstock, 1994; Fredholm *et al.*, 1994). Currently, are described seven subtypes of P2X receptors ($P2X_1$ - $P2X_7$) (Fredholm *et al.*, 1994; Ralevic & Burnstock, 1998; Burnstock, 2018) and eight subtypes of P2Y receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$) (Burnstock & Knight, 2004; Burnstock, 2018).

The P2Y receptors are divided into two subgroups: a) $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ that activate G_q coupled proteins and phospholipase Cβ, leading to the formation of inositol 1,4,5-trisphosphate (IP3) which increases intracellular Ca^{2+}, and diacylglycerol which activates protein kinase C and b) $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ that activate G_i, inhibiting adenylyl cyclase and decreasing intracellular cAMP levels. $P2Y_{11}$ receptor activates both G_q and G_s, which increases both intracellular Ca^{2+} and cAMP (Zimmermann, 2016). The seven P2X receptor subunits assemble to form trimeric homomers and often some combinations of trimeric heteromers (Lewis *et al.*, 1995; Torres *et al.*, 1999) that mediate rapid (within 10 ms) and selective permeability to Na^+, K^+, and Ca^{2+} ions (Khakh & North, 2006), which is in accordance with their role as mediators of ATP action as neurotransmitter or neuromodulator of fast synaptic transmission (Khakh & North, 2012; Boué-Grabot & Pankratov, 2017) in both central

and peripheral nervous systems. These P2X receptors can be located at the presynaptic level (facilitating neurotransmitter release) and at postsynaptic level modulating synapse strength (for a review see North, (2016)). In contrast, P2Y receptors, which involve coupling to G proteins and second-messenger systems present a slower onset of response (less than 100 ms) to ATP (for a review Ralevic and Burnstock, (1998)).

In the CB, the presence of P2 receptors was firstly proposed by McQueen and Ribeiro (1983) in the cat based on experimental data obtained using the ATP analog, the αβ-methylene ATP. Later, the same authors concluded that P2X receptors were present in the rat CB, since P2X agonists activated the carotid chemoreceptor afferents (McQueen *et al.*, 1998). In co-cultures of rat type I cells and petrosal ganglion cells $P2X_2$ receptors were present in the afferent terminals surrounding clusters of type I cells, but not in type I cells themselves, suggesting a postsynaptic localization (Zhang *et al.*, 2000). One year later, a study from the same group showed that $P2X_3$ receptors were also present in chemoafferent CB neurons and that $P2X_2$ and $P2X_3$ colocalize in synaptic terminals opposed to type I cells, forming a heterodimeric receptor (Prasad *et al.*, 2001). In addition, in co-cultures of rat CB and glossopharyngeal neurons it has also been showed that glossopharyngeal neurons expressed at least four different subtypes of P2X receptors ($P2X_2$, $P2X_3$, $P2X_4$, and $P2X_7$) (Campanucci *et al.*, 2006).

Apart from the presence of P2X ATP receptors, P2Y receptors were also described in the CB (Xu *et al.*, 2003; Xu *et al.*, 2005). In rat CB dissociated cells it has been shown that ATP triggers a transient rise in intracellular Ca^{2+} in type II cells, but not in type I cells, and that $P2Y_2$ receptors are localized in type II cells (Xu *et al.*, 2003). Moreover, Xu et al. (2005) described the presence of $P2Y_1$ receptors in the CB since it was observed in CB type I dissociated cells that ATP suppressed the hypoxia-induced intracellular Ca^{2+} rise via the activation of $P2Y_1$ receptors being the order of purinoreceptor agonist potency in inhibiting the hypoxia response in agreement with the involvement of $P2Y_1$ receptors.

1.3.3.4. Adenosine effects on ventilation and on carotid sinus nerve activity

Adenosine increases ventilation in several species, and this effect was attributed to the activation of CB chemoreceptors. In the rat, intracarotid administration of adenosine and its analogs increased in a dose-dependent manner ventilation an effect

abolished after CSN section (Monteiro & Ribeiro, 1987). The effect of adenosine on ventilation was mimicked by its analogues: NECA > CADO > L-PIA, which is compatible with the involvement of A_2 receptors (Monteiro & Ribeiro, 1987). More recently, this involvement was attributed mainly to A_{2A} adenosine receptors, since CGS21680, an A_{2A} selective agonist, increased ventilation in rats by 31% (Conde *et al.*, 2009).

Besides demonstrating the excitatory role of adenosine on ventilation via CB chemoreceptors, in 1981 McQueen and Ribeiro described for the first time that adenosine can stimulate CSN chemosensory activity (McQueen & Ribeiro, 1981). This effect of adenosine on CSN chemosensory activity was mimicked by adenosine analogs and inhibited by theophylline and 8-phenyltheophylline, suggesting the presence and involvement of A_2 receptors (McQueen & Ribeiro, 1983, 1986). Furthermore, McQueen and Ribeiro (1986) also described that intracarotid administration of 8-phenyltheophylline, an adenosine receptor antagonist, in the cat, reduced the CB chemoreceptor response to hypoxia (10% O_2), which could indicate that adenosine released by the CB during hypoxia acts directly on nerve endings or as a modulator in type I cells. In fact, in 2004, Conde and Monteiro demonstrated that the CB releases adenosine in response to hypoxia through the activation of NBTI-sensitive equilibrative nucleoside transporter (Conde & Monteiro, 2004). Moreover, Conde *et al.* in 2006 proved McQueen and Ribeiro contentions by describing that adenosine during moderate hypoxia (5% O_2) acts directly on CSN, on A_{2A} postsynaptic receptors, as SCH58261 decreased by 30% hypoxia-induced CSN activity, but also acts presynaptically as a modulator on A_{2B} receptors present in CB type I cells (Conde *et al.*, 2006). Additionally, in 2012, Conde *et al*. described that adenosine contributes more than ATP to generate CSN activity during moderate hypoxia, an effect that was mediated by A_{2A} and A_{2B} receptors, since ZM241385, in a concentration (300 nM) that block A_2 adenosine receptors, inhibited CSN chemosensory activity with higher efficacy in moderate hypoxia than in intense hypoxia (Conde *et al.*, 2012a). In this work, it was also described that adenosine is preferentially released in response to moderate hypoxia (10% O_2) than in response to higher hypoxic intensities (2% O_2 and 5% O_2), while ATP release from CB has a more pronounced role during high intensity hypoxias (Conde *et al.*, 2012a). Furthermore, it was also shown that during a high-intense hypoxia the main origin of extracellular adenosine is ATP catabolism, whereas in moderate hypoxia the main source of adenosine is its release *per se* by the ENT (Conde *et al.*, 2012a).

1.3.3.5. ATP effects on ventilation and on carotid sinus nerve activity

The first evidence that ATP could affect ventilation was described by Anichkov and Belen'kii, in a work that showed an increase in ventilation when ATP was administrated into the carotid bifurcation of decerebrated cats (Anichkov & Belen'kii, 1963). In the last decades, a lot of literature came out regarding the effects of ATP on CB-mediated ventilation. In 2007, Reyes *et al.* demonstrated in cats that ATP increases ventilation in a dose-dependent manner, an effect that was mediated through P2 receptors since was suppressed by suramin (Reyes *et al.*, 2007a). Additionally, ATP and $P2X_2$ receptors were shown to be involved in the ventilatory responses to hypoxia mediated by the CB, since mice deficient in $P2X_2$ receptors exhibited a prominent diminished ventilatory response to hypoxia (Rong *et al.*, 2003). Moreover, this decrease in the ventilatory response is higher when the partial arterial pressure of oxygen (PaO_2) decreases (Rong *et al.*, 2003). In contrast, mice deficient in $P2X_3$ receptors subunit showed a response to hypoxia comparable with the response of wild-type animals (Rong *et al.*, 2003), suggesting that the $P2X_3$ receptors, that are also present in the CB, do not mediate the ventilatory responses to hypoxia.

The results of ATP effects on ventilation are also consistent with the effects of ATP on CSN chemosensory activity. In the early 1950s, Jarisch and co-authors (1952) described an increase in CSN chemoreceptor discharge following an intracarotid administration of ATP. In the subsequent decades, several were the authors that showed that ATP increases CSN activity in a dose-dependent manner (McQueen & Ribeiro, 1983; Alcayaga *et al.*, 2000; Reyes *et al.*, 2007a; Soto *et al.*, 2010) and that the effect was due to ATP itself and not to its degradation into adenosine (McQueen *et al.*, 1998; Rong *et al.*, 2003; Reyes *et al.*, 2007a). Additionally, P2X receptor agonists induced rapid cardiorespiratory reflexes in anaesthetized rat, suggesting the presence of this receptors in the rat CB (McQueen *et al.*, 1998). These findings were supported by the work by Colin Nurse group, where they showed in a co-culture model of rat type I cell clusters and petrosal neurons that the application of suramin partially inhibited hypoxia-induced postsynaptic responses recorded in petrosal neurons (Zhang *et al.*, 2000) and that both $P2X_2$ and $P2X_3$ receptor subunits were immunolocalized with petrosal afferent terminals in the rat CB (Zhang *et al.*, 2000; Prasad *et al.*, 2001). The importance of $P2X_2$ receptors in the CB-mediated ventilatory responses to hypoxia *in vivo* was also confirmed at CSN level as CSN responses to hypoxia in an *in vitro* CB-petrosal preparation from mice deficient in $P2X_2$ subunits were substantially decreased (Rong *et*

al. (2003). Therefore, is now accepted that ATP is an excitatory neurotransmitter at the synapse between the CB and the CSN and that is involved in the CB response to hypoxia. However, the contribution of ATP for the hypoxic signaling in the CB is dependent on hypoxia intensity, with ATP having a more pronounced role in the response to high intensity hypoxias and adenosine with moderate hypoxias (Conde *et al.*, 2012a), suggesting that the response to hypoxia in the CB are related with alterations in the ATP/adenosine metabolism.

1.3.4. Role of carotid body as a metabolic sensor

1.3.4.1. Is glucose a stimulus for carotid body activation?

In the last decades the CB have been suggested to have metabolic sensing functions, responding to changes in blood glucose and insulin (Prabhakhar & Joyner, 2014). The emerging information on the role of CB as insulin and glucose sensor stands, naturally, on their anatomic location and crucial role as an alarm mechanism to the central nervous system in acute emergency situations that may lead to neuroglycopenia (Conde *et al.*, 2018b).

Several authors dedicated their research efforts to clarify if CB is a glucose sensor and if sense alterations in blood glucose levels, focusing initially on the responses of CB to an acute situation of hypoglycemia. While the initial reports supported a role for CB as low-glucose sensors (Pardal & Lopez-Barneo, 2002), subsequent studies strengthened the hypothesis that CSN activity is not modified by low glucose (for a review see Conde *et al.*, (2014)). Pardal and López-Barneo (2002) described increased sensitivity of chemoreceptor cells to low glucose, as hypoglycemia in CB slices inhibited K^+ currents and increased catecholamine release. Also, Zhang *et al.* (2007) found in cocultures of petrosal ganglions with CB type I cells that low glucose increased afferent action potential frequency in petrosal ganglions. In contrast, Conde *et al*. (2007) showed, using freshly isolated intact rat CB preparations, that the release of catecholamines from type I cells was identical in the presence of physiological (5.55 mM) and low-glucose concentrations (3, 1, and 0 mM). Also, the authors described that both the release of ATP from the CB and the CSN action potential frequency were unaffected by low glucose (Conde *et al.*, 2007). These results support the notion that low glucose is not a direct stimulus for rat CB chemoreceptors (Conde *et al.*, 2007), confirming previous results in the intact CB-CSN preparation

performed by Almaraz *et al.* (1984) and by Bin-Jaliah *et al.* (2004).

More recently, Shirahata *et al.* (2015) also confirmed the lack of effect of hypoglycemia in CSN activity, both in basal conditions and in response to hypoxia. The different low glucose concentrations and partial oxygen pressure (PO_2) values used in the different studies, as well as the type of preparation (intact preparation *vs* cocultures or slices), have been used as putative hypothesis to explain the divergent results. Gallego-Martin *et al.* (2012) found in intact CB cultured during 1 day, but not in freshly isolated organs, that 0 mM of glucose potentiates the release of catecholamines elicited by hypoxia. Moreover, type I cells in culture become transiently more dependent on glycolysis, which suggests that 0 mM of glucose leads the cells to acquire the capacity to increase their hypoxic response (Gallego-Martin *et al.*, 2012) suggesting that type 1 cells may change their phenotype when in culture.

Recently, it was suggested that epinephrine, rather than low glucose, is the stimulus sensed by CB during hypoglycemia being responsible for CB-mediated changes in ventilation and CO_2 sensitivity during hypoglycemia and Thompson *et al*. (2016) observed that the effects on minute ventilation and CO_2 sensitivity induced by hypoglycemia in *Wistar* rats were abolished by preventing epinephrine release or blocking its receptors. It was also demonstrated that physiological levels of epinephrine mimicked the hypoglycemia effect on ventilation and CO_2 sensitivity, providing evidence for a role of this counterregulatory hormone in the ventilatory response to hypoglycemia, as opposed to a direct action of low glucose on the CB (Thompson *et al.*, 2016). Altogether these results do not support a role for the CB at least as a hypoglycemia sensor.

Regarding hyperglycemia, it was observed that 25 mM of glucose did not modified either the basal CSN activity or the CSN chemosensory activity in response to hypoxia (0% O_2) (Conde *et al.*, 2018b). These results suggest that hyperglycemia does not trigger CB activation nor potentiates the response to hypoxia, indicating that hyperglycemia is not one of the key early events involved in CB deregulation that promotes increased sympathetic nervous system activity and insulin resistance.

1.3.4.2. Insulin is a stimulus for CB activation

More than 50 years ago, Pereda *et al.* (1962) observed that insulin administration into the carotid artery of anesthetized dogs induced a higher increase in sympathetic

activity than the systemic administration of insulin. These results suggested for the first time a role for the peripheral nervous system in insulin-mediated increase in sympathetic nervous system activity.

Forty years after the pioneer work by Pereda et al. (1962), Bin-Jaliah *et al*. (2004) in an experimental a work dedicated to evaluate the role of CB in glucose sensing observed that insulin infusion, used to induce hypoglycemia, augmented minute ventilation and the rate of O_2 consumption, an effect that was abolished by CSN denervation. These authors also observed *in vitro* that hypoglycemia did not change the basal CSN chemosensory discharge frequency or increase when glucose was lowered from 10 to 2 mM (Bin-Jaliah *et al.*, 2004), suggesting that these changes are due to modifications in a metabolically derived blood-borne factor rather than glucose *per se*.

CB resection has been shown to blunt the counterregulatory responses to hypoglycemia induced by the hypoglycemic hyperinsulinemic glucose clamp, both in animals (Koyama *et al.*, 2000) and in humans (Wehrwein *et al.*, 2010). Although some of the conclusions obtained in these studies suggested that low-glucose is a potential stimulus for CB, it should be noted however that, in a significant number of *in vivo* animal and human studies, hypoglycemia was generated using high doses of insulin infusions. In fact, in a clinical study designed to determine whether hypo- and hyperglycaemia modulate the ventilatory responses to hypoxia, it was shown that both stimulus obtained under hyperinsulinemic conditions produced an increase in ventilation and in the hypoxic ventilatory response, suggesting that the observed effect on ventilation was due to hyperinsulinemia rather than to altered glucose concentrations (Ward *et al.*, 2007).

The effect of insulin per se on ventilation and on CB activity was later confirmed by Ribeiro *et al*. (2013) in anaesthetized rats, since insulin increased ventilation in a dose-dependent manner during an euglycemic clamp, an effect that it was absent in animals submitted to the CSN resection. Additionally, the authors demonstrated the existence of insulin receptors at the CB and its phosphorylation in response to insulin. These results are in agreement with the work from Gallego-Martin *et al*. (2014) where it was described a higher accumulation of 2-deoxyglucose in whole CB incubated with insulin than in diaphragm muscle. Furthermore, Ribeiro *et al.* (2013) have also showed that insulin, in physiological concentrations, is capable of eliciting a neurosecretory response in type I cells, measured by the increase in intracellular Ca^{2+} concentrations and by the release of dopamine and ATP from whole CB. More recently,

it was showed in humans submitted to hyperinsulinemic-euglycemic clamp, that elevated plasma insulin levels increases minute ventilation independently of alterations in glucose levels (Barbosa *et al.*, 2018).

Taken together, these observations confirm the hypothesis that insulin is a stimulus for CB activation, independently of glucose levels and shift the paradigm of CB stimulus from "low glucose" to "high insulin" as the most important trigger for CB, in terms of glucose homeostasis control (Conde *et al.*, 2018b).

1.3.4.3. Role of carotid body in the pathogenesis of sympathetic mediated diseases

The CB has been implicated in the pathogenesis of chronic pathophysiological conditions such as, essential hypertension, hypertension associated with obstructive sleep apnea, chronic heart failure and T2D (Iturriaga, 2017).

The CB has been proposed to be the link between the reflex increase in sympathetic nervous system activity and the augmented blood pressure associated with obstructive sleep apnea due to chronic intermittent hypoxia (Fletcher *et al.*, 1992; Narkiewicz *et al.*, 1999). Moreover, it was described that chronic intermittent hypoxia, a characteristic feature of obstructive sleep apnea, induce an overactivation of the CB, which is manifested by an increase in the acute hypoxic response (Peng *et al.*, 2003; Rey *et al.*, 2004).

Apart from being involved in the development of hypertension associated with obstructive sleep apnea, a role for the CB in the genesis of essential hypertension has been also proposed. It is well accepted that CB chemoreflex-evoked sympathetic activity responses are increased in human patients and animal models of systemic essential hypertension (Trzebski *et al.*, 1982; Somers *et al.*, 1988; Tan *et al.*, 2010; Abdala *et al.*, 2012; Sinski *et al.*, 2012). The confirmation of role of the CB in the pathogenesis of essential hypertension rise up in the last years, when it was shown that bilateral CB ablation was capable of control the development and maintenance of high blood pressure in spontaneously hypertensive rats and humans (Abdala *et al.*, 2012; McBryde *et al.*, 2013; Narkiewicz *et al.*, 2016). These results are in agreement with the study of Sinski *et al.* (2012), where the authors demonstrate that hyperoxia (100% O_2), which functionally abolishes CB activity, reduced both arterial pressure and sympathetic activity in hypertensive patients.

Aiming to therapeutically target the CB to treat hypertension, without severe adverse effects related with the loss of CB-functions, some authors tested the effect of surgical unilateral CB ablation on hypertensive patients, although without good perspectives. Fudim *et al.* (2015) described in hypertensive patients submitted to unilateral resection of CB due to CB tumors a decreased blood pressure post-surgically. However, over the long term, the effect on pulse pressure and systolic blood pressure were smaller and without statistical significance (Fudim *et al.*, 2015). Also, in a clinical trial (NCT01745172 and NCT01729988, ClinicalTrials.gov) dedicated to evaluate the effect of unilateral CB ablation in essential hypertensive patients a short-term decrease in arterial pressure was described with diminished efficacy 12 months after ablation, suggesting a compensation of the remaining CB (Narkiewicz *et al.*, 2016). These results suggested that other approaches are needed to modulate CB function in cardiovascular diseases. Interestingly, the authors also found that not all hypertensive patients respond homogenously to CB unilateral ablation with a reduction in blood pressure, and characterize a group of responders and non-responders to this procedure (Narkiewicz *et al.*, 2016), suggesting that CB activation may not be an etiological factor in all disease phenotypes.

The CB have also been described to be involved in chronic heart failure in both humans and animal models through an increase in sympathetic nervous system activity (Sun *et al.*, 1999; Ponikowski *et al.*, 2001; Del Rio *et al.*, 2017). Additionally, CB ablation was capable to reduce the hyperventilation and oscillatory breathing, as well as the tonic sympathetic outflow, in both rat and rabbit models of chronic heart failure, leading to the improvement in cardiac function and prolonged survival (Del Rio *et al.*, 2013; Marcus *et al.*, 2014). These results lead to the idea that the modulation of CB activity might be usefull for the treatment of chronic hear failure.

Finally, but not less important, especially in the context of the present work, the CB was shown, more recently, to be involved in the development of insulin resistance. Ribeiro *et al*. in 2013 described for the first time that the CB is a regulator of peripheral insulin sensitivity involved in the genesis of metabolic diseases, since the bilateral CSN resection prevented the development of dysmetabolic changes induced by hypercaloric diets (Ribeiro *et al.*, 2013). Additionally, the authors found that animal models of metabolic dysfunction induced by hypercaloric diets exhibit an overactivation of the CB, demonstrated by an increase in basal ventilation and in ventilatory responses to ischemic hypoxia and by an increase in CB chemoreceptor cell activity as well as by the

enlargement of the CB (Ribeiro *et al.*, 2013). In agreement with this overactivation of the CB in hypercaloric models of metabolic dysfunction, Cramer *et al.* (2014) showed that patients with T2D exhibit CB 20-25% larger than control volunteers. Ribeiro *et al.* (2013) also demonstrated that insulin stimulates the peripheral chemoreceptors in CBs, suggesting that hyperinsulinemia generated by hypercaloric diets might be the key factor initiating CB overactivation, causing an increased adrenergic tone, contributing to the vicious cycle of insulin resistance and glucose intolerance seen in metabolic disorders.

In agreement with the involvement of CBs in the generation of metabolic dysfunction, we have shown that hyperbaric oxygen therapy, that is frequently used to promote wound healing to treat diabetic foot, improves glucose homeostasis in patients with T2D (Vera-Cruz *et al.*, 2015), an effect that can be attributed to the CBs, as hyperoxia dramatically reduces peripheral chemoreceptor activity (Lahiri & DeLaney, 1975).

Altogether, the above-mentioned studies demonstrated that CB dysfunction is implicated in chronic cardiometabolic diseases via modulation of sympathetic nervous system activity and open a new door for the modulation of CB activity as a novel and unexploited therapeutic approach for the treatment of sympathetically mediated diseases.

II

General and specific aims

2. General and specific aims

The CB has been classically described as an organ involved in the cardiorespiratory control, whose dysfunction is associated with the pathogenesis of several sympathetic mediated diseases. However, in the last years, a new role for the CB as a metabolic sensor has emerged being also described that this organ is a peripheral insulin regulator involved in the development of insulin resistance and glucose intolerance and whose activity is increased in animal models of metabolic disorders.

The **general aim** of the present thesis was to investigate if the modulation of CB activity might be a suitable therapeutic to treat metabolic diseases. The mechanisms by which the CB is involved in the deregulation of glucose homeostasis were also investigated.

The **specific aims** of the present work are:

1. To evaluate if the CB functional abolition through the CSN resection reverse pre-established insulin resistance, dyslipidaemia, autonomic dysfunction and hypertension in animal models of metabolic syndrome. Moreover, the effect of CSN resection on insulin signalling pathways and tissue-specific glucose uptake was evaluated in skeletal muscle, adipose tissue and liver.

2. To investigate if the application of kilohertz frequency alternating current (KHFAC) is capable of modulate the CSN neural signals in a diet-induced early stage T2D animal model.

3. To investigate the role of adenosine and ATP, key mediators in the CB, in the CB/CSN overactivity induced by high-fat diet and therefore, the potential therapeutic modulation of CB activity in metabolic diseases through the antagonism of adenosine/ATP actions.

III

This chapter is based on the following manuscript:

Functional abolishment of carotid body activity restores insulin action and glucose homeostasis: key roles for visceral adipose tissue and the liver

Sacramento JF, Ribeiro MJ, Rodrigues T, Olea E, Melo BF, Guarino MP, Fonseca-Pinto R, Ferreira CR, Coelho J, Obeso A, Seiça R, Matafome P, Conde SV. (2017) *Diabetologia*, 60(1):158-168.

3. Functional abolishment of carotid body activity restores insulin action and glucose homeostasis: key roles for visceral adipose tissue and the liver

3.1. Abstract

Aims/hypothesis: We recently described that carotid body (CB) overactivation is involved in the aetiology of insulin resistance and arterial hypertension in animal models of the metabolic syndrome. Additionally, we have demonstrated that CB activity is increased in animal models of insulin resistance, and that carotid sinus nerve (CSN) resection prevents the development of insulin resistance and arterial hypertension induced by high-energy diets. Here, we tested whether the functional abolition of CB by CSN transection would reverse pre-established insulin resistance, dyslipidaemia, obesity, autonomic dysfunction and hypertension in animal models of the metabolic syndrome. The effect of CSN resection on insulin signalling pathways and tissue-specific glucose uptake was evaluated in skeletal muscle, adipose tissue and liver.

Methods: Experiments were performed in male Wistar rats submitted to two high-energy diets: a high-fat diet, representing a model of insulin resistance, hypertension and obesity, and a high-sucrose diet, representing a lean model of insulin resistance and hypertension. Half of each group was submitted to chronic bilateral resection of the CSN. Age-matched control rats were also used.

Results: CSN resection normalised systemic sympathetic nervous system activity and reversed weight gain induced by high-energy diets. It also normalised plasma glucose and insulin levels, insulin sensitivity lipid profile, arterial pressure and endothelial function by improving glucose uptake by the liver and perienteric adipose tissue.

Conclusions/interpretation: We concluded that functional abolition of CB activity restores insulin sensitivity and glucose homeostasis by positively affecting insulin signalling pathways in visceral adipose tissue and liver.

3.2. Introduction

Carotid bodies (CBs) are peripheral chemoreceptors that classically sense hypoxia, hypercapnia and acidosis, responding via increased action potential frequency in their sensory nerve, the carotid sinus nerve (CSN). CSN activity is integrated in the

brainstem to induce respiratory responses, aimed primarily at normalising blood gases via hyperventilation (Gonzalez *et al.*, 1994), and regulating blood pressure and cardiac performance via sympathetic nervous system activation (Marshall, 1994). In the last decade, CBs have been described as a major metabolic sensor, implicated in energy homeostasis (Koyama *et al.*, 2000; Wehrwein *et al.*, 2010). We reported that animal models of the metabolic syndrome displayed abnormal patterns of CB activity (Ribeiro *et al.*, 2013), associated with hyperinsulinaemia. We demonstrated that insulin stimulates the peripheral chemoreceptors in CBs, crucial for triggering CB-induced sympathoadrenal overactivity, contributing to the vicious cycle of insulin resistance and hypertension seen in metabolic disorders (Ribeiro *et al.*, 2013). The involvement of CBs in the control of metabolic homeostasis is also supported by clinical data: T2D patients who underwent functional suppression of CB activity (via hyperbaric oxygen therapy) showed improved glucose tolerance and significantly reduced plasma glucose levels (Desola *et al.*, 1998; Ekanayake & Doolette, 2001; Karadurmus *et al.*, 2010; Wilkinson *et al.*, 2012; Vera-Cruz *et al.*, 2015). It has also been reported that oxygen therapy significantly decreases systolic blood pressure in both type 2 diabetic and hypertensive patients (Peleg *et al.*, 2013) and improves insulin sensitivity in diabetic patients (Ekanayake & Doolette, 2001), without changing insulin levels (Desola *et al.*, 1998). CBs have recently been considered a novel target for regulating peripheral insulin sensitivity.

We tested whether abolition of CB activity would, with sustainable efficacy, reverse pre-established insulin resistance, glucose intolerance, dyslipidaemia and hypertension in animal models of the metabolic syndrome. We also aimed to clarify the mechanisms of action underlying this effect. We hypothesised that CSN transection would improve peripheral insulin action via modification of insulin signalling pathways and tissue-specific glucose uptake in classical insulin-target tissues such as skeletal muscle, adipose tissue and liver. Since the sympathetic nervous system is the natural effector of CB activation, the reversal potential of CSN denervation on pre-existing sympathetic overactivation was evaluated in animal models of diet-induced metabolic disease.

3.3. Methods

3.3.1. Animals and experimental procedure

3.3.1.1. Diets and animal care

Experiments were performed in 3-month-old male Wistar rats (200-420g) obtained from the vivarium of the Faculdade de Ciências Médicas, Universidade Nova de Lisboa, Lisboa, Portugal. After randomisation, the animals were assigned to one of three groups: (1) the high-sucrose diet-fed (HSu) group, fed 35% (wt/vol.) sucrose (PanReac, Madrid, Spain) in drinking water for 28 days representing a lean model of combined insulin resistance and hypertension (Ribeiro *et al.*, 2005; Ribeiro *et al.*, 2013); (2) the high-fat diet-fed (HF) group, fed a 45% fat diet (45% fat + 35% carbohydrate + 20% protein; Mucedola, Milan, Italy) for 21 days. This model combined obesity, dyslipidaemia, insulin resistance and hypertension (Shearer *et al.*, 2009; Panchal & Brown, 2011; Conde *et al.*, 2012b); and (3) an age-matched control group, fed a regular chow diet (7.4% fat + 75% carbohydrate (4% sugar) + 17% protein; SDS diets RM1, Probiológica, Lisboa, Portugal). HSu rats were fed for 28 days because the animals were neither insulin resistant nor hypertensive at 21 days (data not shown). Animals were kept under temperature and humidity control (21 ± 1°C; 55 ± 10% humidity) with a 12 h light/12 h dark cycle and were given ad libitum access to food and water. Body weight was monitored two times per week, and energy and liquid intake were monitored daily. Laboratory care was 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 Faculdade de Ciências Médicas Ethics Committee. In subsequent experiments, experimenters were blinded to group assignment and outcome assessment.

3.3.1.2. CSN transection protocol

After evaluation of insulin sensitivity using an insulin tolerance test (ITT) on day 21 for the HF group and day 28 for the HSu group (Ribeiro *et al.*, 2005; Ribeiro *et al.*, 2013), animals were submitted to bilateral CSN transection under ketamine (30 mg/kg)/xylazine (4 mg/kg) anaesthesia and buprenorphine (10 μg/kg) analgesia (Ribeiro *et al.*, 2013). The controls for each of the feeding groups were submitted to a sham procedure (n = 8/10 per group).

3.3.1.3. Unilateral CSN transection

A group of HF rats (n = 8) was submitted to unilateral CSN transection to evaluate whether this would sufficiently improve insulin sensitivity.

3.3.1.4. In vivo analyses

After surgery, animals continued their respective high-fat and high-sugar diets during both the recovery period and the remaining experimental period (3 weeks) (Fig. 3.1). Fasting glucose, insulin sensitivity and body weight were evaluated weekly during this time.

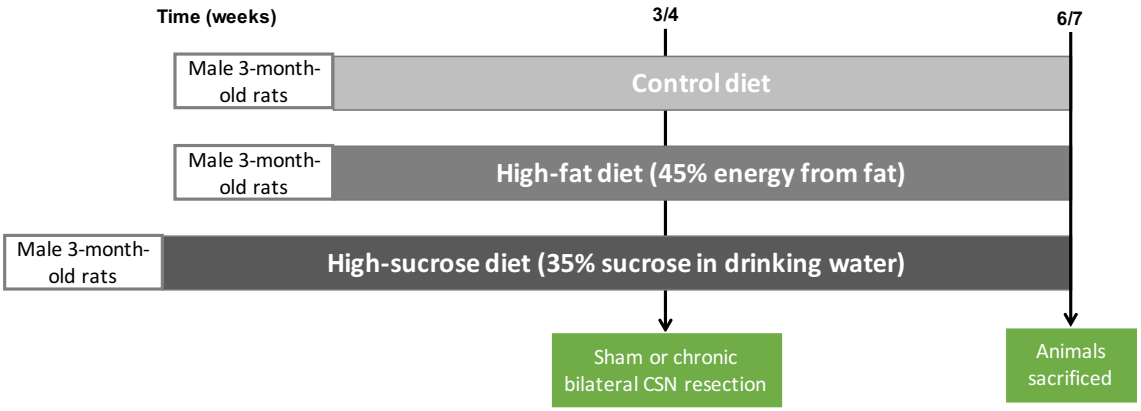


Figure 3.1 - Representation of the carotid sinus nerve (CSN) resection protocol. The animals were submitted to control (3 weeks), high-fat (3 weeks) or high-sucrose (4 weeks) diet before the sham or chronic bilateral CSN resection. After the surgery, the animals continued their respective diets during 3 weeks.

3.3.1.5. Sustained effects of CSN transection

A group of sucrose-fed rats (n = 8) was monitored for 11 weeks after denervation to assess whether the recovery of metabolic variables would be sustained for >3 weeks.

3.3.2. Insulin tolerance test

Insulin sensitivity was evaluated using an ITT (Ribeiro *et al.*, 2013) after overnight fasting. The ITT consists in the administration of an intravenous insulin bolus of 0.1 U/kg body weight in the tail vein, after an overnight fast, followed by the measurement of the decline in plasma glucose concentration over 15 min. The constant rate for glucose disappearance (K_{ITT}) was calculated using the formula $0.693/t_{1/2}$ (Ribeiro *et al.*, 2013). 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. Blood samples were collected by tail tipping and glucose levels were measured with a

glucometer (Precision Xtra Meter, Abbott Diabetes Care, Portugal) and test strips (Abbott Diabetes Care, Lisboa, Portugal).

3.3.3. Quantification of serum biomarkers

Three weeks post-CSN transection, the animals were anaesthetised with pentobarbital (60 mg/kg i.p.), and CSN resection was confirmed by the absence of hypoxia-induced hyperventilation during occlusion of the common carotid artery (Monteiro & Ribeiro, 1989; Ribeiro *et al.*, 2013). Measurement of mean arterial pressure and blood concentrations of insulin, C-peptide, lipids, nitric oxide and catecholamines was performed as previously described (Guarino *et al.*, 2013; Ribeiro *et al.*, 2013; Sacramento *et al.*, 2016). Insulin and C-peptide were measured by ELISA kits (Mercodia AB, Uppsala, Sweden).

To evaluate endothelial function and inflammation, plasma NO/NO_3^- levels were determined at the end of each experiment, in all animals (Guarino *et al.*, 2013; Ribeiro *et al.*, 2013; Sacramento *et al.*, 2016). Blood was collected by cardiac puncture, and immediately centrifuged at 12000g, for 5 min, to obtain plasma samples. Proteins were precipitated from the samples by adding two volumes of ethanol (0°C). After 30 min on ice, samples were centrifuged in a microfuge (Eppendorf, Madrid, Spain) at 12000g for 10 min. NO/NO_3^- concentration was determined by using a specific and sensitive NO/ozone chemiluminescence technique (NO-Analyzer 280, Sievers Research Inc., Boulder Colorado, USA).

Lipid profile was determined using a RANDOX kit (RANDOX, Porto, Portugal) to determine total cholesterol and triacylglycerols by Trinder-based colorimetric end-point assays, and HDL and LDL by a direct-HDL and direct-LDL clearance method, respectively. Catecholamines in serum and in the adrenal medulla have been quantified by HPLC with electrochemical detection as previously described by Ribeiro et al. (2013).

3.3.4. *In vivo* tissue-specific glucose uptake

The effect of CSN resection on glucose uptake by the liver, skeletal muscle, pancreas and adipose tissue was evaluated in a group of HF rats after an intravenous glucose tolerance test (IVGTT) with 2-deoxy-D-[1,2-3H]- glucose (2-deoxy[3H]glucose), based on the method by Kraegen *et al.* (1985). For that, the animals

were fasted overnight and a bolus of 2-deoxy-D-[1,2-^{3}H]-glucose (1 mC/ml; specific activity: 20 Ci/mmol; PerkinElmer, Madrid, Spain) mixed with glucose (100 μCi/kg body weight; 0.5 g/kg body weight) was administered in the tail vein. Blood samples were taken from the tail vein at regular intervals (0, 2, 5, 10, 15, 30 and 60 min). To determine glucose-specific activity, 20μl plasma was deproteinized with 200μl ice-cold perchloric acid (0.4N), centrifuged and radioactivity was measured in a scintillation counter (Tri-Carb 2800TR, Perkin-Elmer, Madrid, Spain). At 60 min, animals were euthanized and the tissues (liver, pancreas, soleus, gastrocnemius, perienteric adipose tissue, epididymal adipose tissue and perinephric adipose tissue) were rapidly excised, frozen into liquid nitrogen, and stored at -80ºC. [^{3}H]2-deoxy-D-glucose incorporation was further investigated in 50-200mg tissue homogenized in 1ml ice-cold perchloric acid (0.4N). The samples were centrifuged and the radioactivity in the supernatant was the measured in a scintillation counter.

Tissue glucose uptake (defined as the glucose metabolic index, Rg′) was calculated using the following equation described by Kraegen *et al.* (1985):

$$Rg' = (CpCm*)/(\int Cp*(t)dt)$$

Cp is the plasma glucose concentration at steady state over the 60-minute period (mmol/l); Cm* is tissue accumulation of 2-deoxy-D-[1,2-^{3}H]-glucose per unit mass at 60 minutes (dpm/mg tissue); Cp* is the 2-deoxy-D-[1,2-^{3}H]-glucose concentration (dpm/ml); and t equals 0 when the tracer is administrated as a bolus. *Rg'* is expressed in mmol.mg tissue.min^{-1}.

3.3.5. Western blots

Skeletal muscle (50mg), liver (50mg) and adipose tissue (100mg) were homogenized in Zurich (10mM Tris-HCl, 1mM EDTA, 150mM NaCl, 1% Triton X-100, 1% sodium cholate, 1% SDS) with a cocktail of protease inhibitors (trypsin, pepstatin, leupeptin, aprotinin, sodium orthovanadate and PMSF). After, samples were centrifuged (Eppendorf, Madrid, Spain) at 13000g for 20 min and the supernatant was collected and frozen at -80ºC until further use. The evaluation of GLUT4, GLUT2, insulin receptor, insulin receptor phosphorylated, Akt and Akt phophorylated were performed according to Guarino *et al.* (2013) and Matafome *et al.* (2014).

Briefly, after blocking, the membranes were incubated overnight at 4°C with the primary antibodies against GLUT4 (1:200), GLUT2 (1:200), insulin receptor (1:200),

insulin receptor phosphorylated (phospho-Tyr1361, 1:500), Akt (1:1000) and Akt phophorylated (phospho-Ser473, 1:1000). The membranes were washed with Tris-buffered saline with Tween (TBST) (0.1%) and incubated with donkey anti-goat (1:2000) or goat anti-mouse (1:2000) or goat anti-rabbit (1:5000) in TBS for 2h at room temperature and developed with enhanced chemiluminescence reagents according to the manufacturer's instructions (ClarityTM Western ECL substrate, BioRad, United States). Intensity of the signals was detected in a Chemidoc Molecular Imager (Chemidoc; BioRad, Madrid, Spain) and quantified using the Quantity-One software (BioRad). The membranes were re-probed and tested for Calnexin, α-Tubulin, β-Actin or GAPDH immunoreactivity (bands in the 85, 55, 42 and 37 kDa region, respectively) to compare and normalize the expression of proteins with the amount of protein loaded. Different loading proteins were used in accordance with the molecular weight of the protein to be studied or with the tissue in where protein expression was analyzed.

3.3.6. Sympathetic activity analysis

The balance between sympathetic and parasympathetic components of the autonomic nervous system was evaluated by power spectral analysis of heart rate variability (Herrington, 1940; Ori *et al.*, 1992; Thireau *et al.*, 2008; Fonseca-Pinto, 2011), and by measurement of circulating and adrenal medullary catecholamines (Thireau *et al.*, 2008; Conde *et al.*, 2012b).

Heart rate (HR) was derived from the mean arterial pressure (MAP) curve obtained by HSE-Harvard Pulmodyn W software, with acquisition frequency of 500Hz. The femoral artery was cannulated under a dissection microscope and the animals were transferred to a heating pad to maintain body temperature at 37.5 ± 0.5 °C, thus avoiding cold stress sympathetic activation (Herrington, 1940). The catheter was connected to a pressure transducer and amplifier to acquire MAP (model 603, HSE-HA GmgH, Harvard Apparatus, Madrid, Spain). The tachogram containing the RR signal was obtained after the identification of the peak of MAP in each cardiac cycle. Subsequently the RR plot was interpolated at 10 Hz (a frequency suitable to catch all oscillations from heart rhythm in rats) using cubic splines. The algorithm used to obtain spectral non-parametric HRV indices was created in Matlab Software (MATLAB version 7.10.0. Natick, Massachusetts, USA: The MathWorks Inc., 2010), using a Fast Fourier Transform approach (Thireau *et al.*, 2008), by Welch spectral estimation

considering a 256 point window and 50% overlapped. Beyond the relative power obtained by the area under the spectral curve associated with slow (High Frequencies–Hf) and Fast (Low Frequencies-Lf) oscillations, the sympathovagal balance was also calculated using the ratio between Lf and Hf. Hf power represents the vagal control of the heart, modulated by breathing, while Lf power (more precisely, its normalized version) reflects primarily the sympathetic modulation of HR (Thireau *et al.*, 2008). Frequencies are presented in normalized units and graphs were obtained using Kubios Software (http://kubios-hrv.software.informer.com/2.2/) (Thireau *et al.*, 2008). In rodents, the lack of a standard protocol has conducted in the recent years to a set of studies regarding methodological issues (Ori *et al.*, 1992; Thireau *et al.*, 2008; Fonseca-Pinto, 2011). Concerning the particular issue of defining the frequency bands associated with Low (Lf) and High (Hf) frequency ranges, it was suggested the use of Lf in [0.15Hz-1.5 Hz] and Hf in [1.5Hz-4 Hz] as a good compromise to gauge the sympathetic and parasympathetic components of HRV (Ori *et al.*, 1992; Thireau *et al.*, 2008; Fonseca-Pinto, 2011). These frequency ranges were used in this work.

3.3.7. Statistical analysis

Data were evaluated using GraphPad Prism Software, version 6 (GraphPad Software, La Jolla, CA, USA) and presented as mean values and SEM. The significance of the differences between the mean values was calculated by one- and two-way ANOVA with Bonferroni multiple comparison tests. Differences were considered significant at $p < 0.05$.

3.4. Results

3.4.1. Body weight and energy intake

Animals were randomly allocated to the HSu and HF groups, submitted to the respective diet protocol and then randomly allocated to CSN transection or sham surgery in which the CSN was left intact. The sham procedure did not alter body weight and energy intake in comparison with the respective controls (data not shown). Bilateral CSN resection was confirmed by the absence of increased ventilatory responses to ischaemic-hypoxia during common carotid artery occlusion (data not shown). Bilateral CSN resection and the sham procedure did not significantly modify animal behaviour or

energy intake, measured as the average energy intake per day during the 3 weeks after CSN denervation (data not shown).

3.4.2. Bilateral CSN transection restores fasting plasma glucose and insulin sensitivity in animals continuously submitted to high-energy diets

Insulin resistance was confirmed before and after surgery by an ITT (data is presented as the constant of the rate of glucose disappearance [K_{ITT}]). Figure 3.2 illustrates the effect of CSN transection on fasting plasma glucose and insulin sensitivity in control (Fig. 3.2 a, b), HSu (Fig. 3.2 c, d) and HF (Fig. 3.2 e, f) animals, 3 weeks after surgery. Bilateral CSN ablation did not modify fasting plasma glucose in either control or HF animals (Fig. 3.2 a, e). However, the high-sucrose diet significantly increased fasting plasma glucose, by 49%, whilst physiological glucose levels were restored as early as 1 week after CSN resection (controls, 4.63 ± 0.1 mmol/l; HSu 1 week after CSN resection, 4.61 ± 0.2 mmol/l) and were sustained in the ensuing 3 weeks (Fig. 3.2c). Compared to controls, the high-sugar and high-fat diets also decreased insulin sensitivity by 47% (Fig. 3.2d) and 40% (Fig. 3.2f), respectively. Two weeks after CSN transection, insulin sensitivity was totally restored in both animal models, an effect that was sustained until the third week after CSN transection (Fig. 3.2 d, f). Insulin sensitivity in control animals was not affected by CSN transection (Fig. 3.2b).

To test whether unilateral CSN transection was sufficient to improve insulin sensitivity, unilateral surgical ablation was performed in an independent subgroup of HF rats. Unilateral transection did not completely restore insulin sensitivity to physiological values in HF rats (Fig. 3.2g). Additionally, insulin sensitivity was monitored in a subgroup of HSu rats for 11 weeks post-surgery to evaluate whether the insulin-sensitising effect induced by bilateral CSN surgical ablation would be maintained. At the end of the 11-week period, insulin sensitivity was maintained at values similar to those of controls (Fig. 3.2h).

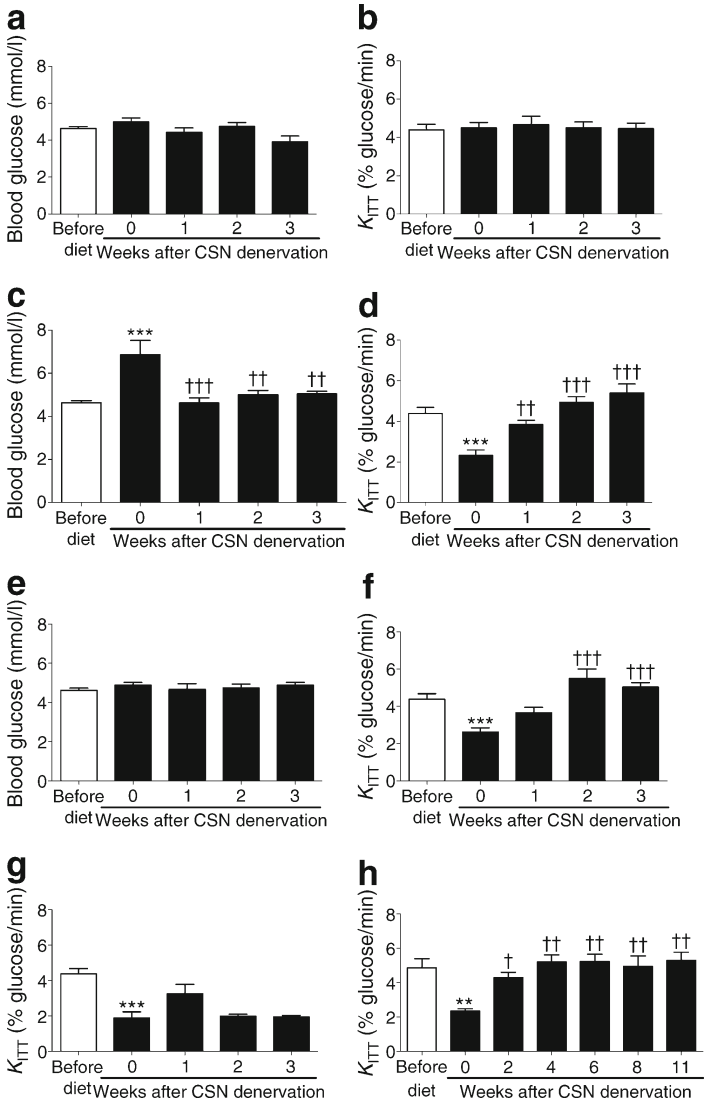


Figure 3.2 - Effect of CSN transection on (a, c, e) fasting plasma glucose and (b, d, f) insulin sensitivity determined by the ITT, expressed as the K_{ITT} in control, HSu and HF animals, respectively (n = 8/10 per group). (g) HF animals were submitted to unilateral CSN resection that was unable to restore insulin sensitivity (n = 8). (h) Effect of bilateral CSN transection on insulin sensitivity over 11 weeks, post-surgical CSN ablation in HSu rats (n = 8). White bars, before the respective diets; black bars, immediately before (0 weeks) and after CSN denervation. Data are presented as mean ± SEM. One-way ANOVA with Bonferroni multiple comparison tests; $^{**}p < 0.01$, $^{***}p < 0.001$ vs control; $\dagger p < 0.05$, $\dagger\dagger p < 0.01$, $\dagger\dagger\dagger p < 0.001$, pre *vs* post CSN resection.

3.4.3. CSN transection restores plasma insulin and C-peptide in animal models of impaired insulin sensitivity

Table 3.1 displays the effect of CSN transection on plasma insulin and C-peptide concentrations. Compared to control animals, HSu and HF animals showed increases in plasma insulin of 123% and 120%, respectively. C-peptide levels were significantly increased, by 66% and 82% in HSu and HF rats, respectively. CSN transection did not modify either plasma insulin or C-peptide levels in control animals. However, in HSu and HF rats, CSN transection restored plasma insulin and C-peptide levels to levels similar to control values, showing that insulin secretion was regulated by CSN ablation (Table 3.1).

Table 3.1 - CSN transection restores plasma insulin and C-peptide levels in HSu and HF rats.

Variable	Control		High-Sucrose Diet		High-Fat Diet	
	Without CSN resection	**With CSN resection**	**Without CSN resection**	**With CSN resection**	**Without CSN resection**	**With CSN resection**
Insulin (µg/L)	1.98±0.28	2.11±0.39	4.41±0.45***	2.21±0.33$^{\#\#}$	4.35±0.54***	1.32±0.40$^{\#\#\#}$
C-peptide (nmol/L)	0.89±0.13	0.86±0.12	1.48±0.21*	0.73±0.15$^{\#}$	1.62±0.29*	0.80±0.10$^{\#\#}$

Data are presented as mean ± SEM Without CSN transection, n = 6–8; with CSN transection, n = 8–9. Two-way ANOVA with Bonferroni multiple comparison tests; *$p < 0.05$, ***$p < 0.001$ vs controls; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$ with vs without CSN transection.

3.4.4. CSN transection improves insulin signalling in skeletal muscle and adipose tissue in animal models of impaired insulin sensitivity

Figure 3.3 shows western blot results for key proteins involved in insulin signalling pathways in insulin-sensitive tissues. In the skeletal muscle of HSu and HF animals, insulin receptor levels decreased significantly by 49% and 48%, respectively (Fig. 3.3a). Levels were restored in HSu and HF rats 3 weeks after CSN transection (Fig. 3.3a). Insulin receptor activity, as assessed by insulin receptor Tyr1361 phosphorylation, also decreased in both HSu and HF groups, by 47% and 31%, respectively (Fig. 3.3b). Chronic CSN transection not only restored, but actually increased, insulin receptor phosphorylation in control, HSu and HF animals, by 98%, 56% and 98%, respectively, relative to controls (Fig. 3.3b). HSu rats were also observed to have reduced levels (46%

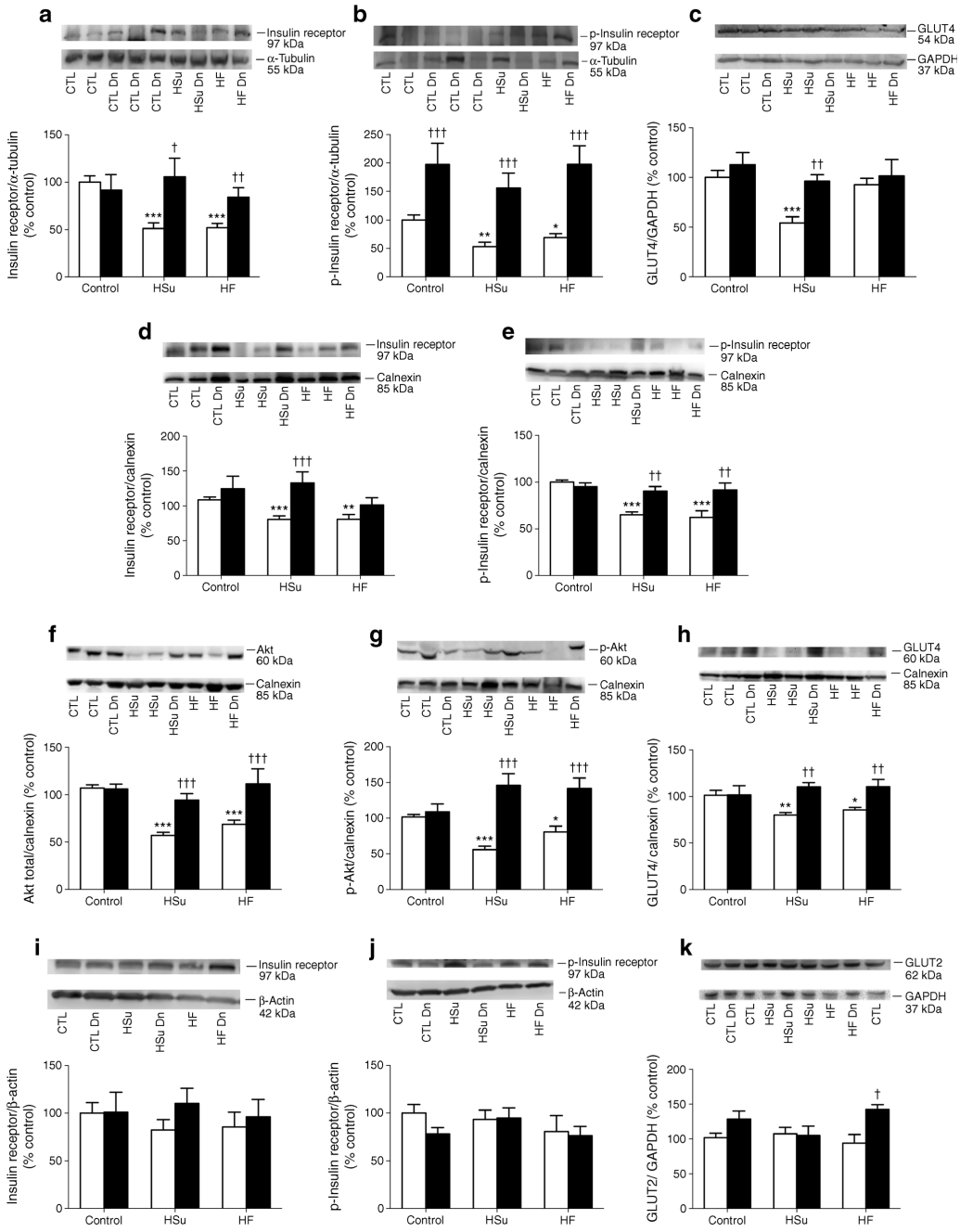


Figure 3.3 - Effect of chronic CSN resection on level and activity of proteins involved in insulin signalling in (a–c) skeletal muscle, (d–h) adipose tissue and (i–k) the liver. Average relative (**a**) insulin receptor levels (97 kDa), (**b**) insulin receptor phosphorylation (97 kDa), and (**c**) GLUT4 (54 kDa) immunoreactivity in skeletal muscle from control, HSu and HF animals with (n = 5–6) or without (n = 5–10) CSN resection. Average relative (**d**) insulin receptor levels, (**e**) insulin receptor phosphorylation, (**f**) Akt levels (60 kDa), (**g**) Akt phosphorylation (60 kDa) and (h) GLUT4 immunoreactivity in adipose tissue from control, HSu and HF animals with (n=5–7) or without (n=7–10) CSN resection. Average relative (**i**) insulin receptor levels, (**j**) insulin receptor phosphorylation and (**k**) GLUT2 (62 kDa) immunoreactivity in liver from control, HSu and HF animals with (n=5–6) or without (n=5–10) CSN resection. White bars without CSN denervation; black bars, with CSN denervation. Data are presented as mean±SEM. Representative western blots for each protein studied are depicted above the respective bar graphs. Dn, denervated/ transected. Two-way ANOVA with Bonferroni multiple comparison tests; *p<0.05, **p<0.01,***p<0.001 vs control; $^{\dagger}p$<0.05, $^{\dagger\dagger}p$<0.01, $^{\dagger\dagger\dagger}p$<0.001 with vs without CSN resection.

decrease) of GLUT4, which were fully restored 3 weeks after CSN transection, to levels similar to healthy controls (Fig. 3.3c).

In HSu and HF rats, insulin receptor levels were significantly decreased in adipose tissue, by 26% (Fig. 3.3d). CSN resection completely restored insulin receptor levels in HSu animals, but not in the HF, group (Fig. 3.3d). Similarly, insulin receptor phosphorylation decreased by 35% and 38% in HSu and HF animals, respectively, and CSN transection completely restored insulin receptor activity (Fig. 3.3e).

The high-sugar and high-fat diets significantly decreased GLUT4 levels in adipose tissue, by 21% and 16%, respectively (Fig. 3.3h), and Akt values by 47% and 36%, respectively (Fig. 3.3f). CSN transection overturned the effect of the high-energy diets on adipose tissue levels of both GLUT4 and Akt (Fig. 3.3 f, h). Akt activity decreased significantly, by 45% and 21% in HSu and HF rats, respectively (Fig. 3.3 g). CSN transection increased Akt phosphorylation by 44% and 39% in HSu and HF animals, respectively (Fig. 3.3g).

In liver, insulin receptor levels and activity were not altered by either high-energy diets or CSN transection (Fig. 3.3 i, j). In contrast, CSN ablation increased GLUT2 levels in HF animals by 40%, compared with controls (Fig. 3.3k).

3.4.5. CSN transection restores whole-body glucose tolerance, and liver and visceral adipose tissue glucose uptake in HF animals

The effect of chronic bilateral CSN resection on glucose uptake was evaluated using 2-deoxy[3H]glucose IVGTT. For these experiments, the HF model was selected because of its obesity phenotype and was compared with controls. Figure 3.4 shows similar IVGTT results to those observed in Fig. 3.4 for the ITT, as shown by the significant increase in the area under the glucose excursion curve in this group (controls, 1.6×107 ±6.5×105; HF rats, 2.1×107 ±1.7×106, Fig. 3.4b). Three weeks after CSN transection, glucose tolerance was restored in HF animals, shown by a decrease in AUC to values similar to those of control rats that had undergone the sham procedure (AUC HF with transected CSN, 1.6×107±7.1×105).

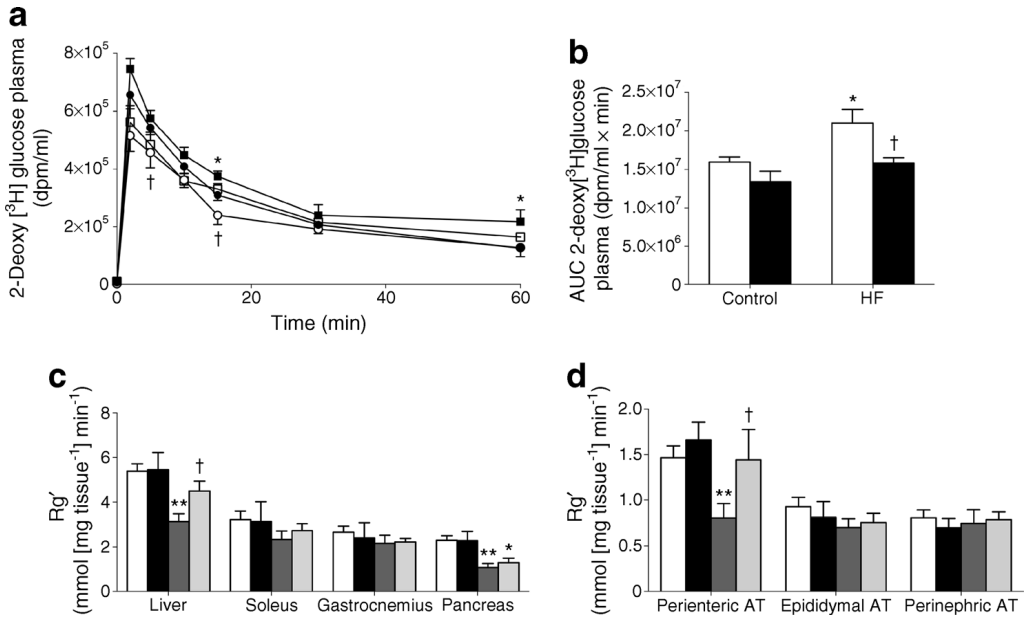


Figure 3.4 - Chronic bilateral CSN resection restores glucose tolerance via its action on visceral/perienteric adipose tissue and liver glucose uptake in HF rats. Effect of chronic CSN transection on glucose tolerance after an IVGTT depicted as (**a**) glucose excursion curves. Black circles, controls without CSN denervation; white circles, controls with CSN denervation; black squares, HF without CSN denervation; white squares, HF with CSN denervation (n = 8 for each). (**b**) AUC obtained from the analysis of glucose excursion curves. White bars, without CSN resection (n = 6–9); black bars, with CSN resection (n = 8). (**c**) Rg′ values, reflecting glucose uptake in the liver, soleus, gastrocnemius and pancreas. (**d**) Rg′ values for perienteric, epididymal and perinephric adipose tissue (AT). Evaluation was performed 3 weeks after CSN transection. (**c, d**) White bars, controls without CSN resection (n = 8); black bars, controls with CSN resection (n = 6); dark grey bars, HF without CSN resection (n = 8); light grey bars, HF with CSN resection (n = 7). Data are presented as mean ± SEM. Two-way ANOVA with Bonferroni multiple comparison tests: $*p < 0.05$, $**p < 0.01$ vs control; $^{\dagger}p < 0.05$ with *vs* without CSN resection CSN transection.

After 6 weeks of the high-fat diet, the glucose metabolic index (Rg′), which evaluates glucose uptake, decreased by 42% and by 43% for the liver and pancreas, respectively. Glucose uptake by the soleus and gastrocnemius muscles did not significantly change, compared with controls (Fig. 3.4c). CSN transection produced a significant increase of 43% in 2-deoxy[3H]glucose uptake by the liver compared with HFD animals with an intact CSN. However, no change in glucose uptake was observed for the pancreas, soleus or gastrocnemius tissues (Rg′ of liver: HF without CSN transection, 3.15 ± 0.33 mmol [mg tissue]$^{-1}$ min^{-1}; HF with CSN transection, 4.50 ± 0.45 mmol [mg tissue]$^{-1}$ min^{-1}; Fig. 3.4c). Finally, in visceral adipose tissue, 6 weeks of the high-fat diet decreased glucose uptake by 45% compared with the control group, and this effect was completely re- stored by CSN transection (Rg′ of perienteric adipose

tis- sue: controls without CSN transection, 1.46 ± 0.13 mmol [mg tissue]$^{-1}$ min^{-1}; HF without CSN transection, 0.81 ± 0.16 mmol [mg tissue]$^{-1}$ min^{-1}; HF with CSN transection, 1.44 ± 0.33 mmol [mg tissue]$^{-1}$ min^{-1}; Fig. 3.4d). No significant results were observed for epididymal and perinephric adipose tissue (Fig. 3.4d).

3.4.6. CSN denervation ameliorates lipid profile in animal models of impaired insulin sensitivity

The HSu and HF animals showed no change in either total cholesterol or LDL-cholesterol (Table 3.2). In contrast, HDL-cholesterol was decreased in HF animals by 28% and was restored to control values by CSN transection (Table 3.2). Additionally, the HSu and HF rats had increased triacylglycerol levels by 75% and 47%, respectively; these values returned to control levels after CSN resection (Table 3.2).

Table 3.2 - Effect of CSN transection on lipid profile in control, HF and HSu rats.

Variable	Control		High-Sucrose Diet		High-Fat Diet	
	Without CSN resection	**With CSN resection**	**Without CSN resection**	**With CSN resection**	**Without CSN resection**	**With CSN resection**
Total cholesterol (mg/dl)	71.89±3.87	68.65±5.02	82.24±4.24	70.84±2.35	67.60±2.94	69.59±2.89
LDL-cholosterol (mg/dl)	4.94±0.27	5.23±0.55	4.85±0.56	4.88±0.18	4.20±0.49	3.81±0.77
HDL-cholesterol (mg/dl)	27.99±1.21	23.86±1.19	28.10±1.56	26.00±0.87	20.23±1.21***	25.01±1.85$^{\#}$
Triacylglycerols (mg/dl)	31.07±3.49	20.39±2.73	54.30±6.27***	22.00±3.67$^{\#\#\#}$	45.41±3.43*	19.87±5.32$^{\#\#}$

Data are presented as mean ± SEM Without CSN transection, n = 8–9; with CSN transection, n = 8–10 Two-way ANOVA with Bonferroni multiple comparison tests; *$p < 0.05$, ***$p < 0.001$ vs control; $^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$ with *vs* without CSN transection.

3.4.7. CSN transection normalises mean arterial pressure, nitric oxide metabolites and sympathetic activity in animal models of impared insulin sensitivity

In the current study, HSu and HF rats had a 39% increase in mean arterial pressure. These results are in line with the values previously obtained in our laboratory

(Conde *et al.*, 2012b; Ribeiro *et al.*, 2013). Three weeks after CSN resection, mean arterial pressure was fully restored to control levels in both HSu (96.7 ± 11.8 mmHg) and HF (92.1 ± 9.6 mmHg) animals (Fig. 3.5a).

The effect of CSN resection on serum nitric oxide and its metabolites (NO + NO_3) is depicted in Fig. 3.5b. Although no significant effects were observed in HSu rats, the HF rats had significantly increased NO+NO_3 levels compared with controls (control, 10.6 ± 1.1 µmol/l; HF, 21.3 ± 2.0 µmol/l), with this effect being completely restored by chronic CSN resection.

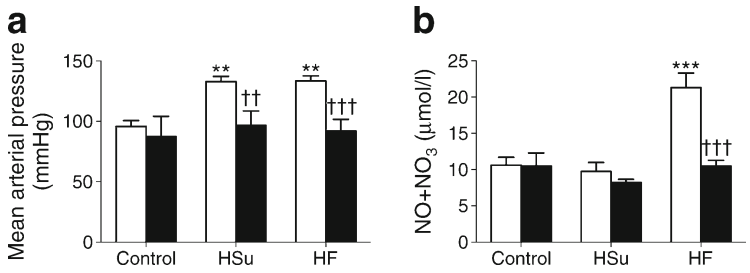


Figure 3.5 - Effect of CSN transection on (a) mean arterial pressure and (b) plasma NO + NO_3 in control, HSu and HF animals. Blood pressure and NO+NO_3 measurement was performed 3 weeks after chronic CSN denervation. White bars, without CSN resection (n = 8–9); black bars, with CSN resection (n = 6–8). Data are presented as mean ± SEM. Two-way ANOVA with Bonferroni multiple comparison tests: $^{**}p < 0.01$, $^{***}p < 0.001$ vs control; $^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$ with vs without CSN transection.

Since CB overactivity contributes to the development of insulin resistance and hypertension through sympathoadrenal overstimulation (Ribeiro *et al.*, 2013), we also analysed sympathetic nervous activity using spectral analysis of heart rate variability and via the measurement of circulating and adrenal medullary catecholamines (norepinephrine and epinephrine). Figure 3.6a shows the analysis of heart rate variability obtained by the Fast Fourier Transform. In a one representative example of Power Spectral Density (PSD) in control animals is shown before (top) and after (bottom) denervation (left panel). In the right panel the PSD plot of a Hf is shown before (top) and after (bottom) denervation. The energy of the low frequency band (Lf) is a marker of the sympathetic component of the autonomic nervous system being increased by 30% in HF animals (Fig. 3.6b), an effect that was normalized by CSN transection. Moreover, chronic CSN surgical ablation decreased sympathetic activity in

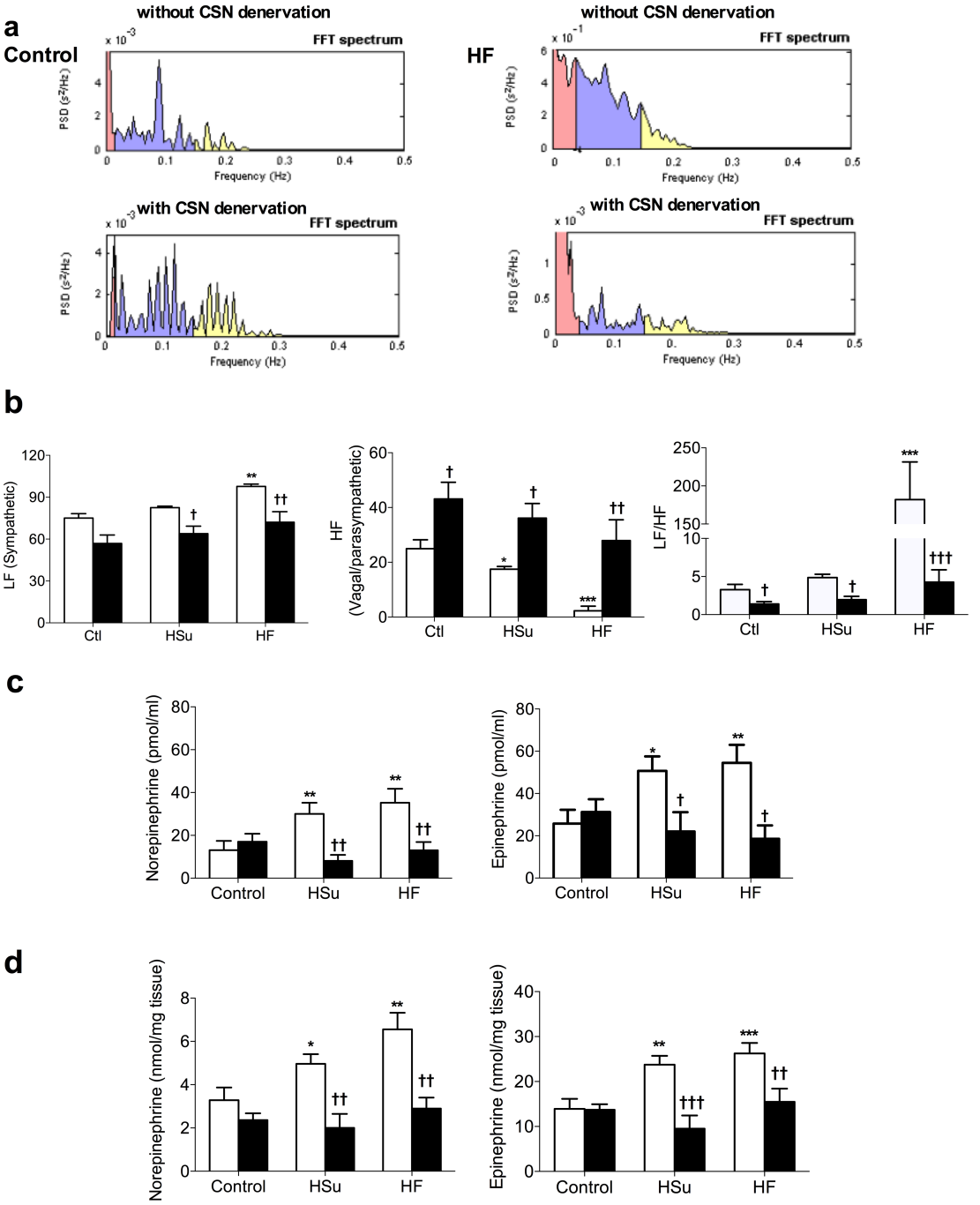


Figure 3.6 - Chronic carotid sinus nerve (CSN) transection restores sympathetic activity in prediabetes animal models. Sympathetic activity was evaluated by spectral analysis of the heart rate (**a** and **b**), and by plasma catecholamines (norepinephrine and epinephrine, n=8-10) (**c**) and adrenal medulla catecholamines content (norepinephrine and epinephrine, n=10-14) (**d**) in control, HSu and HF animal models. In **a** in the left panel one control example of Power Spectral Density is shown before (top) and after (bottom) denervation. On **a** right panel, the Power Spectral Density plot of a High Fat is shown before (top) and after (bottom) denervation. Frequencies are presented in normalized units. White bars represent values of animals without CSN resection and black bars correspond to values in animals with CSN resection. Bars represent mean ± SEM. Two-Way ANOVA with Bonferroni multicomparison tests; *p<0.05, **p<0.01, ***p<0.001 vs control; p<0.05, †p<0.05; ††p<0.01, †††p<0.001 with vs without CSN transection.

HSu animals by 23%, comparing with the HSu-sham group. The high frequency energy band (Hf) represents the parasympathetic component of the autonomic nervous system and it is increased in HSu and HF animals by 30 and 91%, respectively (Fig. 3.6b). Three weeks after chronic CSN resection these values are increased in control and in the disease animal models. The alterations in Lf and Hf bands are reflected in the ratio Lf/Hf, a measure of sympathovagal balance, which shows the increase in sympathetic activity in HF animals and the decrease in sympathetic activity three weeks after the CSN resection in control, HSu and HF animals.

Figure 3.6c shows that plasma norepinephrine significantly increased in both HSu and HF rats by 129 and 169%, respectively, in relation to control animals (13.10 ± 4.34 pmol/ml), as previously reported (Ribeiro *et al.*, 2013). Epinephrine levels also increased by 97 and by 112% in HSu and HF animals, respectively (Control = 25.74 ± 6.58 pmol/ml). Also, as shown in Figure 3.6d, HSu and HF diets significantly increased adrenal medulla norepinephrine by 51 and 100%, respectively, and adrenal medulla epinephrine by 71 and 89%, respectively, compared with control values (norepinephrine control = 3.28 ± 0.59 nmol/mg tissue; epinephrine control = 13.93 ± 2.21 nmol/mg tissue). In control animals, CSN resection did not alter sympathoadrenal activity, however it completely restored the sympathoadrenal overstimulation induced by hypercaloric diets (Fig. 3.6 c and d).

3.5. Discussion

We have established that abolition of CB activity may represent a therapeutic strategy for pre-existing metabolic diseases. In HF and HSu rats, chronic bilateral CSN transection fully restored insulin action, fasting plasma glucose and insulin levels, blood pressure, and lipid profile to physiological values. It also restored insulin signalling pathways in skeletal muscle and adipose tissue. Additionally, CSN transection improved glucose uptake by the liver and perienteric adipose tissue concomitantly with restoration of autonomic imbalance. Another important observation was that the metabolic improvement induced by CSN surgical ablation was maintained, even when animals were continuously fed high-energy diets.

3.5.1. Effect of CSN transection on whole-body insulin action and insulin secretion

We have demonstrated that chronic bilateral CSN resection restored insulin action in animal models of impaired insulin sensitivity, an effect that was sustained over time, as demonstrated by improved insulin sensitivity in HSu animals 11 weeks after the intervention. The time frame of 11 weeks was chosen because the CSN starts to regrow 6 days after being cut and usually completely regenerates after 11-12 weeks (Zapata *et al.*, 1976). We also evaluated the effect of unilateral CSN resection in HF animals, a procedure that was ineffective in restoring insulin sensitivity in this pathological model. Our results are supported by Fudim *et al*. (2015), who demonstrated that the hypotensive effect of unilateral resection of CB tumours in patients with hypertension diminished over the long term. Moreover, Paton *et al.* (2015) also observed that, 12 months after unilateral CB ablation, the significant decrease in blood pressure initially observed was attenuated. These and our own results corroborate the hypothesis that unilateral denervation is compensated by the remaining CB, counteracting the beneficial effect of denervation. This suggests that bilateral denervation is needed to achieve a sustained effect on insulin action and glucose metabolism.

Fasting plasma insulin and glucose were also normalised by bilateral CSN transection. These effects were accompanied by a re-establishment of endogenous insulin secretion, since we observed normalisation of both insulin and C-peptide levels. These results may be either an indirect consequence of improved peripheral insulin sensitivity or a direct effect of the CBs on neurally mediated insulin secretion. Koyama et al have previously demonstrated that CB ablation reduces basal glucagon secretion, and that this surgical approach may also affect insulin secretion (Koyama *et al.*, 2000; Koyama *et al.*, 2001).

3.5.2. Effect of CSN transection on glucose homeostasis

Bilateral CSN transection improved insulin signalling in skeletal muscle in the HF and HSu animal models tested; it fully restored insulin receptor and phosphorylated insulin receptor levels in both animal models, and increased GLUT4 levels in HSu animals. HF animals did not exhibit decreased GLUT4 expression in skeletal muscle, and CSN surgical ablation did not alter these values. The latter result is not unique as GLUT4 protein content is known to be normal in muscle from insulin- resistant

individuals; however, insulin-stimulated GLUT4 translocation is impaired in these patients (Garvey *et al.*, 1998). Accordingly, despite the improvement in insulin receptor levels (phosphorylated and non-phosphorylated) in the skeletal muscle of HF animals after surgery, we did not observe significant changes in 2-deoxy[3H]glucose uptake in either soleus or gastrocnemius muscles, which may be explained by a decrease in insulin-stimulated GLUT4 translocation in the muscle of HF animals. Nevertheless, this hypothesis remains speculative since we only assessed total GLUT4 levels and did not investigate translocation of this molecule. As expected, high-energy diets decreased the expression levels of insulin receptor, GLUT4 and Akt, as well as the phosphorylation of the insulin receptor and Akt in perienteric adipose tissue. Marked impairments in the insulin signalling cascade have been described in fat cells from both animal models and in patients with T2D (Rondinone *et al.*, 1997; Smith, 2002; Matafome *et al.*, 2014; Matafome *et al.*, 2015). In both HSu and HF rats, chronic CSN transection restored perienteric adipose tissue levels of Akt, phosphorylated Akt and GLUT4, so that they were similar to controls. This improvement in insulin receptor activation and protein levels in adipose tissue was accompanied by an increase in 2-deoxy[3H]glucose uptake in the perienteric adipose tissue of HF rats. 2-Deoxy[3H]glucose uptake assays were performed in the HF model, and not the HSu rats, due to their obesity phenotype – resembling the metabolic syndrome and diabetes in humans – and also because the adipose tissue in these rat models was most affected by bilateral CSN transection. These results strongly suggest that visceral/perienteric fat is one of the tissues under the metabolic control of CBs, and that CSN transection improves insulin-stimulated glucose disposal in adipose tissue.

There were no significant changes in hepatic levels of insulin receptors and their phosphorylation in HF or HSu rats. However, GLUT2 levels and Rg′ were significantly increased in the liver of HF rats after surgery. It is conceivable that this may also contribute to the improvements in whole-body insulin action mediated by CSN transection. Cherrington's group (Moore *et al.*, 2012) has shown that both sympathetic and nitrergic innervation of the liver exerts tonic repression of hepatic glucose uptake under feeding conditions. The consumption of high-fat and high-fructose diets impairs net hepatic glucose uptake and glycogen storage, and reduces glucokinase protein level and activity (Coate *et al.*, 2011). According to Cherrington's work, a tonic inhibition of hepatic sympathetic innervation improves hepatic glucose uptake, storage and output, leading to improved glucose homeostasis. This may be achieved by the inhibition of CB

activity since this organ is directly linked to the sympathetic nervous system and has also been shown to be involved in a diminished counterregulatory response, by impacting the release of counterregulatory hormones, such as glucagon (Koyama *et al.*, 2001). According to our results, one of the mechanisms by which sympathetic blockade increases hepatic glucose uptake appears to be an increase in GLUT2 levels in hepatocytes, and although we did not assess this in our work, an increase in glucokinase activity may also be involved. The dynamic IVGTT profiles observed indicate that CSN transection improves first-phase insulin secretion in both control and HF animals by lowering the maximum glucose concentration reached during the test. Additionally, the rapid uptake of glucose by the peripheral tissues may explain the steeper slope observed for the descending phase of the curve (Bergman *et al.*, 1985).

3.5.3. Effect of CSN transection on haemodynamic homeostasis

MAP was fully restored to control levels in HSu and HF animals. This is in accordance with a recent proposal that carotid glomectomy may represent a powerful way to prevent excessive sympathetic discharge in diseases such as hypertension (Paton *et al.*, 2013a; Paton *et al.*, 2013b). Our results also show that CSN transection normalises $NO+NO_3$ in HF animals. Rats fed a high-fat diet are known to have higher levels of inducible nitric oxide synthase and increased inflammatory biomarkers (Stanimirovic *et al.*, 2016). Therefore, CSN resection ameliorates endothelial function, restoring $NO+NO_3$ levels to values similar to control. This may result from improved glucose and lipid homeostasis (Stanimirovic *et al.*, 2016), as well as from decreased MAP and restoration of endothelial function. One of the concerns related to the surgical approach of CSN surgical ablation is that, as well as chemoreceptor activity, CSN carries baroreceptor information. It is well documented that resection of the baroreceptors can cause short-term fluctuations in blood pressure since baroreceptors play a major role in short-term regulation of blood pressure (Cowley *et al.*, 1973; Norman *et al.*, 1981). These short-term fluctuations can be compensated in the long term (Norman *et al.*, 1981; Irigoyen *et al.*, 1995), either by the central nervous system, by renal fluid volume-adjusting mechanisms (Cowley *et al.*, 1973) or by the aortic bodies, as evidenced by recent studies that have shown that the arterial baroreflex is a long-term regulator of blood pressure (Lohmeier *et al.*, 2002; Zoccal *et al.*, 2009; Lohmeier & Iliescu, 2015). These long-term adjustments of the arterial baroreflex may

explain the significant decrease in blood pressure after CSN transection that was observed in the present study.

3.5.4. Effect of CSN transection on lipid homeostasis

Increased plasma triacylglycerols and LDL-cholesterol, together with low HDL-cholesterol levels are central pathophysiological features of metabolic diseases. We observed that CSN denervation fully restored plasma triacylglycerols to control values and significantly increased HDL-cholesterol in the HF model. The major sources of triacylglycerol secretion by the liver are fatty acids from adipose tissue (released by lipolysis) or in the form of remnant lipoproteins, and hepatic de novo lipogenesis, which are all abnormally increased in insulin resistance. Whether the improved lipid profile observed in HF rats was indirectly caused by enhancement of insulin sensitivity or by the direct action of CSN-controlled sympathetic efferent pathways on the liver, as previously suggested (O'Meara *et al.*, 1992; Bruinstroop *et al.*, 2015), remains to be clarified.

3.5.5. Concluding remarks

Bilateral abolition of CB activity restores glucohomeostasis, lipohomeostasis and haemodynamic homeostasis in animal models of pre-established metabolic disease. These effects are sustained for at least 11 weeks after surgery. Additionally, the mechanism behind the repair of glucohomeostasis and lipohomeostasis involves an improvement in glucose uptake by the liver and re-establishment of glucose uptake by perienteric adipose tissue, concomitantly with an improvement in autonomic function. Our data represent proof of principle that abolition of CB activity reverses core features of metabolic disease, and highlight the importance of developing strategies to reduce neural activity in CBs.

IV

This chapter is based on the following manuscript:

Bioelectronic modulation of carotid sinus nerve activity in rat: a potential therapeutic approach for type 2 diabetes

Sacramento JF*, Chew DJ*, Melo BF, Donegá M, Dopson W, Guarino MP, Robison A, Prieto-Lloret J, Patel S, Holinski BJ, Ramnarain N, Famm K, Conde SV. (2018) *Diabetologia*. 61(3):700-710.

4. Bioelectronic modulation of carotid sinus nerve activity in the rat: a potential therapeutic approach for type 2 diabetes

4.1. Abstract

Aims/hypothesis: A new class of treatments termed bioelectronic medicines are now emerging that aim to target individual nerve fibres or specific brain circuits in pathological conditions to repair lost function and reinstate a healthy balance. Carotid sinus nerve (CSN) denervation has been shown to improve glucose homeostasis in insulin-resistant and glucose-intolerant rats; however, these positive effects from surgery appear to diminish over time and are heavily caveated by the severe adverse effects associated with permanent loss of chemosensory function. Herein we characterize the ability of a novel bioelectronic application, classified as kilohertz frequency alternating current (KHFAC) modulation, to suppress neural signals within the CSN of rodents.

Methods: Rats were fed either a chow or high-fat/high-sucrose (HFHSu) diet (60% lipid-rich diet plus 35% sucrose drinking water) over 14 weeks. Neural interfaces were bilaterally implanted in the CSNs and attached to an external pulse generator. The rats were then randomized to KHFAC or sham modulation groups. KHFAC modulation variables were defined acutely by respiratory and cardiac responses to hypoxia (10% O_2 + 90% N_2). Insulin sensitivity was evaluated periodically through an ITT and glucose tolerance by an OGTT.

Results: KHFAC modulation of the CSN, applied over 9 weeks, restored insulin sensitivity (constant of the insulin tolerance test [K_{ITT}] HFHSu sham, 2.56 ± 0.41% glucose/min; K_{ITT} HFHSu KHFAC, 5.01 ± 0.52% glucose/min) and glucose tolerance (AUC HFHSu sham, 1278 ± 20.36 mmol/l × min; AUC HFHSu KHFAC, 1054.15 ± 62.64 mmol/l × min) in rat models of type 2 diabetes. Upon cessation of KHFAC, insulin resistance and glucose intolerance returned to normal values within 5 weeks.

Conclusions/interpretation: KHFAC modulation of the CSN improves metabolic control in rat models of type 2 diabetes. These positive outcomes have significant translational potential as a novel therapeutic modality for the purpose of treating metabolic diseases in humans.

4.2. Introduction

The principal defects in type 2 diabetes (T2D) are peripheral insulin resistance, abnormal hepatic glucose metabolism and progressive pancreatic beta cell failure. Glucose control in T2D deteriorates progressively over time and, after failure of diet and exercise alone, needs on average a new intervention with glucose-lowering agents every 3–4 years in order to obtain/retain good control. Despite combination therapy and/or insulin treatment, a sizeable proportion of individuals remain poorly controlled (EMA, 2012). The global burden of disease continues to increase and the number of individuals with T2D will exceed 500 million by 2040 (IDF, 2015; WHO, 2016). There remains a need for novel therapeutic approaches with different mechanistics. We have previously shown that the abolition of carotid body (CB) activity through surgical resection of its sensitive nerve, the carotid sinus nerve (CSN), restores insulin sensitivity and glucose tolerance in high-energy-fed animal models of insulin resistance and glucose intolerance via a re-establishment of sympathetic nerve activity (Ribeiro *et al.*, 2013; Sacramento *et al.*, 2017). Additionally, we described that this re-establishment of metabolic variables was related to the recovery of insulin signalling in insulin-sensitive tissues, as well as the improvement of glucose uptake by the liver and visceral adipose tissue (Sacramento *et al.*, 2017). Herein, we tested the proof-of-principle that inhibition of CSN activity is a sustainable therapeutic strategy for early T2D by bilaterally resecting the CSN in rats previously exposed to a high-fat/high-sucrose (HFHSu) diet for 14 weeks. However, knowing in advance that the surgical resection of the CSN is prone to cause side effects related to loss of the peripheral hypoxic response and decreased sensitivity to CO_2 (Timmers *et al.*, 2003; Dahan *et al.*, 2007), impaired response to exercise (Forster *et al.*, 1983; Dempsey & Smith, 1994; Forster & Pan, 1994) and fluctuations in blood pressure (Paton *et al.*, 2013b), we sought to determine whether a reversible approach could induce long-term glycaemic control in rats with diet-induced early diabetes without significant side effects.

A new type therapeutic approach that allows a precise detection and modulation of electrical signalling patterns in the peripheral nervous system, known as bioelectronic medicine, is emerging (Famm *et al.*, 2013; Birmingham *et al.*, 2014; Adameyko, 2016). Acknowledging that the therapeutic approaches currently available for metabolic diseases do not provide long-term control of the disease, combined with significant side effects, a bioelectronic medicine approach could bring significant improvement in the standard of care for T2D by targeting nodal metabolic pathways and avoiding systemic

effects. Additionally, bioelectronic medicines might have high acceptance among patients given that they require only minimally invasive procedures, while providing high adherence and negligible interference with daily activities (Camilleri *et al.*, 2009; Famm *et al.*, 2013; Birmingham *et al.*, 2014; Adameyko, 2016).

In the present work, we tested the use of bilateral kilohertz frequency alternating current (KHFAC) modulation to inhibit CSN activity, since this method has been shown to produce an effective and reversible block of nerve conduction in somatic nerves (Kilgore & Bhadra, 2004; Williamson & Andrews, 2005; Kilgore & Bhadra, 2014). It has been proposed that the effect of KHFAC is due to direct inhibition of the action potential conduction and that this is achieved within the frequency range 1-100 kHz (Kilgore & Bhadra, 2004), with the highest frequencies evaluated in vivo being 50 and 70 kHz (Cuellar *et al.*, 2013; Patel *et al.*, 2017).

4.3. Methods

4.3.1. Ethical statement

All animal studies were carried out in accordance with the European Union Directive for Protection of Vertebrates Used for Experimental and Other Scientific Ends (2010/63/EU) and the GlaxoSmithKline (GSK) Policy on the Care, Welfare and Treatment of Animals. All surgery was performed following the LASA guiding principles for preparing and undertaking aseptic surgery (LASA., 2010).

4.3.2. Surgical procedures

Terminal experiments were performed at GlaxoSmithKline (Stevenage, UK) in male Crl:CD (SD) rats (230–280 g, aged 14–16 weeks) obtained from Charles River (Margate, Kent, UK). Recovery experiments were performed at NOVA Medical School Lisbon (Lisbon, Portugal) in male Crl:WI (Wistar Han) rats (200–300 g, aged 8-9 weeks) obtained from the NOVA Medical School animal house. In both animal houses, the rats were kept under temperature and humidity control (21 ± 2°C, 55 ± 10% humidity) with a 12 h light/dark cycle. For recovery experiments animals were blinded divided into two groups: group 1 received a standard chow diet (7.4% fat, 17% protein and 75% carbohydrate (4% sugar) (Dietex International Limited, France) and group 2 were fed an high-fat high-sucrose (HFHSu) diet to induce T2D (60% lipid rich diet: 34% fat+33% carbohydrate+23% protein; Mucedola, Milan, Italy) plus sucrose (35%) in drinking

water) over 14-15 weeks. Body weight was periodically recorded, and diet and water consumption were monitored daily. In subsequent experiments, experimenters were blinded to group assignment and outcome assessment. Inclusion criteria for study animals was constant of the insulin tolerance test (K_{ITT}) at 14 weeks of diet of 2.8% glucose/min for HFHSu animals and 4.0% glucose/min for control animals.

4.3.2.1. CSN resection

After insulin sensitivity and glucose tolerance evaluation using the insulin tolerance test (ITT) and oral glucose tolerance test (OGTT), respectively, animals were subjected to bilateral CSN transection under ketamine (30 mg/kg, Nimatek, Dechra, Lostock Gralam, UK) and xylazine (4 mg/kg, Rompun, Bayer, Leverkusen, Germany) anaesthesia and buprenorphine (10 µg/kg, Bupaq, Richter pharma, Wels, Austria) analgesia as previously described (Ribeiro *et al.*, 2013; Sacramento *et al.*, 2016). The control groups were subjected to a sham surgical procedure (n = 8-10 each group) (Fig. 4.1).

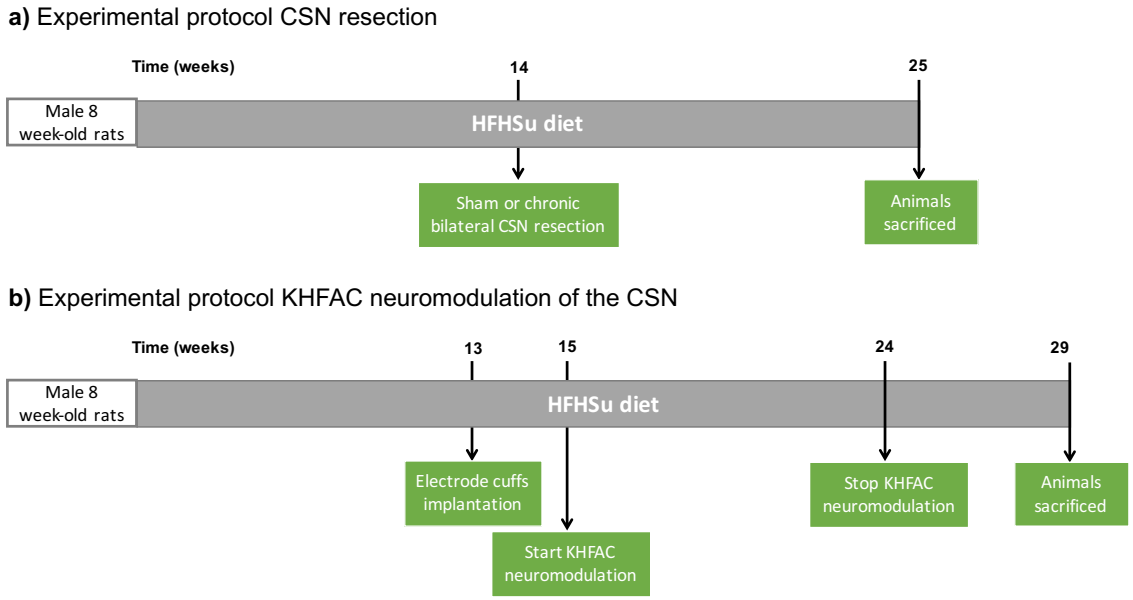


Figure 4.1 - Representation of the carotid sinus nerve (CSN) resection protocol (a) and of CSN kilohertz frequency alternating current (KHFAC) neuromodulation protocol (b).

4.3.2.2. CSN cuff electrode implantation

To evaluate the impact of continuous KHFAC modulation of the CSN, animals from group 1 (non-disease model) and group 2 (diabetes-disease model) were implanted with CSN cuff electrodes. Rats were anaesthetised with urethane (i.p. 1.5 g/kg, Sigma, Gillingham, UK) for acute experiments or medetomidine/ketamine for recovery

experiments and bipolar sling cuff electrodes (90% platinum and 10% iridium, 100 µm inner diameter × 1 mm length, electrode surface area 0.4 × 0.5 mm^2, 0.45 mm inter-electrode centre-to-centre distance, CorTec, Freiburg, Germany) were connected to the CSN bilaterally (Fig. 4.2). Fibrin glue (Tisseel, Baxter Healthcare, Compton, Newbury, UK) was used to secure the cuff to the CSN and to prevent current spread from the ends of the cuff. Wires from the cuff electrodes were tunnelled subcutaneously dorsally on the neck and exteriorised on the head via a skull-mounted percutaneous connector (MS363, Plastics One, Roanoke, VA, USA) and encapsulated with epoxy to form a head cap (Fig. 4.2). Immediately after implantation, correct electrode placement was confirmed by an increase in respiratory rate during stimulation at 5 Hz and 300 µA for ~2 s. Anaesthesia was reversed with atipamezole (0.25 mg/kg in 2 ml, i.p., Antisedan, Esteve, Finland). Rats were treated post- operatively with analgesic buprenorphine (10 µg/kg, s.c.) and for 2–3 days with anti-inflammatory carprofen (5 mg/kg, s.c, Rimadyl, Pfizer, Zaventem, Belgium). The animals were allowed to recover for 10 days prior to acclimatisation to tethering and initiating the KHFAC modulation.

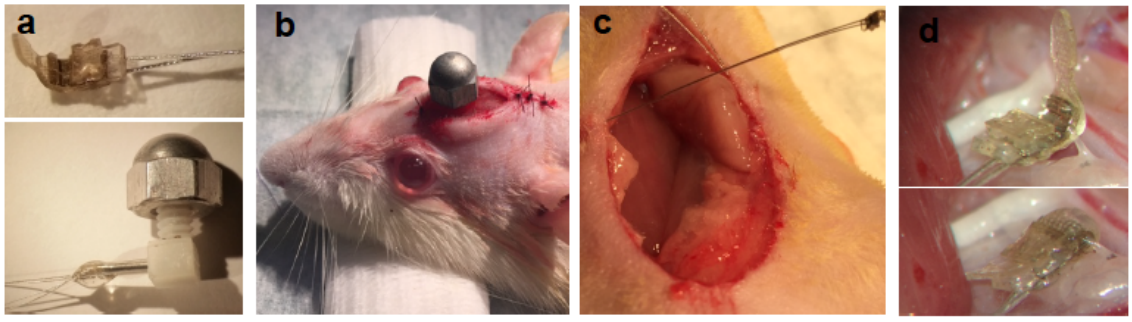


Figure 4.2 - Implantation of cuffs electrodes in rats. a) shows CorTec cuffs connected to plastic one headcaps; **b**) shows headcaps cemented in place; **c)** shows the wires trocated behind the ear to the neck; and **d)** shows the electrical cuffs placed bilaterally on CSN.

4.3.2.3. EMG and ECG recording

Intercostal platinum wires for electromyography (EMG) and electrocardiogram (ECG) electrodes were placed subcutaneously across the diaphragm. EMG and ECG data were differentially recorded using Plexon Multichannel Acquisition Processor Data Acquisition System (Plexon, Dallas, TX, USA) and analysed in MatLab.

4.3.3. KHFAC modulation of the CSN

KHFAC modulation was applied to the cuff electrodes bilaterally as rectangular pulses: at a current of 1 mA for the frequencies 20 kHz, 30 kHz, 40 kHz or at a current of 2 mA for 50 kHz. KHFAC was applied using a commercial current source (Keithley 6221, Tektronix, Bracknell, UK) connected to the percutaneous connector via a tether (305-305, Plastics One). In the percutaneous connector, wires from two cuffs were attached in parallel, so the current was split between the cuffs according to their impedance. To ensure near-equal current split, the cuff electrode impedances were measured in saline (154 mmol/l NaCl) prior to implantation and the cuffs were matched for each animal based on <10% difference in their impedance values. The current values are reported as peak-to-peak for each cuff, assuming equal 50/50% split from the current source output. The current source was only used in the range 0 - 4.2 mA (peak-to-peak), as higher ranges provided noticeable current attenuation at frequencies about 10 kHz due to poor calibration of the Keithley 6221 device at these frequencies. No capacitors or inductors were used to compensate for possible direct current (DC) offset, so the confounding effect of DC contribution in this study cannot be ruled out. At the same time, omission of output capacitors/inductors avoided distortion of the rectangular pulse shape.

Baseline respiratory frequency and the response to hypoxia (10% O_2 balanced N_2) were recorded to determine the effectiveness of KHFAC modulation of the CSN. Respiratory and cardiac variables were measured by intercostal EMG and ECG surface electrodes, respectively.

The effect of continuous KHFAC modulation on insulin sensitivity and glucose tolerance was tested in animals from group 2 (diabetes-disease model). After CSN electrodes implantation, animals from group 2 were randomly divided into two groups. Half of the animals were submitted to continuous KHFAC modulation of the CSN for 9 weeks. KHFAC modulation was not applied to animals in the sham group. To evaluate the reversibility of CSN activity after 9 weeks of KHFAC modulation, the animals were monitored for 5 weeks after cessation of KHFAC modulation for insulin sensitivity, glucose tolerance and ventilatory variables (Fig. 4.1).

4.3.4. Experimental design for animal tests

At baseline and before submitting the animals to surgical procedures, animals were evaluated periodically (every 2 or 3 weeks) for fasting glucose, insulin sensitivity

and glucose tolerance (Sacramento *et al.*, 2017). After the surgical procedure the animals in group 2 were kept on the HFHSu diet to continue exposure to disease-promoting factors. Fasting glucose, insulin sensitivity, glucose tolerance and weight were evaluated at several time points. Blood was collected from the tail vein at the end of the OGTT to quantify serum mediators. At the terminal experiment, animals were anaesthetised with pentobarbitone (60 mg/kg, i.p.) and mean arterial pressure (MAP) was measured (Conde *et al.*, 2012b). Blood was then collected by heart puncture for quantification of soluble biomarkers (Conde *et al.*, 2012b; Ribeiro *et al.*, 2013). Fat depots were collected after an abdominal laparotomy and weighted.

4.3.4.1. Insulin Tolerance Test

Insulin sensitivity was evaluated through an ITT (Monzillo & Hamdy, 2003) in conscious animals as previously described (Ribeiro *et al.*, 2013; Sacramento *et al.*, 2017). The ITT is one of the earliest methods developed to assess insulin sensitivity in vivo and provides an estimate of overall insulin sensitivity, correlating well with the 'gold standard' hyperinsulinaemic–euglycaemic clamp (Monzillo & Hamdy, 2003). The ITT consists in the administration of an intravenous insulin (Humulin® R 100IU/ml, Lilly) bolus of 0.1 U/kg body weight in the tail vein, after an overnight fast (approx. 16 hours), followed by the measurement of the decline in plasma glucose concentration over a 15 min period. The constant rate for glucose decline (K_{ITT}) was calculated using the formula $0.693/t_{1/2}$ (Monzillo & Hamdy, 2003; Conde *et al.*, 2012b). 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. Blood samples were collected by modified tail snip technique and glucose levels were measured with a glucometer (Precision Xtra Meter, Abbott Diabetes Care, Portugal) and test strips (Abbott Diabetes Care, Portugal) (Ribeiro *et al.*, 2013; Sacramento *et al.*, 2017).

4.3.4.2. Oral Glucose Tolerance Test

Glucose tolerance was evaluated through an OGTT. For that, the animals were fasted overnight and a bolus of glucose (2 g/kg, Sigma, Madrid, Spain) was administered by oral gavage. In animals with electrodes implanted at the CSN, in order to protect the CorTecTM electrode wires running subcutaneously from the headcap dorsally round to the CSN, the animals were orally dosed for the OGTT test using a

flexible rubber nelaton dosing catheter, model 7.17.1; 10 French g, 39cm long (Harvard Apparatus UK, Cambridge, UK). This allowed a suitable handling and restraint method for dosing procedure, eliminating the need to scruff the animals and prevented damage to the subcutaneous electrodes. Blood samples were collected by modified tail snip at intervals of 0, 15, 30, 60, 120, and 180 min. Glucose levels were measured with a glucometer (Precision Xtra Meter, Abbott Diabetes Care, Portugal) and test strips (Abbott Diabetes Care, Portugal).

4.3.4.3. Whole-body plethysmography recordings of ventilation

Ventilation was measured in conscious freely moving rats by whole-body plethysmography. The system (Emka Technologies, Paris, France) consisted of 5-litre methacrylate chambers continuously fluxed (2 l/min) with gases. Tidal volume (VT; ml/kg) respiratory frequency (f; breaths/min; bpm) and minute ventilation (VE; ml/min/Kg) were monitored. Each rat was placed in the plethysmography chamber and allowed to breathe room air for 20 min to allow adaptation to chamber environment and to acquire a standard resting behavior. Animal acclimatized well during this period and enabled subsequent ventilatory parameters to be recorded according to the protocol used. The protocol consisted in submitting the animals to 20 min acclimatization followed by 10 min normoxia (20% O_2 balanced N_2) followed by 10 min hypoxia (10% O_2 balanced N_2), followed by 10 minutes normoxia, followed by 10 min hypercapnia (20% O_2 + 5% CO_2 balanced N_2) and then 10 min normoxia. The pressure change within the chamber reflecting tidal volume (VT) was measured with a high-gain differential pressure transducer. Ideally, the frequency of pressure fluctuations is identical to breathing movements; spurious fluctuations of the pressure due to animal movements were electronically rejected. The amplitude of the pressure oscillations is proportionally related to VT; a calibration of the system by injections of 0.2 to 0.5 ml air into the chamber allowed a direct estimation of VT. Pressure signals were fed to a computer for visualization and storage for later analysis with EMKA software (Emka Technologies, Paris, France).

4.3.4.4. Quantification of biomarkers: plasma insulin, C-peptide, glucagon, corticosterone, nitric oxide and lipid profile

Insulin and C-peptide concentrations were determined with commercial ELISA kits (Mercodia Ultrasensitive Rat Insulin ELISA Kit and Mercodia Rat C-peptide

ELISA Kit, respectively, Mercodia AB, Uppsala, Sweden) as previously described (Conde *et al.*, 2012b; Ribeiro *et al.*, 2013; Sacramento *et al.*, 2017). Corticosterone was obtained with DetectX corticosterone immunoassay kit (Arbor Assays, Madrid, Spain). To evaluate endothelial function and inflammation, plasma NO/NO_3^- levels were determined in all animals (Conde *et al.*, 2012b; Ribeiro *et al.*, 2013). For that, proteins from serum samples were precipitated by adding two volumes of ethanol (0°C). After 30 min on ice, samples were centrifuged in a microfuge (Eppendorf, Madrid, Spain) at 12000g for 10 min. NO/NO_3^- concentration was determined by using a specific and sensitive NO/ozone chemiluminescence technique (NO-Analyzer 280, Sievers Research Inc., Boulder Colorado). The lipid profile was assessed using a RANDOX kit (RANDOX, Irlandox, Porto, Portugal) to determine total cholesterol and triglycerides by Trinder-based colorimetric end-point assays, and HDL and LDL by a direct-HDL and direct-LDL clearance method, respectively.

4.3.4.5. Measurement of electrode impedance

Impedance was measured at 0 and 1 day post-implantation and just prior to animal's sacrifice using a handheld potentiostat (pocketSTAT, Ivium Technologies B.V., Eindhoven, Netherlands) connected to a computer running the electrochemical impedance spectroscopy (EIS) software (IviumSoft, Ivium Technologies). The impedances were measured using a two-pole setup: working and sense electrodes were connected to form the first pole, while the counter and reference electrodes were connected to form the second pole, ground electrode not connected. The EIS was performed at frequencies from 10 to 10,000Hz.

4.3.4.6. Histology

Carotid artery bifurcations were collected and processed for microcomputed tomography (microCT) and histology analysis using H&E staining or toluidine blue staining.

For histology analysis, the carotid artery (CA) area and the cuff electrodes were bilaterally dissected and immersion-fixed in PFA 4%. Samples were embedded into paraffin for routine H&E staining (4 μm thick coronal sections). Some samples were instead frozen and cut with a cryostat (20-30 μm thick) for H&E or toluidine blue staining. Some samples were used for electron microscopy analysis. Fixed samples were

post fixed in 1% aqueous osmium tetroxide and processed into Agar 100 resin.1 mm toluidine blue stained survey sections were prepared and examined by light microscopy to locate the areas of interest. Ultra-thin sections (1 μm thick) were stained with uranyl acetate and lead citrate and examined in a Hitachi H7500 transmission electron microscope. The AMT XR41 Digital Camera System v600.202 was used to capture TEM digital images. Representative digital images were taken.

For Micro CT analysis, fixed samples were incubated in 0.3% phosphotungstic acid solution (in 70% EtOH). Contrast images were then acquired with a SkyScan1176 (Bruker microCT, US) at 18μm resolution. Images were then reconstructed using NRecon Software v1.6.10.4.

4.3.5. Statistical analysis

Statistical analyses were performed using GraphPad Prism software, version 6 (GraphPad Software, La Jolla, CA, USA) and by MatLab Statistics and Machine Learning Toolbox, version 8.5 (Natick, MA, USA). The significance of the differences between the mean values was calculated by one- and two-way ANOVA with Dunnett's and Bonferroni multicomparison test, respectively. Differences were considered significant at $p < 0.05$.

4.4. Results

4.4.1. Effect of chronic bilateral CSN resection in an animal model of diet-induced T2D

We have previously demonstrated that chronic bilateral resection of CSN reverses insulin resistance and glucose intolerance in rats with metabolic syndrome (Ribeiro *et al.*, 2013; Sacramento *et al.*, 2017). However, from a clinical perspective, the modulation of CSN would have a higher impact if it showed beneficial effects in an animal model of T2D. Herein we have used a model of T2D obtained by submitting Wistar rats to a HFHSu diet for 14 weeks. Bilateral chronic CSN resection restored insulin sensitivity to baseline values (Fig. 4.3 a-c), an effect that was preserved over 11 weeks post-resection. Furthermore, CSN resection restored fasting glucose (Table 4.1) and improved glucose tolerance (Fig. 4.3 d-f, Table 4.2), and fasting plasma insulin and C-peptide (Table 4.1).

CSN resection also normalised mean blood pressure and nitric oxide levels (Fig. 4.3 h), as well as LDL-cholesterol and triacylglycerols (Table 4.3).

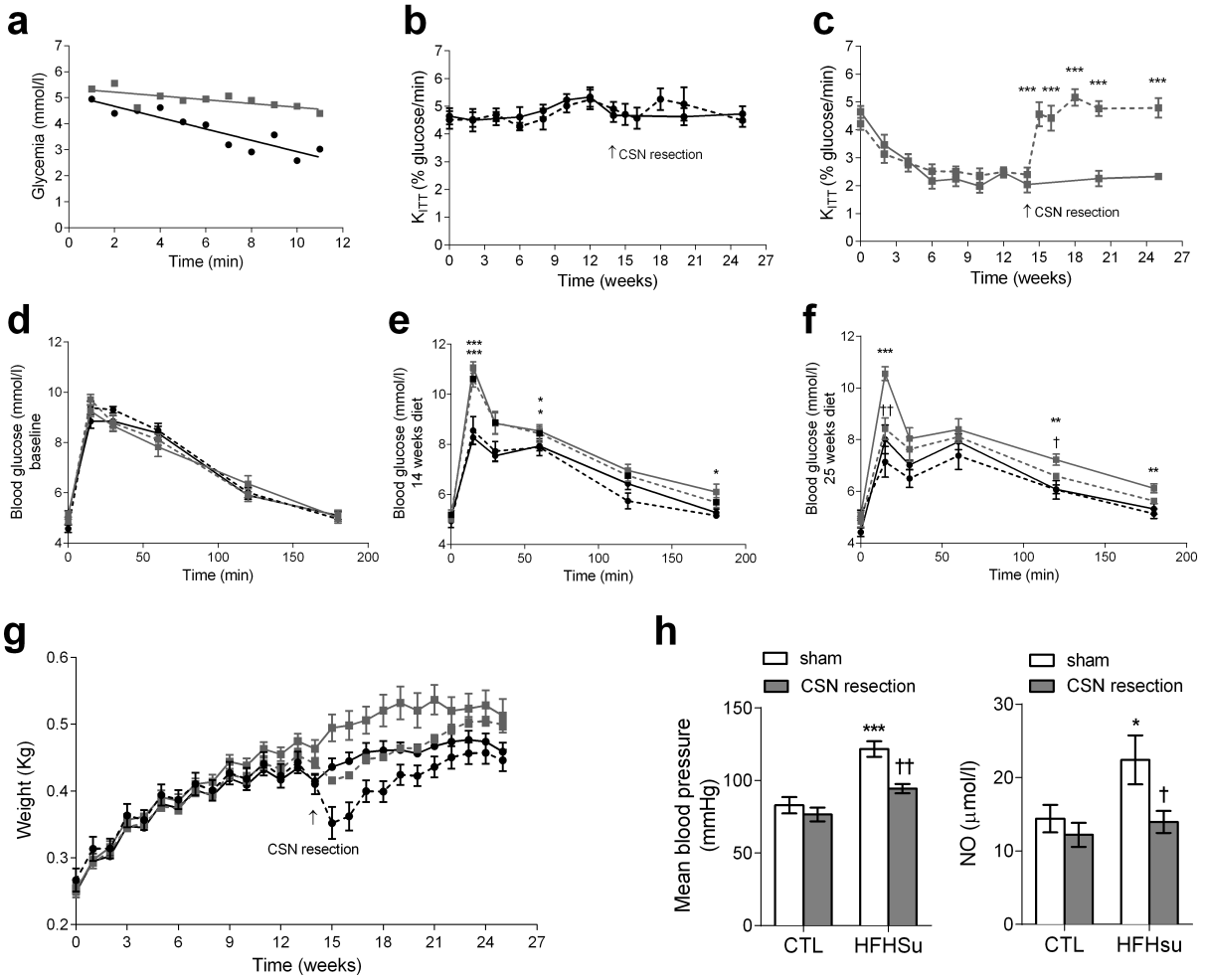


Figure 4.3 - Effect of chronic carotid sinus nerve (CSN) bilateral resection on cardiometabolic parameters in a high-fat/high-sucrose (HFHSu)-induced type 2 diabetes (T2D) animal model. a, depicted representative glucose excursion curves for insulin tolerance test in a control rat and in the HFHSu animal. **b, c,** shown the effect of CSN resection on insulin sensitivity assessed by an insulin tolerance test and expressed as the constant rate for glucose disappearance (K_{ITT}) in control (b) and HFHSu (c) animals. **d-f,** Effect of CSN resection on glucose tolerance depicted as glucose excursion curves in control and HFHSu animals at baseline (d), before CSN resection (14 weeks of diet) (e) and 11 weeks-post-CSN resection (25 weeks) (f). **g,** Effect of CSN resection on weight gain in control and HFHSu animals. **h,** Effect of CSN resection on mean arterial pressure (MAP) and endothelial function and inflammation measured as plasma NO/NO_3^- levels. CSN resection was performed at 14th week of diet and animals were maintained under their respective diets until the 25th week. (a-g) Black line, control sham; Black dotted line, control with CSN resection; Grey line, HFHSu sham; Grey dotted line, HFHSu with CSN resection. (h) White bars, without CSN resection; grey bars with CSN resection. Data are means ± SEM of 8-10 animals. One and Two-Way ANOVA with Dunnet's and Bonferroni multicomparison test: $^{**}p<0.01$, $^{***}p<0.001$ vs control; †$p<0.05$, ††$p<0.01$, with vs without CSN resection.

The surgical intervention had no effect on metabolic and haemodynamic variables in the group fed a standard diet (Fig. 4.3 a-h). All together, these results demonstrate that bilateral CSN resection improves insulin sensitivity and glucose

metabolism in a model of T2D. However, the surgical transection of these peripheral nerves is unlikely to be well-accepted by patients or clinicians. Therefore, we envisaged and applied a novel modality of 'on demand' and reversible inhibition of the nerve through KHFAC.

Table 4.1 - Effect of carotid sinus nerve (CSN) bilateral resection on fasting blood glucose and insulin and C-peptide in control (CTL) and early-T2D (HFHSu) animals.

Treatments	Models	Baseline	14 weeks of diet	25 weeks of diet
Blood glucose (mmol/l)	**CTL Sham**	5.08 ± 0.13	4.60 ± 0.13	4.81 ± 0.13
	CTL – CSN resection	5.07 ± 0.10	4.86 ± 0.29	4.74 ± 0.30
	HFHSu Sham	4.80 ± 0.15	5.40 ± 0.10***	5.47 ± 0.19***
	HFHSu – CSN resection	4.80 ± 0.19	5.38 ± 0.12**	4.90 ± 0.16†,‡
Insulin (pmol/l)	**CTL Sham**	98.0 ± 19	161.0 ± 19.5$_{*}$	199.6 ± 23.3*
	CTL – CSN resection	103.5 ± 20.8	190.2 ± 27.2*	191.9 ± 49.2*
	HFHSu Sham	110.9 ± 17.1	421.8 ± 62.5***	595.9 ± 116.4***
	HFHSu – CSN resection	104.6 ± 18.3	370.0 ± 32.1***	381.8 ± 41.7$^{***,\#}$
C-peptide (nmol/l)	**CTL Sham**	0.42 ± 0.08	0.65 ± 0.08**	0.85 ± 0.10**
	CTL – CSN resection	0.43 ± 0.13	0.79 ± 0.12**	0.76 ± 0.16**
	HFHSu Sham	0.44 ± 0.09	1.57 ± 0.27***	2.03 ± 0.39***
	HFHSu – CSN resection	0.43 ± 0.08	1.54 ± 0.19***	1.89 ± 0.30***

Data are means ± SEM of 8/10 animals. One and Two-Way ANOVA with Dunnet's and Bonferroni multicomparison test: *p<0.05, **p<0.01, ***p<0.001 *vs* control; †p<0.05, with *vs* without CSN resection; ǂp<0.05, ǂǂp<0.01 with *vs* without CSN resection at 25 weeks of diet.

Table 4.2 - Area under the curve (AUC) obtained through the analysis of the glucose excursion curves in control (CTL) and early-T2D (HFHSu) animals with or without carotid sinus nerve (CSN) bilateral resection.

Area under curve (mmol/l x min)	Baseline	14 weeks of diet	25 weeks of diet
CTL Sham	1254 ± 27	1233 ± 26	1192 ± 25
CTL – CSN resection	1276 ± 17	1193 ± 43	1141 ± 58
HFHSu Sham	1256 ± 38	1387 ± 28**	1372 ± 31**
HFHSu – CSN resection	1252 ± 29	1353 ± 26**	1263 ± 22†

Data are means ± SEM of 8/10 animals. One and Two-Way ANOVA with Dunnet's and Bonferroni multicomparison test: **p<0.01, *vs* control; †p<0.05, with *vs* without CSN resection.

Table 4.3 - Effect of carotid sinus nerve (CSN) bilateral resection on total, visceral/perienteric, epididymal and perinephric fat and on the lipid profile (total cholesterol, HDL, LDL and triacylglycerols) in control (CTL) and early-T2D (HFHSu) animals.

	CTL Sham	CTL – CSN resection	HFHSu Sham	HFHSu – CSN resection
Total fat (g/kg)	66.44 ± 3.93	64.14 ± 5.16	113.9 ± 9.73***	104.2 ± 7.64
Perienteric fat (g/kg)	15.58 ± 0.82	14.42 ± 1.37	24.44 ± 2.80**	22.97 ± 1.74
Perinephric fat (g/kg)	25.78 ± 1.75	26.03 ± 1.74	52.57 ± 4.45***	44.02 ± 3.22†
Epididymal fat (g/kg)	25.08 ± 1.52	23.68 ± 2.77	36.90 ± 2.83**	37.17 ± 3.19
Total cholesterol (mmol/l)	1.90 ± 0.25	1.98 ± 0.11	2.00 ± 0.16	1.74 ± 0.13
LDL (mmol/l)	0.24 ± 0.03	0.19 ± 0.02	0.40 ± 0.05*	0.22 ± 0.03†
HDL (mmol/l)	0.63 ± 0.04	0.65 ± 0.04	0.59 ± 0.04	0.60 ± 0.06
Triacylglycerols (mmol/l)	0.90 ± 0.14	0.93 ± 0.16	1.53 ± 0.13**	0.89 ± 0.12††

Data are means ± SEM of 8/10 animals. One and Two-Way ANOVA with Dunnet's and Bonferroni multicomparison test: *p<0.05, **p<0.01, ***p<0.001 vs control; †p<0.05, ††p<0.01with vs without CSN resection.

4.4.2. Establishing KHFAC parameters for CSN inhibition

We established the KHFAC modulation parameters required to inhibit CSN activity by testing the effect of KHFAC on the classical physiological stimulus of the CB: hypoxia (Gonzalez *et al.*, 1994). A 1 min exposure to hypoxic air (10% O_2 + 90% N_2) resulted in a reproducible and significant ($p = 3.04 \times ^{-10}$) increase in respiratory rate from baseline to about 30-60 s after induction in the sham group (100.4 ± 1.55 bpm *vs* 130.9 ± 4.08 bpm) (Fig. 4.4 a, b). Notably, no significant difference was seen over the first 30 s of hypoxia, probably due to the time required for blood saturation (Fig. 4.4). KHFAC, applied for 1 min (increasing in frequency from 30-50 kHz), significantly reduced the respiratory response to hypoxia compared with the sham group (30 kHz, 111.7 ± 2.87; 40 kHz, 109.8 ± 3.13; 50 kHz, 104.1 ± 2.86; 50 kHz at 2 mA, 103.4 ± 2.54) Fig. 4.4 a-c).

Figure 4.4c shows a dose-response relationship between the frequency of KHFAC and efficiency at preventing the respiratory effects of hypoxia. Frequencies of 30 kHz and 40 kHz did not provide a 100% suppression of the respiratory rate, as evidenced by a remaining significant difference in ventilatory response in the final 30 s compared with baseline. However, at 50 kHz, the respiratory rate during hypoxia was not significantly different from baseline, indicating an almost complete suppression.

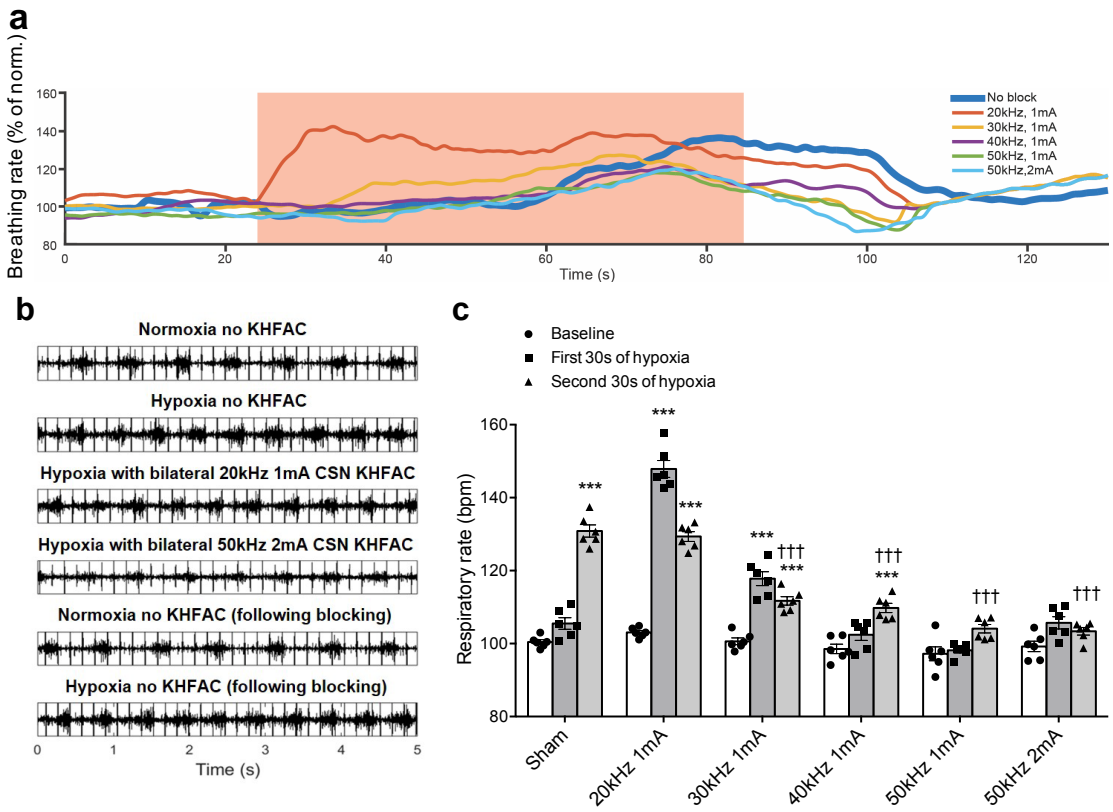


Figure 4.4 - Effect of KHFAC modulation of the CSN on cardiorespiratory responses to hypoxia. (**a**) Cardiorespiratory responses to hypoxia over time with and without KHFAC modulation (period of hypoxia shown by red shaded area). Norm., normoxia. (**b**) Raw data traces of EMG and ECG in normoxic and hypoxic conditions with and without KHFAC modulation. (**c**) Quantification of respiratory rate change in response to hypoxia with and without KHFAC modulation. Data points represent individual animals and bars represent overall mean respiratory frequency. Data represent means ± SEM. One- and two-way ANOVA with Bonferroni multicomparison test: ***p<0.001 vs baseline within the stimulation group; †††p<0.001 *vs* sham.

At 20 kHz, no inhibition of respiration frequency was observed compared with sham levels. Furthermore, at 20 kHz an exacerbation of the hypoxic respiratory response occurred over the first 30 s (43.5 ± 6.29%) significantly higher than in sham stimulations (p < 0.05) (Fig. 4.4 c), suggesting neural activation rather than inhibition. A smaller exacerbation in response was seen at 30 kHz. The KHFAC applied at 50 kHz and 2 mA produced the most robust inhibition of cardiorespiratory responses to hypoxia without any onset cardiac or respiratory responses (Fig. 4.4 c); therefore, 50 kHz was used for long-term KHFAC modulation to provide superior safety and efficacy.

After each KHFAC application, the reversibility of its ef- fect on the CSN was confirmed by the recovery of respiratory responses to hypoxia (Fig. 4.4 b). KHFAC modulation had no effect on respiratory response during normoxic air breathing (data

not shown).

These results demonstrate that KHFAC modulation applied at specific frequencies and amplitudes, specifically 40-50 kHz at 1-2 mA, can suppress the hypoxia response without causing an abnormal physiological onset response, and that the observed effect is fully reversible.

4.4.3. Effect of chronic KHFAC modulation of the CSN on animal behaviour and ventilation

Previously, there have only been a few studies on the effects of chronic KHFAC modulation (Cuellar *et al.*, 2013; Kilgore & Bhadra, 2014; Patel *et al.*, 2017), none of which reported the effect on the CSN. To evaluate the impact of KHFAC modulation on the CSN, 50 kHz square pulses at 2 mA (peak-to-peak) were applied continuously for 1 week in healthy animals fed a standard diet. No behavioural alterations were observed and the animals responded well to headcaps and tethers. Following cessation of KHFAC modulation, animals were exposed to a hypoxic stimulus (10% O_2 balanced N_2) and the respiratory response was evaluated at two time points: 20 min and 1 week post-KHFAC. At 20 min post-KHFAC, animals presented a decrease in basal ventilation and did not respond to the hypoxic challenge, demonstrating that a functional neuromodulation was maintained (Fig. 4.5).

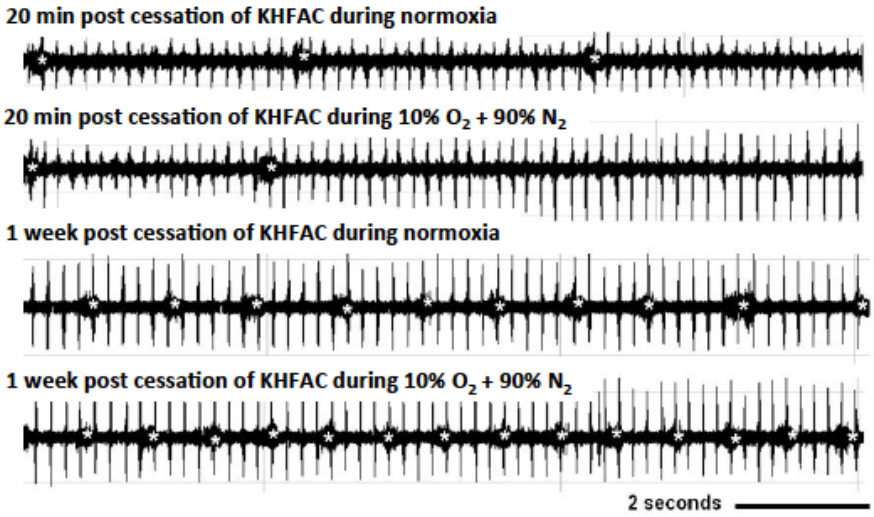


Figure 4.5 - Effect of one-week of kilohertz alternating frequency current (KHFAC) on EMG recording in a standard-diet animal. Panel shows respectively, from the top to the bottom, EMG recording 20 min after electrical block cessation, in normoxia and hypoxia and EMG recording one-week after block cessation in normoxia and hypoxia. *represents respiratory burst.

At 1 week post-KHFAC, the basal ventilation and the response to hypoxia returned to baseline levels (increase in respiratory rate of ~20%) (Fig. 4.5). The decrease in basal ventilation and abolishment of hypoxic ventilatory responses demonstrate that suppression of the CB response was produced in conscious animals via KHFAC modulation of the CSN. Reversibility of the suppression was also demonstrated by the recovery of hypoxia sensitivity, suggesting that long- term application of KHFAC did not cause permanent pathological changes in the nerve conduction properties.

4.4.4. Effect of chronic KHFAC modulation of the CSN on insulin sensitivity and glucose homeostasis and its reversibility in an animal model of diet-induced T2D

Effect of KHFAC modulation of the CSN on insulin sensitivity and glucose homeostasis was evaluated in HFHSu-fed animals (Fig. 4.6 a-f, Table 4.4). As expected, HFHSu-fed animals were insulin-resistant (data not shown) and exhibited increased fasting insulin and C- peptide levels (Table 4.4). Electrode implantation on the CSN had minimal impact upon K_{ITT} (Fig. 4.6a; K_{ITT} HFHSu sham, 2.87 ± 0.22% glucose/min; HFHSu KHFAC, 2.70 ± 0.24% glucose/min) and on fasting insulin and C-peptide levels (Table 4.4) measured 2 weeks post-implantation. Furthermore, glucose tolerance post-surgery, measured by an OGTT, confirmed that the glycaemic response to a stimulus was not impacted by implantation surgery (Fig. 4.6 b, c).

The electrical impedance spectroscopy at 1 day post-implantation confirmed the stability of the cuff-nerve interface, with the average impedance of 8.8 ± 1.5 kΩ at 1 kHz (Fig. 4.7).

HFHSu-fed animals were then randomised and KHFAC modulation was applied in half of the animals. After 1 week of KHFAC, a significant increase in insulin sensitivity (Fig. 4.6 a) and a decrease in glucose intolerance was observed (Fig. 4.6 b, c), with no effects on insulin or C-peptide levels (Table 4.4). These effects on glucose metabo- lism were maintained over 9 weeks of KHFAC modulation (K_{ITT} HFHSu sham, 2.56 ± 0.41%glucose/min; K_{ITT} HFHSu KHFAC, 5.01 ± 0.52%glucose/min; AUC glucose excursion curve HFHSu sham, 1278 ± 20.36 mmol/l × min; AUC glucose excursion curve HFHSu KHFAC, 1054.15 ± 62.64 mmol/l × min) and were subsequently reversed over the 5 weeks following cessation of KHFAC, evidenced by the re-emergence of insulin resistance and glucose intolerance (Fig. 4.6 a-f, Table 4.4).

Full reversibility of KHFAC- induced suppression of respiratory responses to hypoxia was observed at 10 days after the cessation of KHFAC modulation (Fig. 4.8, Table 4.5).

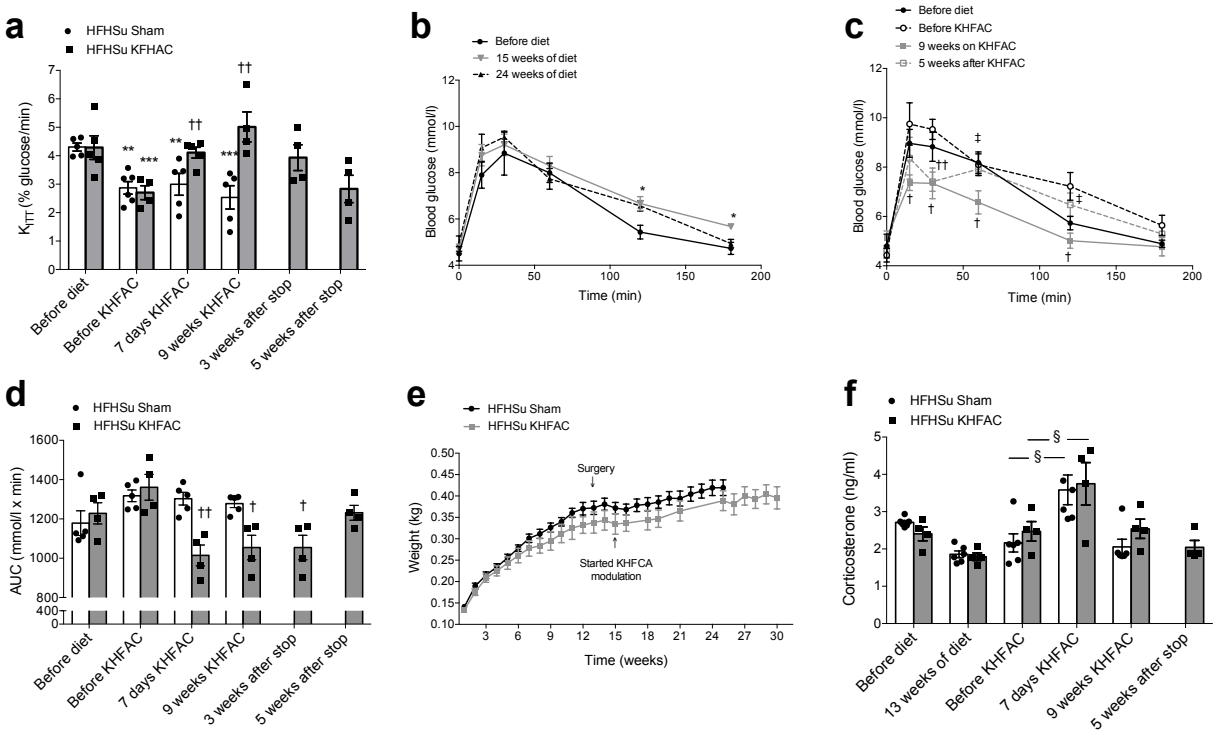


Figure 4.6 - Effect of KHFAC modulation of the CSN on cardiometabolic variables and stress responses in HFHSu-induced type 2 diabetes and its reversibility. (**a**) Effect of KHFAC on insulin sensitivity assessed by an ITT and expressed as the constant rate for glucose disappearance (K_{ITT}) in HFHSu-fed animals. (**b**) Glucose excursion curves in HFHSu sham animals before diet, after 15 weeks of diet and at 24 weeks of diet. (**c**) Glucose excursion curves in HFHSu KHFAC animals before diet, prior to the initiation of KHFAC, during 9 weeks of KHFAC, and 5 weeks post-KHFAC. (**d**) Effect of KHFAC modulation on the AUC obtained through the analysis of glucose excursion curves. (**e**) Effect of KHFAC modulation on weight gain curves in HFHSu sham and HFHSu KHFAC animals. (**f**) Effect of KHFAC modulation of the CSN on corticosterone levels in HFHSu-fed animals. Sling cuffs were implanted at week 13 of the HFHSu diet; 2 weeks post-surgery KHFAC stimulation was started. Sham animals represent HFHSu-fed animals with electrical cuff implants but no KHFAC stimulation. Data are means ± SEM of 4–5 animals. One- and two-way ANOVA with Dunnett's and Bonferroni multicomparison tests: *p < 0.05, **p < 0.01, ***p < 0.001 vs chow-fed controls; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ *vs* sham; $^{\ddagger}p < 0.05$, $^{\ddagger\ddagger}p < 0.01$ *vs* 9 weeks of KHFAC; § before KHFAC *vs* 7 days of KHFAC.

Table 4.4 - Effects of KHFAC modulation of the CSN on fasting blood glucose, insulin and C-peptide in HFHSu-fed animals.

	Models	Before diet	13 weeks of diet	Before KHFAC	9 weeks block	5 weeks after stop
Blood glucose (mmol/l)	HFHSu sham	4.14 ± 0.31	5.06 ± 0.22*	4.83 ± 0.24	6.15 ± 0.26***,†	
	HFHSu block	4.45 ± 0.22	5.24 ± 0.32	4.96 ± 0.47	5.54 ± 0.32	4.83 ± 0.23
Insulin (pmol/l)	HFHSu sham	99.69 ± 4.65	388.34 ± 25.83*	372.95 ± 31.63*	488.83 ± 107.45**	
	HFHSu block	103.41 ± 6.47	426.38 ± 13.45*	445 ± 61.21**	410.48 ± 72.71*	425.81 ± 90.26*
C-peptide (nmol/l)	HFHSu sham	0.44 ± 0.44	1.22 ± 0.27*	1.37 ± 0.133*	2.45 ± 0.34**	
	HFHSu block	0.50 ± 0.05	1.12 ± 0.14*	1.22 ± 0.15*	2.33 ± 0.34**	2.675 ± 0.18**

Data are means ± SEM of 4-5 animals. One- and two-way ANOVA with Dunnett's and Bonferroni multicomparison test: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs chow-fed controls; $^{\dagger}p < 0.05$ vs HFHSu KHFAC.

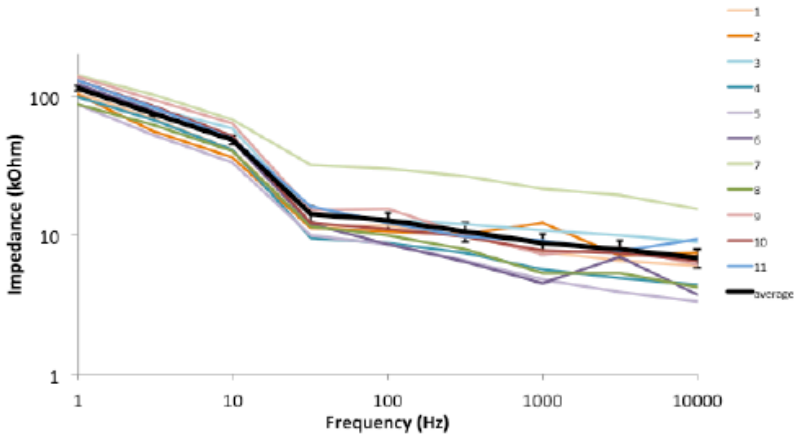


Figure 4.7 - Electrical impedance of the eleven cuff electrodes implanted bilaterally at the carotid sinus nerve (CSN).

Corticosterone levels were evaluated periodically to assess the stress response to KHFAC modulation. As seen in Fig. 4.6f, corticosterone levels were increased 1 week after initiation of KHFAC modulation. This effect, however, cannot be directly attributed to KHFAC, as the non-stimulated sham group also presented an increase in hormonal levels. Furthermore, at 9 weeks after KHFAC modulation, corticosterone values returned to the baseline level and remained low after cessation of KHFAC. No behavioural alterations were observed.

Table 4.5 - Effects of KHFAC modulation of the CSN on basal ventilation (frequency, VT and VE) in chow-fed controls and in HFHSu-fed animals with or without KHFAC modulation.

	Control	HFHSu sham	HFHSu KHFAC [a]
Frequency (bpm)	83.38 ± 7.65	84.68 ± 6.44	81.20 ± 6.32
VT (ml/kg)	6.72 ± 0.28	5.67 ± 0.33	6.06 ± 0.33
VE (ml min^{-1} kg^{-1})	562.0 ± 62.3	488.5 ± 10.6	471.5 ± 57.7

Data are means ± SEM of 4–5 animals. [a] Basal ventilatory recordings were performed immediately post-KHFAC.

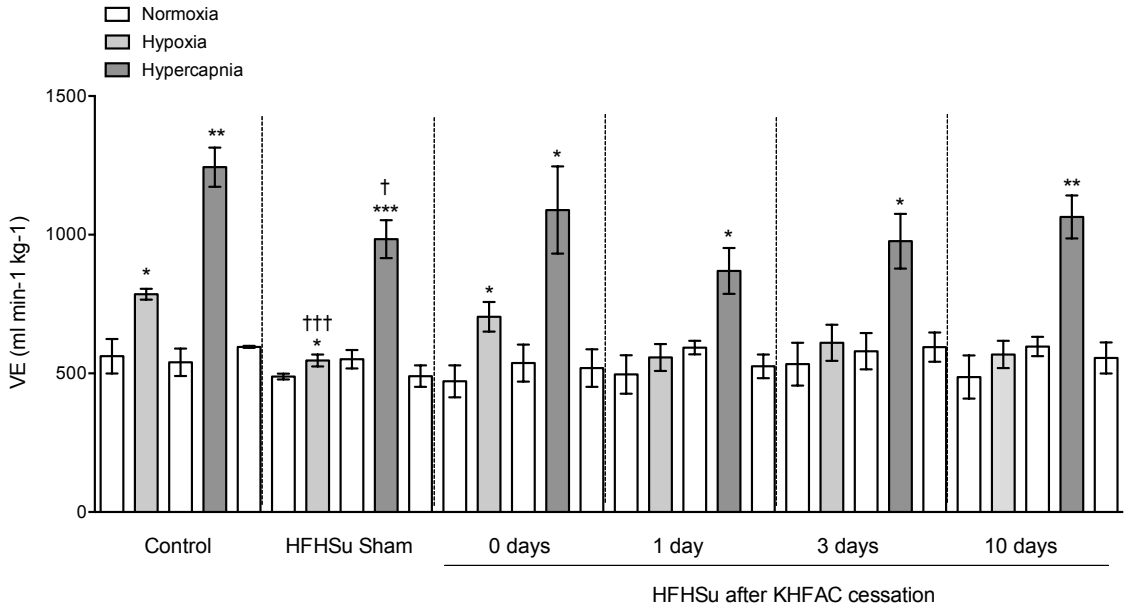


Figure 4.8 - Impact of KHFAC modulation of the CSN on ventilatory responses to hypoxia and hypercapnia in age-matched chow-fed control, HFHSu sham and HFHSu KHFAC animals. Ventilatory recordings were performed immediately post-KHFAC and at 1 day, 3 days and 10 days after cessation of KHFAC. Ventilatory recordings were performed in freely moving animals and the protocol consisted of 10 min normoxia (20% O_2), followed by 10 min hypoxia (10% O_2), followed by 10 min normoxia, followed by 10 min hypercapnia (20% O_2 + 5% CO_2) and finally by 10 min normoxia. Sham animals represent HFHSu animals with electrical cuffs implanted but not submitted to KHFAC. Data are means ± SEM of 4–5 animals. One- and two-way ANOVA with Dunnett's and Bonferroni multicomparison test: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ vs normoxia applied immediately before hypoxic or hypercapnic stimuli; $^{\dagger}p < 0.05$, $^{\dagger\dagger\dagger}p < 0.001$ vs age-matched control animals.

4.4.5. Effect of chronic KHFAC modulation on the axonal and myelin integrity of the CSN

Correct placement of and tissue response to the sling cuffs were evaluated post-mortem by light and electron microscopy, histology and by contrast microCT imaging.

The microCT imaging was used in HFHSu sham and HFHSu KHFAC animals to visualise placement of the electrical cuffs on the CSN in the dissected tissue block with common carotid artery bifurcation (Fig. 4.9).

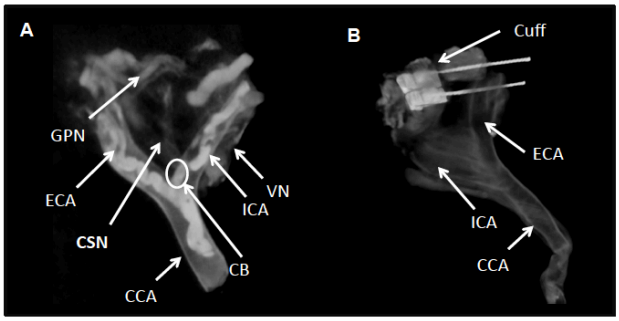


Figure 4.9 - Micro CT of carotid artery bifurcations with (A) or without (B) electrode cuff implantations at the carotid sinus nerve (CSN). CCA – common carotid artery; ECA – external carotid artery; GPN – glossopharyngeal nerve; ICA – internal carotid artery; VG – vagus nerve.

Next, a qualitative histology analysis was performed to evaluate possible damage to the CSN by a foreign body response to the implanted cuffs and long-term application of KHFAC. Transverse haematoxylin/eosin staining of sections from sham-stimulated animals evidenced no discernible signs of the nerve damage (Fig. 4.10 a, b). In Fig. 4.10b intact nerve tissue can be seen in intimate contact with the sling cuff around the CSN. Evaluation of the CSN adjacent to the cuff by electron microscopy indicated a similarly minor amount of myelin damage in both the sham and stimulated animals (Fig. 4.10 c, d). In Fig. 4.10 e-h, frozen and paraffin-embedded sections stained with toluidine blue and haematoxylin/eosin, respectively, demonstrate long-term infiltration of immune cells (e.g. macrophages and neutrophils), as well as chronic adipose tissue deposition that occurs during the foreign body reaction process to implanted material (see white and black arrowheads in Fig. 4.10 f-h).

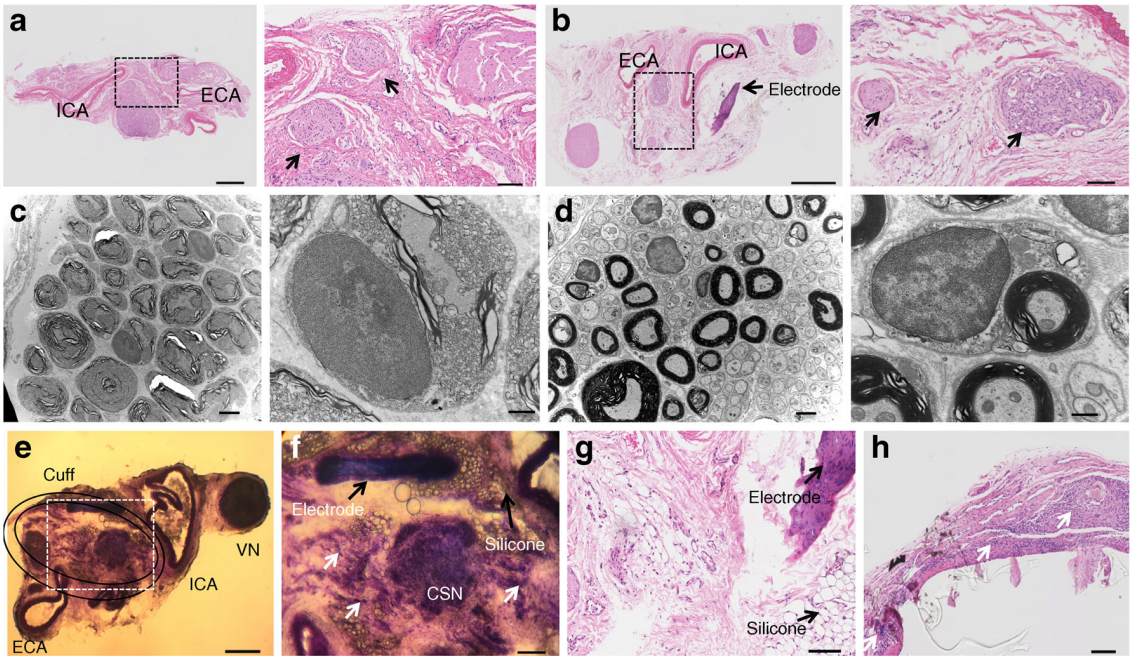


Figure 4.10 - Impact of KHFAC modulation of the CSN on CSN histology. Low and high magnification pictures of 4 µm thick sections stained with haematoxylin–eosin of the left and right carotid bifurcation area isolated from rats in the (**a**) HFHSu sham group and (**b**) HFHSu KHFAC group (scale bars, 500 µm and 100 µm for low and high magnification, respec- tively); black arrows indicate putative CSN sections. Electron microscopy images of the CSN at low and high magnification of the left and right CSN isolated from rats in the (**c**) HFHSu sham group and (**d**) HFHSu KHFAC group (scale bars, 2 µm and 500 nm for low and high magnification, respectively). (**e**) Low magnification image of a 30 µm thick section of the carotid bifurcation area, stained with toluidine blue, isolated from an HFHSu animal implanted with an electrical cuff (scale bar, 500 µm). (**f–h**) High magnification images of the carotid bifurcation area (scale bars, (f) 200 µm; (g, h), 100 µm); black arrows roughly indicate the position of the implanted cuff as well as fragments of elec- trodes and silicone; white arrows indicate areas of fibrosis characterised by high density of cells. ECA, external carotid artery; ICA, internal carotid artery; VN, vagus nerve.

4.5. Discussion

We describe that electrical modulation of the CSN restores metabolic homeostasis in an animal model of early T2D. Neuromodulation of the CSN was achieved by application of bilateral KHFAC through surgically implanted cuff electrodes. The beneficial effects of KHFAC on glucose tolerance and insulin sensitivity were reversed after discontinuation of the electrical stimulus. Together, these findings sup- port a potential role for bioelectronic medicines in the treatment of T2D.

In this study, we provide further evidence that CSN overactivity is linked to the development of diet-induced insulin resistance and glucose intolerance (Ribeiro *et al.*, 2013; Sacramento *et al.*, 2017). The CB chemoreceptors have been previously

associated with the aetiology of cardiometabolic diseases both in animal models (Abdala *et al.*, 2012; Del Rio *et al.*, 2013; Marcus *et al.*, 2014) and in humans (Niewinski *et al.*, 2013; Niewinski *et al.*, 2014; Narkiewicz *et al.*, 2016).

Approaches that specifically target the CB may represent a putative therapeutic strategy to pharmacological- treatment-resistant metabolic diseases. However, surgical procedures that are currently offered present several drawbacks. Unilateral ablation of a single CB has been shown to reduce arterial pressure in individuals with hypertension; however, efficacy is diminished 12 months after ablation, suggesting a compensation of the remaining CB and the need for bilateral ablation to achieve a sustained therapeutic effect (Narkiewicz *et al.*, 2016). Additionally, bilateral surgical removal of the CB or the CSN is prone to side effects related to both the chemosensory and baroreceptor functions of the carotid sinus. Furthermore, even bilateral ablation may be non-sustainable in the long term, since the CSN nerve fibres regenerate with time. Herein, we show that bilateral KHFAC modulation of the CSN may be an alternative approach since it mimics the beneficial metabolic effects of the bilateral surgical resection of the CSN combined with the advantages of being tuneable and fully reversible.

KHFAC modulation at 10-70 kHz has been suggested as an effective method to suppress nerve conduction, since action potentials are arrested as they pass the depolarising charge field of the electrode (Kilgore & Bhadra, 2014; Patel *et al.*, 2017). In this study, we demonstrated that acute application of KHFAC induces nearly complete inhibition of the relevant physiological response: decreased ventilation and hypoxic response. The effect of KHFAC modulation lasted for 20 min and caused no permanent nerve dam- age, as indicated by a full recovery of ventilation and hypoxic responses to pre-KHFAC values at 1 week after cessation of KHFAC modulation. The ‘onset response’, a transitory end-organ activation that commonly occurs at 10 kHz (and below) in motor nerves (Bhadra & Kilgore, 2005; Lothet *et al.*, 2014), was evident as an enhanced cardio-respiratory response at 20 kHz modulation of the CSN, less evident at 30 kHz and not observed at 50 kHz. Also, the stress response to 9 week KHFAC modulation of the CSN at 50 kHz with a 2 mA current was minimal, as assessed by corticosterone plasma levels.

Effective nerve modulation with KHFAC requires an electrode design that guarantees a uniform delivery of current to the nerve. Herein, we used sling cuff electrodes consisting of an insulating outer layer and nearly circumferential metal electrode contacts inside the cuff, as similar cuffs have been shown to be effective in

KHFAC modulation of other nerves (Kilgore & Bhadra, 2004; Foldes *et al.*, 2011). The long-term stability of the cuff placement was confirmed by qualitative histology analysis and by evaluating electrode impedance. Reversibility of KHFAC was also dem- onstrated, since the CSN recovers its ability to conduct action potentials 1 week after cessation of chronic KHFAC pulsing.

A lower KHFAC frequency of 5 kHz has been evaluated in human clinical trials. Intermittent KHFAC modulation (5 min on and 5 min off, amplitudes ranging from 1–6 mA) of the intra-abdominal vagus nerve, using an implanted spiral cuff has been recently trialled in nearly 200 individuals as a therapy for appetite suppression and obesity control (Camilleri *et al.*, 2009; Sarr *et al.*, 2012). Subjects were followed for 1 year and their weight loss was found to be linearly correlated with the duration of KHFAC modulation. There were no significant adverse events related to the KHFAC therapy. KHFAC modulation has also been used for temporary (10 min) pain relief with a cuff placed on the sciatic and tibial nerves (Soin *et al.*, 2015). Therefore, the use of KHFAC modulation could provide an effective and safe option for treatment of various chronic disorders.

In conclusion, the present study shows that KHFAC modulation of the CSN improves metabolic control in a rodent model of early T2D, an effect that is long-lasting and that persists despite the continued influence of disease- promoting factors, such as a high-energy diet. According to our results, KHFAC modulation of the CSN has significant potential as a means of controlling CSN activity for the purpose of treating metabolic diseases.

V

This chapter is based on the following manuscript:

Modulation of adenosine and/or ATP signalling in the carotid body: potential therapeutic target for type 2 diabetes

Sacramento JF, Cunha-Guimarães JP, Prego CS, Quintão C, Conde SV. (2018) (submitted)

5. Modulation of adenosine and/or ATP signalling in the carotid body: potential therapeutic target for type 2 diabetes

5.1. Abstract

Aims/hypothesis: The carotid body (CB) is a metabolic sensor involved in energy homeostasis whose overactivation is known to be involved in the genesis and maintenance of metabolic diseases. Knowing that adenosine and ATP are key mediators in CB hypoxic chemotransduction, herein we investigated the role of adenosine and ATP in CB/CSN overactivity induced by high-fat (HF) diet aiming a possible therapeutic intervention in the context of metabolic diseases.

Methods: Rats fed a chow or HF (60% energy from fat) diet over 21 days. CSN activity was characterized in HF and control rats in normoxia and in response to hypoxia (0 and 5%O_2) and hypercapnia (10%CO_2). Basal and hypoxic CSN chemosensory activity were evaluated in the absence and in the presence of adenosine and ATP receptors antagonists.

Results: Four different types of CSN action potentials were identified in control and HF animals. HF diet increased by 63.65% basal CSN discharges. CSN discharges evoked by both hypoxic intensities and hypercapnia were similar between control and HF animals. AF-353, a $P2X_3$ receptor antagonist, and ZM241385, and SCH58261, an A_2 and A_{2A} adenosine receptor antagonists, respectively, restored CSN discharges in HF animals. ATP exhibited a higher contribution to CSN response to 0%O_2 in both control and HF animals whereas adenosine presented a higher contribution to the response to 5%O_2. The different action potentials identified in control and HF animals followed this profile.

Conclusion: ATP and adenosine are involved in basal CSN overactivity induced by the HF diet, mainly via $P2X_3$ and A_{2A} receptors, respectively. HF diet did not modify CB hypoxic and hypercapnic responses, but increased the contribution of ATP and adenosine to hypoxic CB chemotransduction. Since, ATP is mainly involved in the response to high intensity hypoxias that are less prone to appear, we suggest that modulation of ATP signalling in the CB via $P2X_3$ receptors could be a therapeutic target for type 2 diabetes.

5.2. Introduction

The CB is a peripheral sensor for arterial blood O_2, CO_2 and pH, initiating respiratory and cardiovascular reflex responses to maintain homeostasis (Gonzalez *et al.*, 1994; Marshall, 1994). Adenosine and ATP have been described as key excitatory neurotransmitters involved in the hypoxic carotid body (CB) chemotransduction process (Buttigieg & Nurse, 2004; Conde *et al.*, 2012a). It has been described in *in vivo* and *in vitro* studies that ATP increases carotid sinus nerve (CSN), the CB sensitive nerve, activity, in a dose-dependent manner (Dontas, 1955; McQueen & Ribeiro, 1983; Alcayaga *et al.*, 2000). This excitatory effect of ATP on the CSN chemosensory activity was shown to be due to ATP itself and not to its degradation into adenosine since the non-degradable ATP agonist, αβ-methylene ATP, increased CSN discharges in rats (McQueen *et al.*, 1998) and mice (Rong *et al.*, 2003). ATP exerts its effects on post-synaptic $P2X_2$ and $P2X_3$ receptors as it has been shown that these subtypes of ATP receptors are present in rat CB afferent terminals (Zhang *et al.*, 2000; Prasad *et al.*, 2001). The confirmation of the significance of ATP to CB responses to hypoxia come up from the findings that mice deficient in $P2X_2$ subunits showed a substantial decrease in the CSN responses to hypoxia as well as decreased hypoxic ventilatory responses (Rong *et al.*, 2003). More recently, Pijacka *et al.* (2016) also demonstrated the importance of ATP on pathological conditions by showing that $P2X_3$ receptor mRNA expression is upregulated in CB chemosensory afferent neurons in spontaneous hypertensive rats and that the pharmacological blockade of $P2X_3$ receptors reduced arterial pressure and basal sympathetic activity and normalized CB hyperreflexia present in these hypertensive rats.

Adenosine, a catabolic product and precursor of ATP, has also been described as an excitatory neurotransmitter in the CB (Conde *et al.*, 2017a). Like ATP, adenosine also increased the CSN discharge (McQueen & Ribeiro, 1981; Vandier *et al.*, 1999) in a dose-dependent manner (Runold *et al.*, 1990) being also involved in CB responses to hypoxia (Conde *et al.*, 2009; Conde *et al.*, 2012a; Conde *et al.*, 2017a). The effect of adenosine on CSN chemosensory discharges elicited by hypoxia was shown to be mediated by both, A_{2A} and A_{2B} adenosine receptors (Conde *et al.*, 2006; Conde *et al.*, 2012a), present post and presynaptically, respectively and depending on the hypoxic intensity (Conde *et al.*, 2012a). In response to hypoxia, adenosine was shown to contribute to generate CSN activity in moderate hypoxias while ATP showed a more pronounced role during high-intensity hypoxias (Conde *et al.*, 2012a). Apart from the

role of adenosine in mediating CB physiological responses to acute hypoxia, it has been shown that adenosine is also involved in CB sensitization to chronic sustained (Conde *et al.*, 2012c; Salman *et al.*, 2017) and intermittent hypoxia (Sacramento *et al.*, 2015).

In the last years, the CB has also been described as a metabolic sensor involved in the control of energy homeostasis (Koyama *et al.*, 2000; Wehrwein *et al.*, 2010; Ribeiro *et al.*, 2013; Conde *et al.*, 2017b). We have previously shown that the abolition of CB activity through CSN resection or by chronic continuous kilohertz frequency alternate current application in the CSN, restored insulin sensitivity and glucose tolerance in metabolic syndrome and type 2 diabetes (T2D) animal models, via a re-establishment of sympathetic nervous system activity (Sacramento *et al.*, 2017; Sacramento *et al.*, 2018a). However, CSN resection will not be well accepted as a therapy as it is prone to produce side effects (Wade *et al.*, 1970; Honda, 1985; Timmers *et al.*, 2003; Dahan *et al.*, 2007) and CSN bioelectronic modulation will require an invasive intervention, and therefore might not be suitable for all patients.

Herein, we evaluated the role of ATP and adenosine on the dysfunction of CB/CSN produced by hypercaloric diets and responsible for the deregulation of glucose homeostasis. We hypothesize that the modulation of ATP and/or adenosine signalling in the CB might normalize the overactivated CB in prediabetes and T2D animal models, representing this approach a new treatment for T2D.

5.3. Methods

5.3.1. Animals and diet

Experiments were performed in male Wistar rat (3-4-month-old) obtained from the vivarium of the NOVA Medical School|Faculdade de Ciências Médicas, Universidade Nova de Lisboa, Lisboa, Portugal. Two groups of animals were used: the control (CTL) group that fed a regular chow diet (7.4% fat, 17% protein and 75% carbohydrate (4% sugar); Dietex International Limited, France), and the high-fat (HF) group that fed a lipid-rich diet (60% energy from fat: 34.9% fat, 23.1% protein, 25.9% carbohydrate and 6.5% fiber; Test Diet, San Luis, USA) for 21 days. The HF model combined obesity, dyslipidaemia, insulin resistance and hypertension (Conde *et al.*, 2012b; Sacramento *et al.*, 2017).

Animals were kept under temperature and humidity control (21 ± 1°C; 55 ± 10% humidity) with a 12 h light/12 h dark cycle and were given ad libitum access to food

and water. Body weight was monitored every week, and energy and liquid intake were monitored daily. Laboratory care was 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 NOVA Medical School|Faculdade de Ciências Médicas Ethics Committee.

5.3.2. Insulin tolerance test

Insulin sensitivity was evaluated through an insulin tolerance test (ITT) (Monzillo & Hamdy, 2003) in conscious animals as previously described (Ribeiro *et al.*, 2013; Sacramento *et al.*, 2017). Briefly, the ITT consists in the administration of an intravenous insulin (Humulin® R 100IU/ml, Lilly, Portugal) bolus of 0.1 U/kg body weight in the tail vein, after an overnight fast (approx. 16 hours), followed by the measurement of the decline in plasma glucose concentration over a 15 minutes' period. The constant rate for glucose decline (K_{ITT}) was calculated using the formula $0.693/t_{1/2}$ (Monzillo & Hamdy, 2003; Conde *et al.*, 2012b). 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. Blood samples were collected by modified tail snip technique and glucose levels were measured with a glucometer (Precision Xtra Meter, Abbott Diabetes Care, Portugal) and test strips (Abbott Diabetes Care, Portugal) (Ribeiro *et al.*, 2013; Sacramento *et al.*, 2017).

5.3.3. Recordings of CSN activity

Animals were anesthetized with pentobarbital sodium (60 mg/kg i.p.), tracheostomized, and the carotid bifurcation was removed to a Lucite chamber containing ice-cold/95% O_2-equilibrated Tyrode bicarbonate (in mM: 116 NaCl, 5 KCl, 2 $CaCl_2$, 1.1 $MgCl_2$, 5 glucose, 23 $NaHCO_3$; pH 7.4). After the collection of the carotid bifurcation the animals were euthanized by an intracardiac overdose of pentobarbital. The preparation CB-CSN was identified under a dissecting microscope and the tissue surrounding CB and CSN was dissected. The CB-CSN preparation was digested in collagenase type I (1mg/ml; Worthington Biochemical Corporation, Lakewood, USA).

For CSN activity recordings, the CB-CSN preparation was transferred to a recording chamber mounted on a dissection microscope (Nikon) and perfused with Tyrode bicarbonate equilibrated with 20% O_2 + 5% CO_2 + balanced N_2 at 37°C. Extracellular recordings from single- or multiple-fiber filaments of CSN were made

using a suction electrode. The pipette potential was amplified (Neurolog Digimiter, Hertfordshire, UK), filtered with low pass (5 kHz) and high pass (10 Hz), digitized at 5 kHz (Axonscope, Axon Instruments, Molecular Devices, Wokingham, UK), and stored on a computer. Chemoreceptor activity was identified (spontaneous generation of action potentials at irregular intervals) and confirmed by its increase in response to hypoxia (0% O_2 + 5% CO_2 + balanced N_2). CSN unit activity was converted to logic pulses, which were summed every second and converted in a voltage proportional to the sum.

The effect of hypoxia (0 and 5% O_2 + 5% CO_2 + balanced N_2) and hypercapnia (20% O_2 + 10% CO_2 + balanced N_2) on the CSN chemosensory activity was evaluated in control and HF animals (Fig. 5.1a). Additionally, the effect of ZM241385 (300nM; A_2 antagonist), SCH58261 (20nM; selective A_{2A} antagonist), suramin (50µM; P2 receptor antagonist) and AF-353 (0.3 and 1µM; $P2X_3$ and $P2X_{2/3}$ selective antagonist) on the CSN activity in normoxia and hypoxia (0 and 5%O_2)-evoked CSN action potentials was evaluated (Fig. 5.1b). The effect of AF-353 (1µM) was also evaluated in control animals in the presence of adenosine (10 µM) and α,β-methylene ATP (30 µM) (Fig. 5.1c). The effect of adenosine on CSN activity in the presence and absence of AF-353 was tested in the presence of erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA, an inhibitor of adenosine deaminase; 25µM).

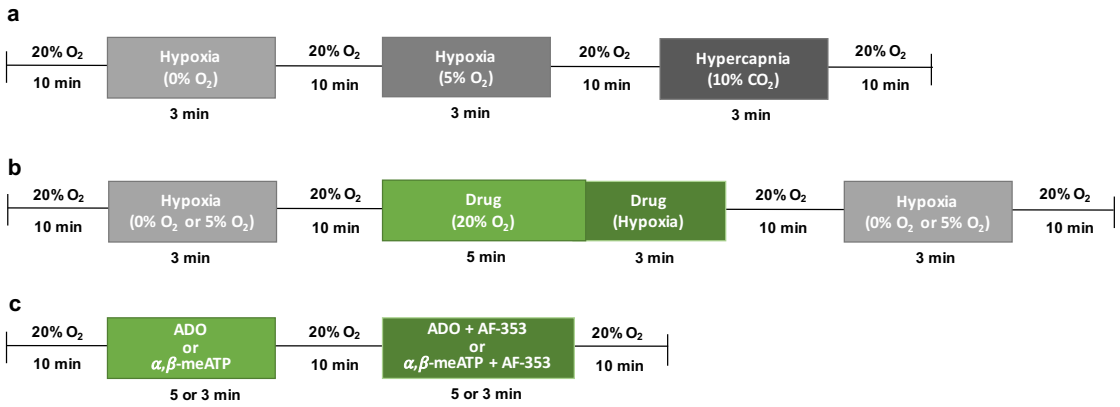


Figure 5.1 - Representation of carotid sinus nerve (CSN) activity recordings protocols. a) Protocol used to test the intense (0% O_2) and moderate (5% O_2) hypoxia and hypercapnia (10% CO_2) on the frequency of action potentials of the CSN in control and high-fat (HF) animals. **b)** Protocol used to investigate the effect of the different adenosine and ATP antagonists on the CSN discharge in normoxia (20% O_2) and in response to hypoxia (0 and 5% O_2) in control and HF animals. **c)** Protocol used to evaluate the effect of AF-353 (1µM) on the CSN action potentials elicited by adenosine or α,β-methylene ATP in control animals. Adenosine (AD0; 10µM) was applied during 5 minutes. α,β-methylene ATP (α,β-meATP; 30µM) was applied during 3 minutes.

5.3.4. Analysis of CSN activity recordings

The analysis of CSN recordings was performed using MATLAB software. The recordings were divided in two periods: basal and response (hypoxia, hypercapnia). The response period includes 1) the latency time that was measured from the application of the stimulus until the moment when the nerve activity was higher than the baseline activity, 2) time to peak that was measured from the end of the latency time until the moment the preparation reached maximal activity and 3) the recovery time, measured from the peak to the point where it was observed the return to the basal CSN frequency of discharge. A Savitzky-Golay filter was applied for smoothing noise data. Identification of the action potentials in the CSN frequency of discharge in control and HF animals was performed using an algorithm developed by Cunha-Guimarães (2018). The identification of the action potentials originated the creation of a template for each CSN action potential in control and HF animals.

5.3.5. Statistical analysis

Statistical analysis was performed using GraphPad Prism software, version 6 (GraphPad Software, La Jolla, CA, USA). The significance of the differences between the mean values was calculated by Student's t-test, and one- and two-way ANOVA with Dunnett's and Bonferroni multicomparison test, respectively. Differences were considered significant at $p < 0.05$.

5.4. Results

5.4.1. Effect of HF diet on metabolic parameters

As previously described by our group (Conde *et al.*, 2012b; Ribeiro *et al.*, 2013), the consumption of HF diet during 21 days decreased insulin sensitivity by 56.95% without significant changes in plasma fasting glycaemia (Table 5.1).

Table 5.1 - Effect of high-fat diet on insulin sensitivity and plasma fasting glycaemia.

	Control	High-fat diet
K_{ITT} (%glucose/min)	4.39 ± 0.11	1.89 ± 0.08****
Plasma glucose (ml/dl)	83.45 ± 2.60	90.17 ± 2.59

Insulin sensitivity was determined by an insulin tolerance test (ITT) and expressed as constant for glucose disappearance (K_{ITT}). Data are means ± SEM of 22-28 animals. Student's t-test: ****$p <$ 0.0001.

5.4.2. Identification of action potentials in the CSN

Figure 5.2a shows typical recordings of CSN chemosensory activity in basal conditions and in response to intense (0% O_2) and moderate (5% O_2) hypoxia and hypercapnia (10% CO_2) in control and HF animals. Note that in the raw data is possible to observe the increase in CSN activity produced by the different physiological stimuli and also that HF animals seem to have a higher baseline CSN activity.

The analysis of the CSN activity recordings allowed the identification of four types of action potentials both in control and HF animals, being these CSN action potentials similar between the two groups (Fig. 5.2b).

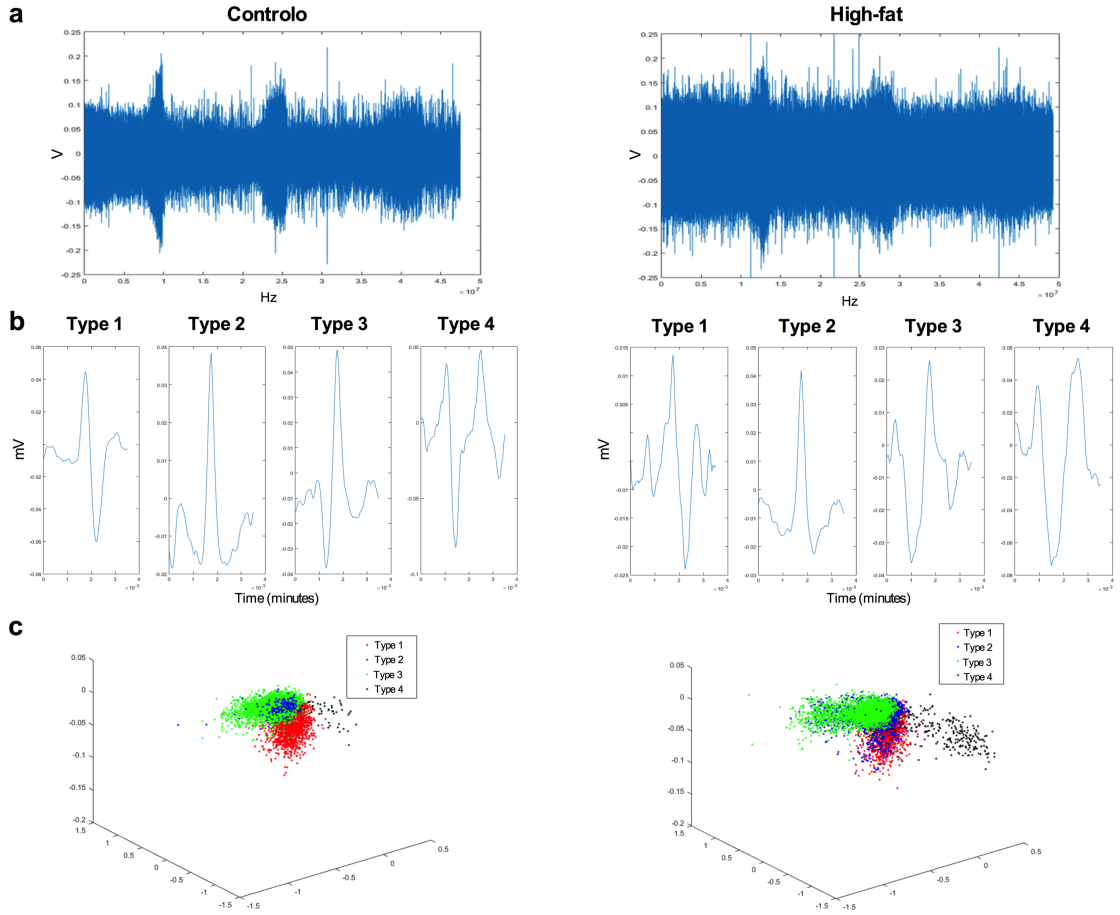


Figure 5.2 - Identification of different action potentials in the carotid sinus nerve (CSN) from control and high-fat (HF) animals. a) Typical recordings of the effect of hypoxia (0 and 5% O_2) and hypercapnia (10% CO_2) applied for 3 minutes on the CSN frequency of action potentials in control and HF animals. **b)** Different action potentials identified in control and HF animals. **c)** Cluster of the different action potentials identified in control and HF animals.

Despite the similarity, since this classification in four types of action potentials was based in correlations, for further analyses it was assumed separately the four types

of action potentials for the control and HF group. Figure 5.2c shows the clustering of the different four action potentials and where it was observed clearly the presence of tree action potential types, the type 1, 3 and 4, with the type 2 overlapping with the type 3 (Fig. 5.2c).

5.4.3. Effect of HF diet on basal CSN chemosensory activity

Figure 5.3 presents the effect of HF diet on CSN basal activity. As it was suggested by figure 5.2a HF diet increased by 63.65% the basal CSN chemosensory activity (Fig.5.3 a, b), an effect that was already described by our group (Ribeiro *et al.*, 2017). As previously described in figure 5.2, analysis of CSN recordings showed the presence of four types of action potentials both in control and HF animals (Fig. 5.3 c-c1). The number of type 1 and 3 CSN action potentials per second at baseline, were not significantly different between control and the HF group. In contrast, HF diet increased the number of type 2 and 4 CSN action potentials by 80.36 and 74.28%, respectively (Fig. 5.3 c, c1).

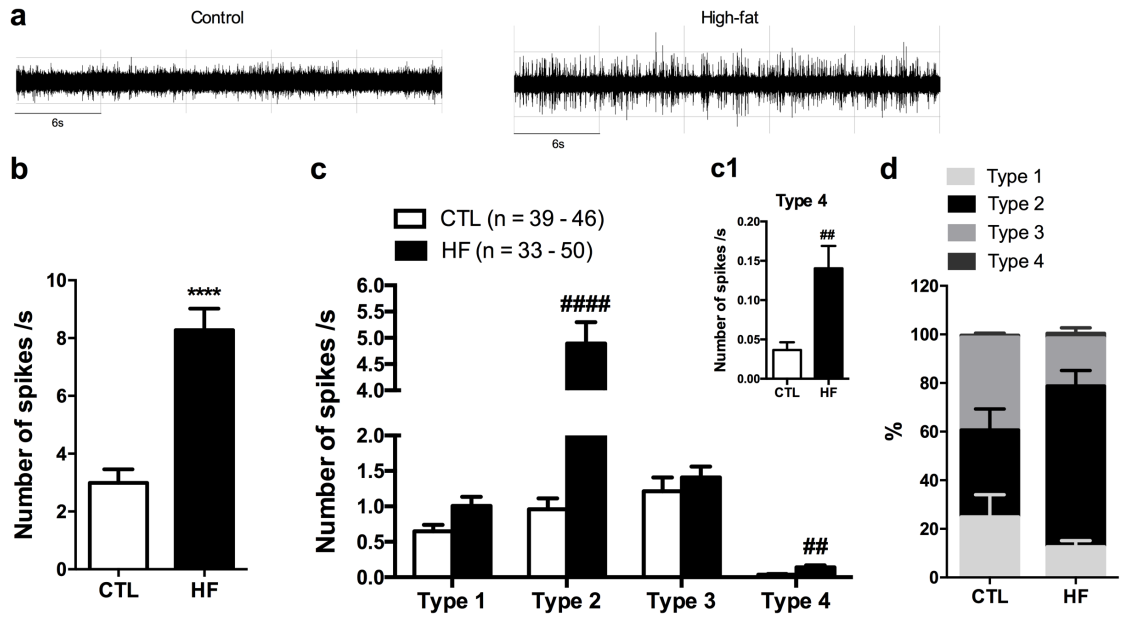


Figure 5.3 - High-fat (HF) diet increases basal carotid sinus nerve (CSN) chemosensory activity. a) Typical recordings of CSN basal frequency of action potentials in control and HF animals. **b)** Mean basal action potentials of the CSN activity in control (CTL) and HF animals. **c)** Basal CSN activity divided into the four action potentials in CTL and HF animals. **c1)** Magnification of the CSN action potential type 4. **d)** Percentage of the four different action potentials in CTL and HF animals. Data represent mean ± SEM. Student's t-test: ****$p < 0.0001$. Two-way ANOVA with Bonferroni multicomparison test: $^{\#\#}p < 0.01$ and $^{\#\#\#\#}p < 0.0001$ compared with CTL within the same group.

Figure 5.3d displayed the percentage of the four types of action potentials in both control and HF group. As stated previously, it could be clearly noted that the most pronounced effect of hypercaloric diet on CSN activity is at the level of type 2 action potentials.

5.4.4. Effect of HF diet on the CSN response to hypoxia and hypercapnia

Figure 5.4 shows the effect of HF diet on CSN action potential frequency evoked by intense (0% O_2) and moderate (5% O_2) hypoxia and by hypercapnia (10% CO_2). Typical increases of CSN chemosensory activity in response to hypoxia and hypercapnia are displayed in Fig. 5.4a and it can be noted that CSN responses to hypoxia and hypercapnia were similar between control and HF animals (Fig. 5.4 a, b). It is very well described in the literature that there is a linear correlation between PaO_2 and CSN activity, with the slope between PO_2 and CSN discharges changing abruptly from linear to exponential below 10-15 mmHg (Gonzalez *et al.*, 1994). Our results herein described confirmed this correlation as we showed that the increase in the CSN activity evoked by hypoxia was as higher as the hypoxia intensity applied (ΔCSN frequency: CTL 0%O_2 = 10.96 ± 1.27; CTL 5%O_2 = 7.60 ± 0.72; HF 0%O_2 = 10.24 ± 1.19; HF 5%O_2 = 6.57 ± 0.73 times over basal) (Fig. 5.4b). As expected CSN response to hypercapnia was smaller when compared to the response to hypoxia (ΔCSN frequency: CTL 10% CO_2 = 2.42 ± 0.40; HF 10% CO_2 = 3.12 ± 0.49 times over basal) (Fig. 5.4b).

In Figure 5.4c is depicted the effect of HF diet on the four types of CSN action potentials in response to the different stimuli. As noted for the compound electrical activity (figure 5.4b) the frequency of each type of action potential is as higher as the intensity of hypoxia. Also, responses to hypercapnia followed the same profile than the compound activity. In general, HF diet did not produce significant alterations in any of the four types of CSN action potentials for the different stimuli applied (Fig. 5.4c), except for the type 2 action potential that showed a tendency to decrease in response to intense and moderate hypoxia (29.32 and 36.45%, respectively), and for the type 3 action potentials that increased non-significantly by 26.32% in HF animals in response to intense hypoxia.

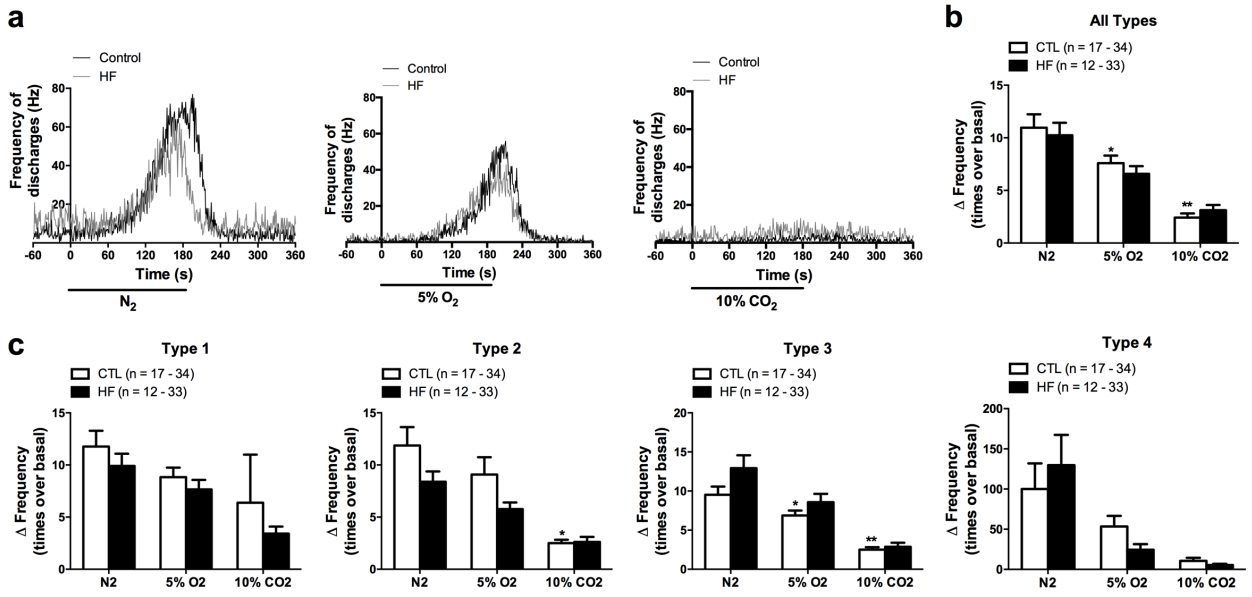


Figure 5.4 - Effect of the high-fat (HF) diet on the carotid sinus nerve (CSN) chemosensory activity evoked by hypoxia and hypercapnia. **a)** Typical recordings on the frequency of action potentials of the CSN in response to hypoxia (0 and 5% O_2) and hypercapnia (10% CO_2) in control (CTL) and HF animals. **b)** Mean responses to intense and moderate hypoxia and to hypercapnia obtained in preparations of CTL and HF animals. **c)** CSN activity in response to hypoxia (0 and 5% O_2) and hypercapnia (10% CO_2) divided into the four types of action potentials in CTL and HF animals. Data represent mean ± SEM. One- and Two-way ANOVA with Bonferroni multicomparison test, respectively: *$p < 0.05$ and **$p < 0.01$ *vs* CTL 0% O_2.

5.4.5. Effect of AF-353, a $P2X_3$ antagonist, on the CSN activity

AF-353 is a recently described $P2X_3$ and $P2X_{2/3}$ selective antagonist, with pIC_{50} at $P2X_3 = 8.1$, $P2X_{2/3} = 7.4$, $P2X_{1,2,4,5,7} < 5$, that binds allosterically to ATP receptors, and consequently behaves as a non-competitive antagonist (Gever *et al.*, 2010). Aiming to evaluate the effect of $P2X_3$ and $P2X_{2/3}$ receptors on CB overactivation induced by hypercaloric diets, we first tested the effect of the selective antagonist $P2X_3$ and $P2X_{2/3}$ ATP receptors, AF-353 (0.3 and 1μM), on basal CSN discharge and on CSN response to hypoxia (0 and 5% O_2) in control animals. The doses that were tested were based on the work of (Gever *et al.*, 2010) and on the doses used by Pijacka *et al*. (2016) in the CB (Fig. 5.5). AF-353 in our CB/CSN preparation did not modify the basal CSN chemosensory activity or the activity elicited by moderate hypoxia in any of the doses tested, however it modified the response to intense hypoxia (Fig. 5.5 a, b). The absence of effects in moderate hypoxia together with the decrease by 54.74 and 66.91% in the presence of 0.3 and 1μM of AF-353, respectively, in response to intense hypoxia (0% O_2), suggested that the drug was blocking an ATP-mediated effect. To confirm that the

effect of AF-353 was due to a selective block of ATP receptors and knowing that some purinergic antagonists lack selectivity, we evaluated the effect of AF-353 on CSN activity evoked by adenosine (10μM) and by the non-degradable ATP agonist, α,β-methylene ATP (30μM). AF-353 did not modify CSN activity (Fig.5 d, d1) evoked by adenosine but decreased by 76.95% the CSN activity-elicited by α,β-methylene ATP (Fig. 5.5 e, e1).

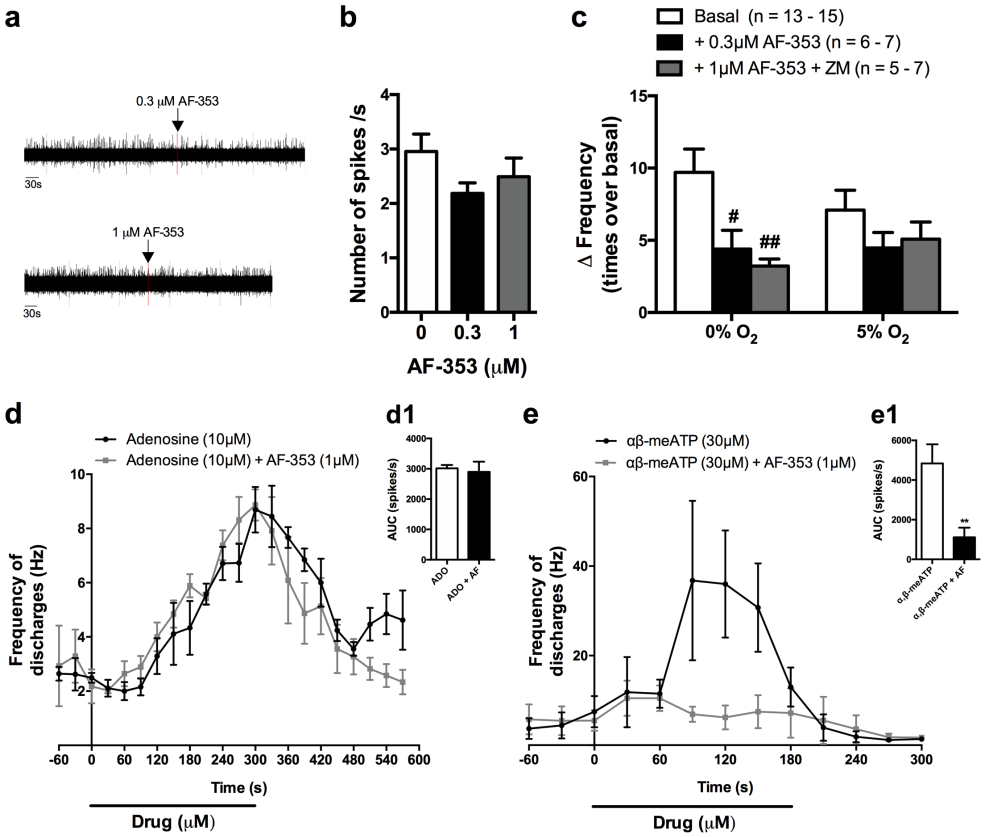


Figure 5.5 - Effect of AF-353, a selective $P2X_3$ antagonist, on the carotid sinus nerve (CSN) activity in control animals. a) Typical recordings of the effect of AF-353 (0.3 and 1μM) on the basal frequency of action potentials of CSN in control animals. **b)** Effect of 0.3 and 1μM of AF-353 applied during 5 minutes on the basal CSN discharge (n = 5 - 15). **c)** Effect of AF-353 (1μM) on the CSN activity in response to intense (0% O_2) and moderate (5% O_2) hypoxia. **d)** Effect of AF-353 (1μM) on the CSN activity-evoked by adenosine (ADO; 10μM) applied for 5 minutes and **d1)** the respective area under the curve (AUC) (n = 4). The experiments were performed in the presence of 25μM EHNA, an inhibitor of adenosine deaminase. **e)** Effect of AF-353 (1μM) on the CSN activity-evoked by α,β-methylene ATP (α,β-meATP; 30μM) applied for 3 minutes and **e1)** the respective area under the curve (AUC) (n = 5). AUC was measured since the application of the stimulus until the moment in which the activity of the nerve returned to baseline level. Data represent mean ± SEM. Student's t-test: $^{**}p < 0.01$. Two-way ANOVA with Bonferroni multicomparison test: $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with basal condition values within the same group.

5.4.6. Effect of ATP and adenosine antagonists on basal CSN chemosensory activity

To evaluate the role of ATP and adenosine on basal CSN frequency of discharge, we tested the effect of different ATP and adenosine antagonists applied alone or together on basal CSN activity (Fig. 5.6). Both the ATP and adenosine receptor antagonists tested did not modify basal CSN chemosensory activity in control animals, except for ZM241385, an A_2 antagonist, that decreased CSN basal activity by 30.23% in CSN type 3 action potentials (Fig. 5.6d, middle panel). Suramin, a P2 receptor antagonist, applied alone or together with ZM241385 did not changed significantly nor the CSN compound chemosensory activity neither the increase in each of the different types of action potentials produced by HF diet. However, it showed a tendency to decrease the CSN compound chemosensory basal activity in the HF animals by 29.91 and 30.81%, respectively (Fig. 5.6, left and right panels). In contrast, the $P2X_3$ receptor antagonist, AF-353, decreased significantly by 57.89%, to control values, the CSN compound chemosensory basal activity in HF animals, the same happening for all the types of action potentials, except for type 4 (figure 5.6 a-e, left panels).

The A_2 and A_{2A} adenosine receptor antagonists, ZM241385 and SCH58621, decreased CSN compound chemosensory basal activity in HF animals by 52.09 and 48.63 % to control levels in HF animals, an effect that was mainly due to an action on type 2 and type 4 action potentials, although these antagonists also affected type 3 action potentials (Fig. 5.6 b-e, middle panel). Additionally, no significant differences were observed between the A_2 and A_{2A} adenosine receptor antagonists, suggesting that the effect of HF diet on the increase in CSN activity in basal conditions is mainly due to A_{2A} adenosine receptors.

The application of AF-353 together with ZM241385 decreased basal CSN compound chemosensory activity in HF animals by 62.77% to a level below control baseline activity (Fig. 5.6a, right panel). Moreover, the application of these antagonists together also decreased the type 1, 2 and 3 CSN action potentials in HF animals (Fig. 5.6 b-e, right panel), without affecting significantly type 4 CSN action potentials.

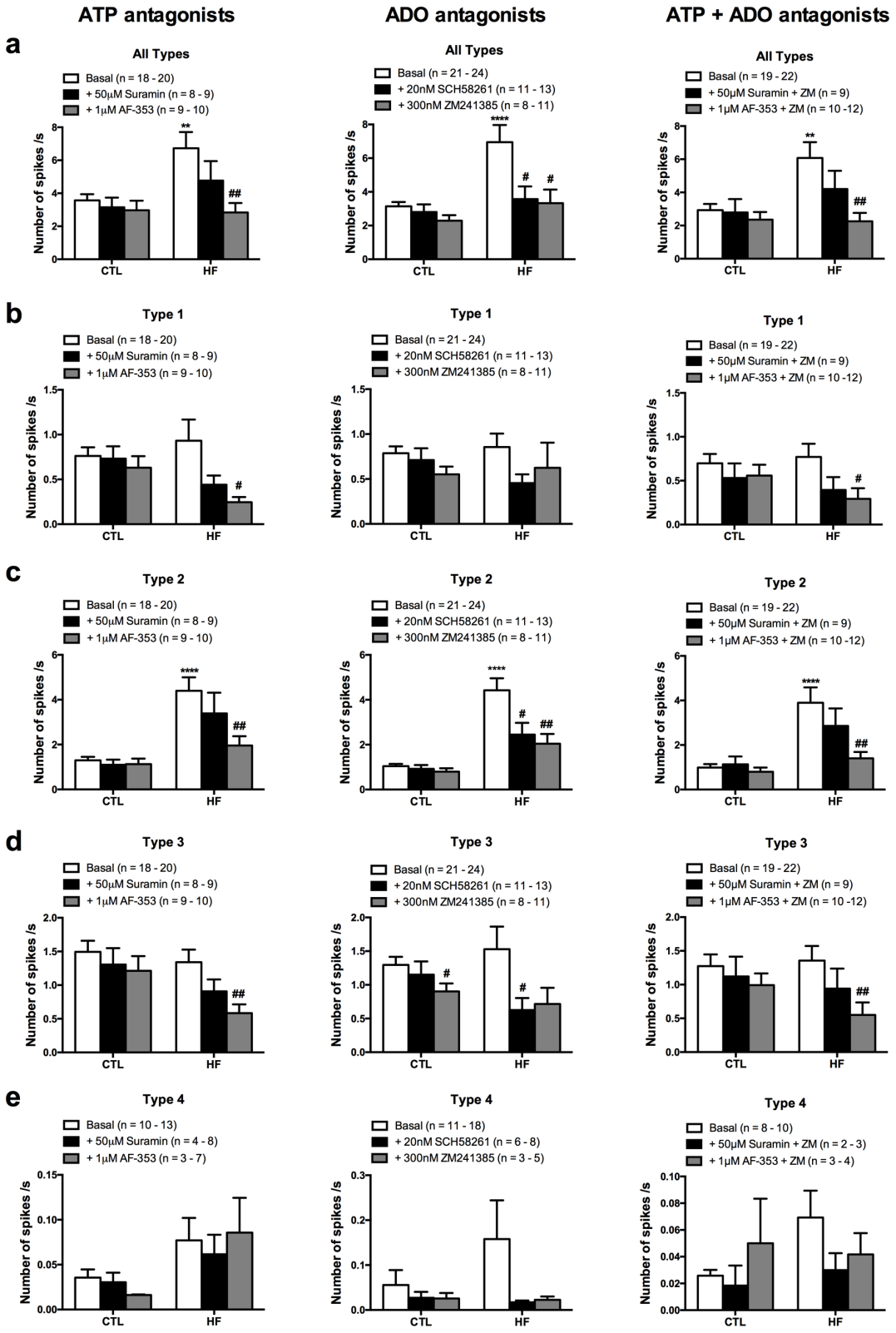


Figure 5.6 - Effect of ATP and adenosine receptor antagonists applied alone or together on basal carotid sinus nerve (CSN) frequency of discharge in control and high-fat (HF) animals. Mean basal CSN activity for the all types of action potentials **(a)** and for type 1 **(b)**, 2 **(c)**, 3 **(d)** and 4 **(e)** action potentials detected in CTL and HF animals in response to ATP (left panel) and adenosine (ADO, middle panel) antagonists applied alone or together (right panel). Data represent mean ± SEM. One- and Two-way ANOVA with Bonferroni multicomparison test: $^{**}p < 0.01$ and $^{****}p < 0.0001$ *vs* control animals; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with basal condition values within the same group.

5.4.7. Effect of ATP and adenosine antagonists on the CSN chemosensory activity in response to intense and moderate hypoxia

As previously described by Conde *et al*. (2012a) in control animals, the contribution of ATP and adenosine for CB output is dependent on the hypoxic intensity, with adenosine having a higher contribution in response to moderate hypoxias and ATP in response to high intensity hypoxias. Therefore, as expected herein we observed that suramin and AF-353, a P2 and $P2X_3$ ATP receptor antagonists, decreased significantly by 74.37 and 69.99%, respectively, the CSN compound chemosensory response to intense hypoxia in control animals, while did not alter significantly the response to moderate hypoxia, meaning that ATP highly contribute to generate CSN activity during intense hypoxia (Fig. 5.7a and 5.8a, left panel). Moreover, SCH58261 and ZM241385 reduced significantly by 58.90 and 80.82%, the CSN chemosensory activity in response to moderate hypoxia in control animals, without affecting the response to intense hypoxia, confirming the more relevant contribution of adenosine to CSN chemotransduction during moderate hypoxia (Fig. 5.7a and 5.8a, middle panel). Also note, that in control animals the decrease in response to intense hypoxia was of the same magnitude when applying suramin and AF-353 (Fig. 5.7a, left panel), which suggests mainly the involvement of $P2X_3$ receptors, while in the response to moderate hypoxia the application of ZM241385 decreases more than SCH58261 the CSN activity (Fig. 5.8a, middle panel), confirming the contribution of both A_{2A} and A_{2B} adenosine receptors to CSN response to moderate hypoxia previous described by Conde *et al.* (2006). The same profile was observed when the CSN compound chemosensory activity was separated in the different types of action potentials in control animals (Fig. 5.7 b-e and 5.8 b-e, left and middle panel).

A different profile of contribution of adenosine and ATP for CSN chemosensory responses to moderate and intense hypoxias was observed in HF animals, with both ATP and adenosine being involved in the responses in HF animals, however with a higher contribution of adenosine (Fig. 5.7 and 5.8, left and middle panel). During intense hypoxia, the inhibition of the CSN compound chemosensory activity in HF animals was 50.10 and 74.70% in the presence of suramin and AF-353, respectively (Fig. 5.7a, left panel), and 49.36 and 54.45% in the presence of SCH58261 and ZM241385, respectively (Fig. 5.7a, middle panel). During moderate hypoxia, inhibitions of the CSN activity in HF animals in the presence of suramin and AF-353 were 41.33 and 48.80%, respectively (Fig. 5.8a, left panel), and in the presence of

SCH58162 and ZM241385 was 68.81 and 59.84%, respectively (Fig. 5.8a, middle panel). The subtypes 1, 2 and 3 CSN action potentials identified in HF animals followed the profile described above. During intense hypoxia, the higher and significant decreases were observed in the presence of ATP antagonists, suramin and AF-353 (Fig. 5.7 b-d, left panel). In contrast, adenosine is contributing more for the CB output on moderate hypoxia, since the application of SCH58261 and ZM2411385 decreased significantly the CSN type 1, 2 and 3 action potentials (Fig. 5.8 b-d, middle panel). It was not possible to conclude the effect of the application of ATP and adenosine antagonists on the CSN type 4 action potentials in both intense and moderate hypoxia (Fig. 5.7 and 5.8 e), since the frequency of this type of CSN action potential during the period of the recording analysis (1 minute) was almost always zero. This type of action potential was much less frequent than the type 1, 2 or 3 of CSN action potentials during hypoxia.

The application of suramin and ZM241385 together or AF-353 and ZM241385 together, decreased the CSN chemosensory activity both in control and HF animals, being the decrease as higher as the hypoxic intensity (Fig. 5.7a and 5.8a, right panel). During intense hypoxias, the inhibition of the CSN compound chemosensory activity in control animals was 83.26 and 72.87% in the presence of suramin together with ZM241385 and AF-353 together with ZM241385, respectively (Fig. 5.7a, left panel). In the HF animals, the decrease was 75.00 and 77.65% in the presence of suramin together with ZM241385 and AF-353 together with ZM241385, respectively (Fig. 5.7a, left panel). In moderate hypoxia, when applied together, suramin and ZM241385 or AF-353 with ZM241385 decreased the CSN compound chemosensory activity by 56.01 and 80.19% in control animals, respectively, and by 55.50 and 53.13% in HF animals, respectively (Fig. 5.8a, left panel). The CSN type 1, 2 and 3 action potentials followed this profile (Fig. 5.7 and 5.8 b-d, left panel).

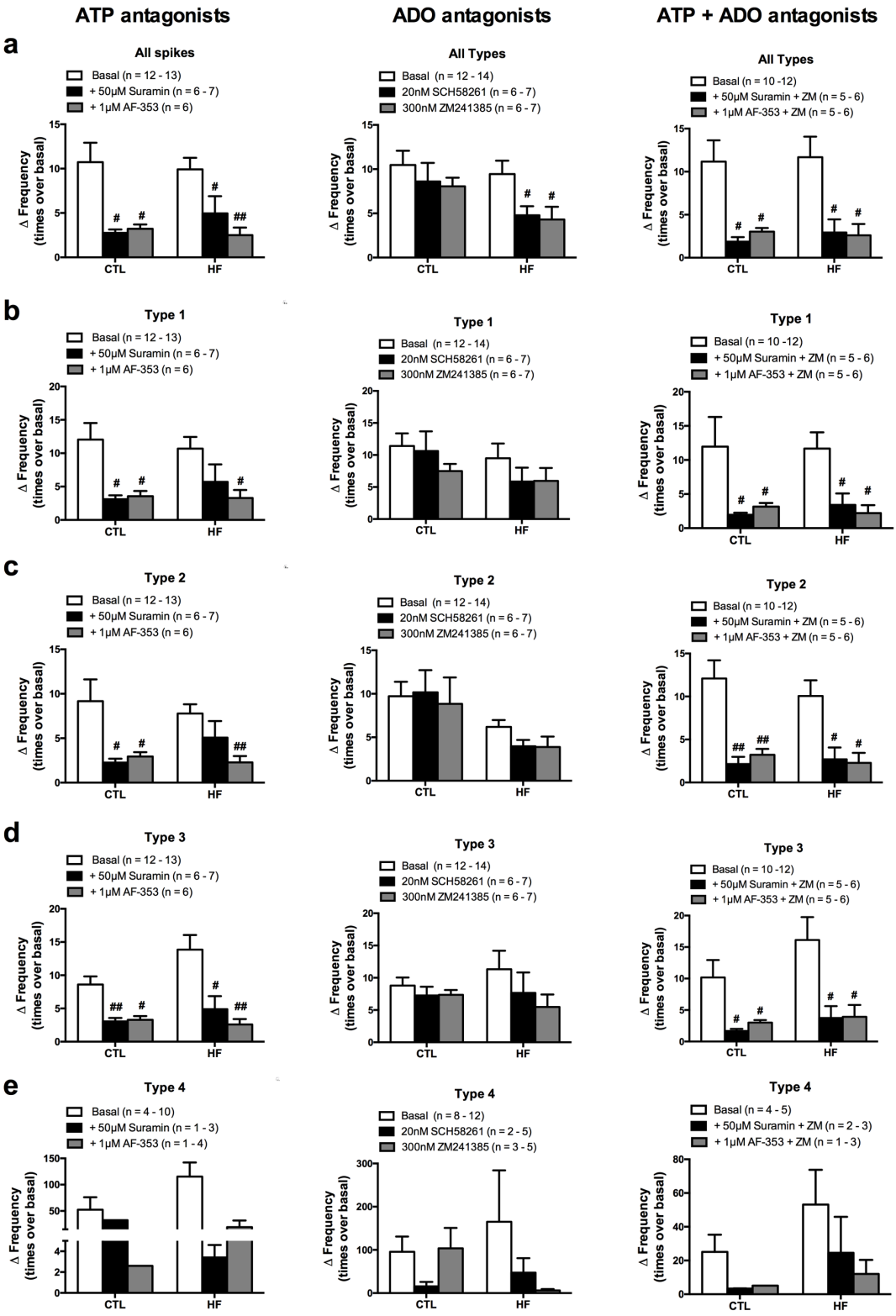


Figure 5.7 - Effect of ATP and adenosine receptor antagonists on the carotid sinus nerve (CSN) chemosensory activity in response to intense (0% O_2) hypoxia in control and high-fat (HF) animals. Effect of ATP (left panel) and adenosine antagonists (ADO, middle panel) applied alone or together (right panel) on the CSN activity evoked by intense hypoxia and discriminated in all action potentials **(a)** and in type 1 **(b)**, 2 **(c)**, 3 **(d)** and 4 **(e)** action potentials detected in CTL and HF animals. Data represent mean ± SEM. Two-way ANOVA with Bonferroni multicomparison test: $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with basal condition values within the same group.

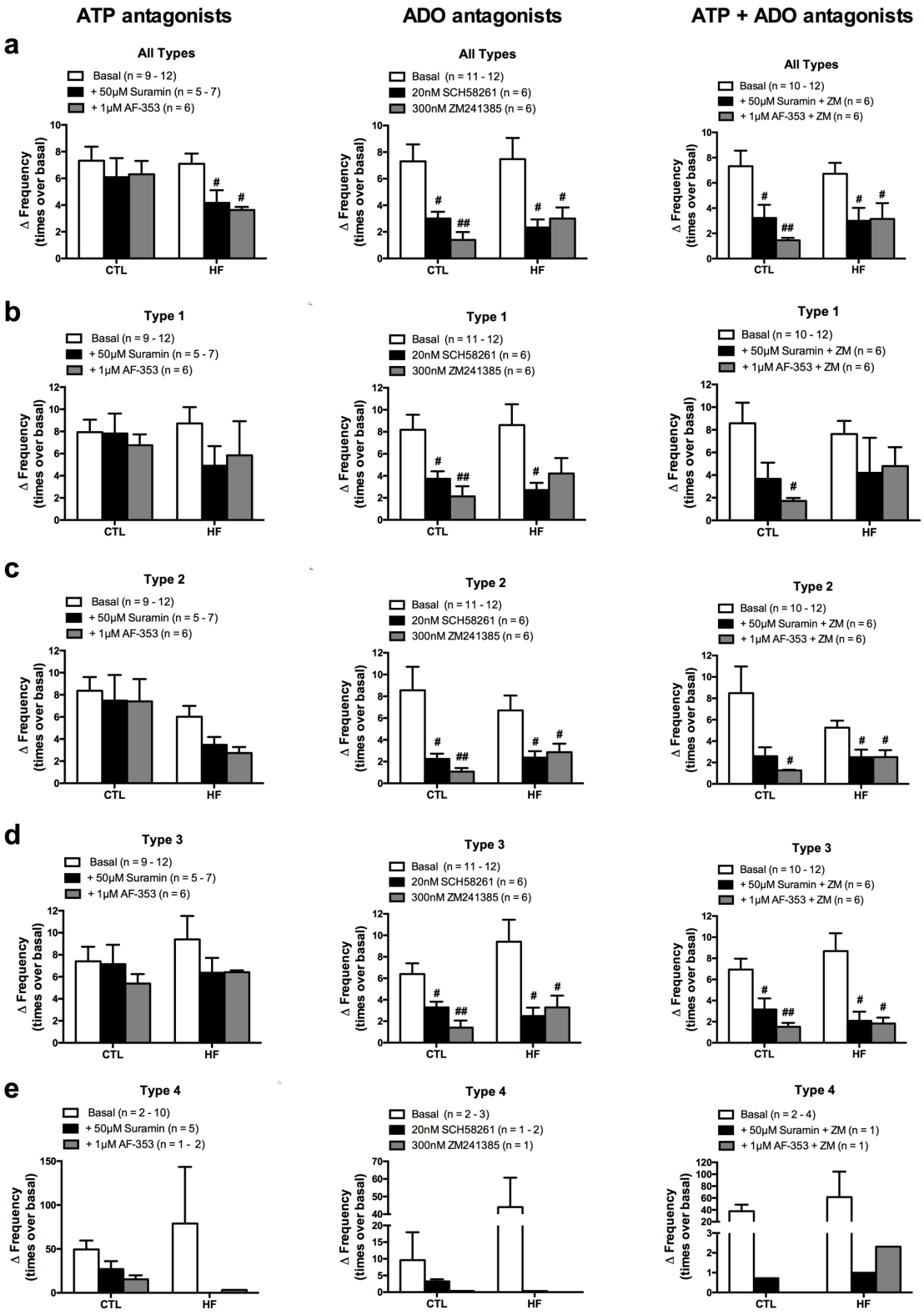


Figure 5.8 - Effect of ATP and adenosine receptor antagonists on the carotid sinus nerve (CSN) chemosensory activity in response to moderate (5% O_2) hypoxia in control and high-fat (HF) animals. Effect of ATP (left panel) and adenosine antagonists (ADO, middle panel) applied alone or together (right panel) on the CSN activity evoked by intense hypoxia and discriminated in all action potentials **(a)** and in type 1 **(b)**, 2 **(c)**, 3 **(d)** and 4 **(e)** action potentials detected in CTL and HF animals. Data represent mean ± SEM. Two-way ANOVA with Bonferroni multicomparison test: $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with basal condition values within the same group.

5.5. Discussion

In the present work, we found that 3 weeks of HF diet alter baseline CSN activity but did not change CSN responses to hypoxia and hypercapnia. We identified 4 different types of action potentials in CSN compound chemosensory activity and described for the first time that the overactivation of CSN in HF diet animals come mainly from an increase in the frequency of type 2 action potentials. Additionally, herein we demonstrated for the first time that ATP and adenosine are involved in CB/CSN overactivation induced by HF diet. ATP and adenosine has also been shown to be involved in the CB chemotransduction in hypoxia in control and HF animals, with a more relevant contribution of ATP during intense hypoxia, while adenosine contributes more to generate CSN activity in moderate hypoxia. We also conclude that the excitatory effects of ATP and adenosine on the basal CSN activity and CSN chemosensory responses to hypoxia occur mainly via an action on $P2X_3$ and A_{2A} receptors, respectively.

As previously described by our group, CB is overactivated in HF animals (Ribeiro *et al.*, 2013; Ribeiro *et al.*, 2017). The increase in basal CSN discharge observed herein and by Ribeiro *et al*. (2017) corroborates our previous results, that showed an increase in spontaneous ventilation and in dopamine release in response to hypoxia (5% O_2), that runs with an increase in the expression of tyrosine hydroxylase and with an increase in CB weight in 3 weeks HF animals (Ribeiro *et al.*, 2013). Some of these alterations that run with an overactivation of CB were not only seen in prediabetes models but also in T2D models as we have recently described that animals with early T2D, obtained by submitting the rats to 14 weeks of high-fat-high sucrose diet, exhibit an higher percentage of type I cells concomitantly with an increased expression of tyrosine hydroxylase (Dos Santos *et al.*, 2018). These last results are in agreement with the increase in CB weight observed by Cramer *et al.* (2014) in patients with T2D. The overactivation of CB in metabolic diseases was also supported by results from a clinical study, in which we evaluated CB chemosensitivity by the Dejours test in prediabetic and non-prediabetes patients and found that prediabetic patients exhibit an increased CB chemosensitivity, which correlates inversely with insulin resistance and insulin levels (unpublished data, Conde *et al.*, 2018a).

In the present chapter, we described for the first time the presence of four different types of action potentials in the CSN. Moreover, we found that these action

potentials were also present in HF animals and that they were similar between both groups, with type 2 and 4 of CSN action potentials being increased in HF animals, which suggest that these two types of action potentials mediate the increase in the basal CSN chemosensory activity in this animal model. In fact, from the increase in percentage of action potentials per second in the compound activity of the HF animals, we can suggest that mainly type 2 actions potentials are responsible for the increased activity in the disease model. Also, these results suggest that HF diet did not alter the type of activity but instead the frequency of discharge that could be due to the excited state of the CB induced by the hypercaloric diet. This kind of mechanism of increased frequency of discharge due to an increased overactivity is not new as in crayfish an increased excitement of the animal lead to increased frequency of discharge of the optomotor fibres (Wiersma & Fiore, 1971).

Despite the increase in the basal CSN discharge in the HF animals, no significant changes were observed in the CSN chemosensory activity in response to hypoxia (intense and moderate) and hypercapnia. Moreover, the four types of CSN action potentials did not change significantly with the stimuli applied. Previously, we described that in hypoxia the CB release of dopamine is increased (Ribeiro *et al.*, 2013) and the release of adenosine is decreased (Ribeiro *et al.*, 2017) in HF animals, suggesting that the mechanisms involved in the CSN response to hypoxia are altered in HF animals. However, when compared with control animals, these alterations are not transduced in differences in the CSN activity evoked by hypoxia and hypercapnia, suggesting adaptations in neurotransmitters dynamics to compensate these alterations.

As previously described by Conde *et al.* (2012a), suramin and ZM241385 applied alone or together did not modify the basal CSN chemosensory activity in control animals. Herein, we have confirmed those data as basal CSN activity in control animals did not change in the presence of other ATP and adenosine receptor antagonists, which confirms that ATP and adenosine do not contribute to the steady basal CSN chemosensory activity in the adult rat (Reyes *et al.*, 2007b; Conde *et al.*, 2012a). In HF animals, the application of SCH58261, an A_{2A} antagonist, and ZM241385, an A_2 antagonist, restored basal CSN activity, showing that adenosine via A_{2A} receptors mediates basal CSN overactivity in this animal model. Suramin is a P2 antagonist that was previously used to inhibit ATP responses in the CB (Zhang *et al.*, 2000; Prasad *et al.*, 2001; Reyes *et al.*, 2007b; Conde *et al.*, 2012a) and its use in

electrophysiological experiments suggested that suramin acts via P2X receptors in the CB (Zhang *et al.*, 2000), namely on $P2X_2$ and $P2X_3$ receptors that were detected by immunohistochemistry in petrosal somas and CB afferent terminals (Zhang *et al.*, 2000; Prasad *et al.*, 2001; Yokoyama *et al.*, 2016). However, in the present manuscript we found that suramin only decreased CSN basal overactivation in HF animals by 29.91%, while AF-353, a selective $P2X_3$ and $P2X_{2/3}$ antagonist, restored the basal CSN chemosensory activity in HF animals to control values. Knowing that suramin could also act on $P2Y_1$ (pIC_{50} = 5.5), $P2Y_2$ (pIC_{50} = 4.3) and $P2Y_{12}$ (pIC_{50} = 5.5) receptors (von Kugelgen, 2006), and that these receptors are described in the CB (Xu *et al.*, 2003; Xu *et al.*, 2005; Carroll *et al.*, 2012; Zhang *et al.*, 2012) we can postulate that the lower effect of suramin *vs* AF-353 is due to an action on $P2Y_1$ and $P2Y_{12}$ receptors. In fact, it was shown that ATP can inhibit the hypoxia-induced intracellular Ca^{2+} increase in type I cells via $P2Y_1$ and $P2Y_{12}$ receptors, without affecting resting intracellular Ca^{2+} (Xu *et al.*, 2005; Carroll *et al.*, 2012). Therefore, in HF animals, in where an increase in CSN basal activity is present, suramin might act also to inhibit $P2Y_1$ and $P2Y_{12}$ receptors, leading to a decrease in the release of neurotransmitters, like ATP. Another plausible explanation could be due to the inhibitory effect of suramin on $P2Y_2$ receptors in type II cells (Xu *et al.*, 2003; Zhang *et al.*, 2012). It was suggested that type II cells may function as ATP signal amplifiers through a mechanism of ATP-induced ATP release (Zhang *et al.*, 2012; Murali & Nurse, 2016; Nurse *et al.*, 2018) and therefore, if suramin is acting on $P2Y_2$ it can cause a decreased response from type I cells. Hence, the lack of selectivity of suramin could explain the smaller effect of suramin compared to AF-353 in the basal and CSN activity in response to intense hypoxia in HF animals. Our data on CSN activity with AF-353 suggests that besides adenosine, ATP is also mediating the basal CSN overactivity in HF animals, mainly via $P2X_3$ and/or $P2X_{2/3}$. AF-353 in those experiments was tested with the dose of 1μM, and from that we cannot discriminate if the effect of AF-353 is only on homomeric $P2X_3$ receptor and/or on the heteromeric $P2X_{2/3}$ (Gever *et al.*, 2010).

Herein we showed that ATP and adenosine also mediate CSN chemotransduction in response to hypoxia in both control and HF animals, being the contribution of each neurotransmitter dependent on the hypoxia intensity. The finding that both neurotransmitters contribute to hypoxic chemosensory process is not new as Conde *et al.* (2012a) showed in control animals that ATP has a more relevant contribution during intense hypoxia, while adenosine has a preponderant role during

moderate hypoxia, a correlation that we herein observe in HF animals. ATP contributes to the CSN chemosensory activity through an action on $P2X_3$ and/or $P2X_{2/3}$ receptors both in control and HF animals, which is in concordance with the already described role for $P2X_2$ and $P2X_3$ receptors in the CB hypoxic sensing (Zhang *et al.*, 2000; Prasad *et al.*, 2001; Rong *et al.*, 2003; Niane *et al.*, 2011). Rong *et al.* (2003) described that the CSN discharged evoked by hypoxia was reduced in $P2X_2$ knockout mice and attenuated in $P2X_2/P2X_3$ double knockout mice. Additionally, it was described that A317491, a selective $P2X_3$ and $P2X_{2/3}$ antagonist, decreased the ventilatory responses during hypoxic exposures in newborn rats (Niane *et al.*, 2011).

We also found that in control animals, adenosine contributes to CSN discharge evoked by moderate hypoxia (5% O_2) through an action on A_{2A} and A_{2B} adenosine receptors. This is in accordance with the previous findings by Conde *et al.* showing that caffeine (Conde *et al.*, 2006), a non-selective adenosine receptor antagonist, and ZM241385, that inhibits A_{2A} and A_{2B} adenosine receptors (Conde *et al.*, 2012a), decreased CSN chemosensory activity in response to moderate hypoxia. Herein we found that in HF animals, the effect of adenosine on CSN chemosensory activity evoked by moderate hypoxia is via mainly A_{2A} receptors.

As expected and previously demonstrated (Conde *et al.*, 2012a), in the present work the combined application of ATP and adenosine antagonists did not completely inhibit the hypoxic CB chemoreceptor response. We can attribute the remaining CSN chemosensory activity in hypoxia to other neurotransmitter besides ATP and adenosine that are involved in the response of CB to hypoxia. From the several neurotransmitters that have been described to be involved in the response to hypoxia (Iturriaga *et al.*, 2007; Nurse, 2014) acetylcholine has been the one that was proposed to be co-released with ATP and mediating the ventilatory and the CSN chemosensory response to hypoxia (Zhang *et al.*, 2000; Varas *et al.*, 2003). However, controversy remains about the hypothesis of ATP-acetylcholine co-signalling in the CB, as the co-application of antagonists of both neurotransmitters only blocked approximately by 50% the CSN hypoxic response (Reyes *et al.*, 2007b). Apart from the involvement of other neurotransmitters, we can also postulate the involvement of other ATP receptors that could contribute to the remaining CSN chemosensory in hypoxia. For example, in type II cells, ATP activates $P2Y_2$ receptors, leading to intracellular Ca^{2+} increase (Xu *et al.*, 2003) and opening of pannexin-1 channels, that induces ATP release from these cells

(Zhang *et al.*, 2012). This ATP, released by type II cells, may act on excitatory P2X receptors on the CSN (Murali & Nurse, 2016; Nurse *et al.*, 2018) and/or may be broken down by extracellular 5'ectonucleotidase into adenosine (Conde *et al.*, 2012a; Salman *et al.*, 2017), that can stimulate A_2 receptors in type I cells (Xu *et al.*, 2006) and/or may bind to the A_{2A} on the postsynaptic side at the CSN nerve endings, leading to the increase in the CSN discharge (Conde *et al.*, 2012a).

We also have described that the drugs that inhibit adenosine and ATP receptors affect the four types of CSN action potentials identified in CSN compound activity, which suggest that all types of CSN action potentials are involved in the effect of ATP and adenosine in the CSN basal overactivity and in the CSN chemosensory response to hypoxia.

The use of ATP and adenosine antagonists has shown herein to be effective in restoring basal CSN activity in HF animals, suggesting that the pharmacological inhibition of ATP and/or adenosine receptors could be used to treat metabolic diseases, like T2D. However, we have also described herein that type 2 and 4 are the action potentials that are increased in HF animals and that adenosine and ATP contribute to all action potentials (Fig 5.6, 5.7, 5.8), being probably involved in main functions of the CB. Moreover, it is clear that both neurotransmitters mediate the CSN chemotransduction in hypoxia, being ATP the major contributor to the CSN chemosensory activity in intense hypoxia and adenosine involved in the responses to mild/moderate hypoxia. Intense hypoxias are less common and less compatible with life and appear in very exacerbated pathological conditions. At high altitudes, the partial oxygen pressure (PO_2) decreased in proportion to the lower ambient pressure, leading to impaired oxygen supply, which compromises cellular functions (Burtscher *et al.*, 2018). In patients, hypoxia could be caused by hypoventilation, impaired oxygen diffusion in the lungs (e.g. pulmonary edema, chronic obstructive pulmonary disease), anemia and impaired cardiovascuçar function (Burtscher *et al.*, 2018). Therefore, we can postulate that the modulation of ATP signalling in the CB, via $P2X_3$ receptors, could be a better therapeutic approach aiming to decrease CB activity for metabolic diseases as adenosine could still mediating the CB responses to moderate hypoxia. Additionally, adenosine is known to mediate hypercapnic response in the rat CB (Holmes *et al.*, 2015; Sacramento *et al.*, 2018b), an effect that is due to an action via A_{2A} and A_{2B} receptors (Sacramento *et al.*, 2018b) and therefore, hypercapnic responses will be also maintained.

The clinical importance of modulating $P2X_3$ receptors in the CB has been also

described recently, for essential hypertension. Pijacka *et al*. (2016) showed that purinergic receptor $P2X_3$ mRNA expression is upregulated substantially in chemoreceptive petrosal sensory neurons conributing to high blood pressure in hypertension. Additionally, they also found that the antagonism of $P2X_3$ receptors was able to reduce arterial pressure and sympathetic activity in spontaneous hypertensive rats, without any effect in normotensive rats (Pijacka *et al.*, 2016). All these data suggest that $P2X_3$ receptors in the CB could be a new target for the control of hypertension. However, the authors found that the antagonism of $P2X_3$ receptors reduced the petrosal neurons firing response to chemical hypoxia induced by sodium cyanide (NaCN) in spontaneous hypertensive rats (Pijacka *et al.*, 2016), with these data suggesting that ATP antagonism might compromise hypoxic ventilatory responses. Another adverse effect that was described for the use $P2X_3$ antagonists was taste disturbances (Abdulqawi *et al.*, 2015; Vandenbeuch *et al.*, 2015), but appropriate dosing should be able to decrease or eliminate this effect.

In conclusion, ATP and adenosine are involved in the CSN overactivity induced by the HF diet, via $P2X_3$ and A_{2A} receptors, respectively. ATP and adenosine are key neurotransmitters responsible for the CB chemotransduction during hypoxia in both control and HF animals, with a higher contribution of ATP for intense hypoxias and adenosine to moderate hypoxias. For the clinical purpose and knowing that intense hypoxias are less prone to appear, the modulation of ATP signalling in the CB via $P2X_3$ receptors could be a new therapeutic target for metabolic diseases, like T2D.

VI

General discussion

6. General discussion

T2D is one of the most common chronic diseases in the world. The prevalence of T2D continues to increase, being expected to affect 629 million people in the world in 2045 (IDF, 2017), and Portugal is not an exception. The main goal in the treatment of T2D is glycemic control. To achieve this goal, it is recommended a lifestyle intervention that includes weight loss and exercise (Inzucchi *et al.*, 2012). Apart from lifestyle and nutritional interventions, several oral antihyperglycemic agents are available in the market, however even with all the therapeutic options, the control of plasma glucose levels deteriorates progressively over time (EMA, 2012). Therefore, there is a need for new therapeutic approaches for the treatment of T2D.

In the last years, the CB has been described as a metabolic sensor involved in the control of energy homeostasis (Koyama *et al.*, 2000; Wehrwein *et al.*, 2010; Conde *et al.*, 2017b). It was described that CB overactivity is involved in the genesis of insulin resistance and hypertension induced by the hypercaloric diets (Ribeiro *et al.*, 2013).

In the present thesis, we demonstrated for the first time that CSN resection not only prevents the development of insulin resistance and hypertension (Ribeiro *et al.*, 2013) but that was capable to reverse pre-established insulin resistance, glucose intolerance and hypertension in animal models of metabolic syndrome and T2D (Fig. 3.2 and 3.5, chapter III; Fig. 4.3, chapter IV). The mechanisms behind the restoration of insulin sensitivity and glucose metabolism involved an improvement in the insulin signalling pathways in liver and visceral adipose tissue and the restore of glucose uptake in these tissues (Fig. 3.3 and 3.4, chapter III). Since the surgical resection of the CSN is unlikely to be well-accepted by patients, as can cause several adverse effects related with the loss of other CB-mediated function, e.g. the loss of hypoxic responses, we aimed to test two different approaches to modulate the activity of the CSN. We tested the use of a novel bioelectronic application, the kilohertz frequency alternating current (KHFAC) to modulate CSN activity and showed that this method was capable to restore the insulin sensitivity and the glucose tolerance in a T2D animal model (Fig. 4.6, chapter IV). Additionally, knowing that a bioelectronic intervention, although efficacious might not be accepted by all patients, as it is an invasive procedure, we aimed to investigate the possibility of a pharmacological intervention to modulate CSN activity. For that, we first investigated the contribution of adenosine and ATP for CB/CSN overactivation seen in metabolic diseases. We showed that ATP and adenosine

are involved in the CSN overactivation induced by the HF diet, via mainly $P2X_3$ and A_{2A} receptors (Fig. 5.6, chapter V), respectively. Also, as previously described both neurotransmitters were also are involved in the CSN activity evoked by hypoxia, being adenosine more relevant in moderate hypoxia, while ATP is preponderant in intense hypoxia (Fig. 5.7 and 5.8, chapter V). As intense hypoxias are less common and less compatible with life, this lead us to suggest that the modulation of ATP signalling in the CB via $P2X_3$ might be use as a therapeutic target to treat metabolic diseases, as metabolic syndrome and T2D.

The main achievements of this thesis (summarized in Fig. 6.1) contribute to strengthen that the modulation of CB/CSN activity represents a novel therapeutic approach for metabolic diseases, as metabolic syndrome and T2D.

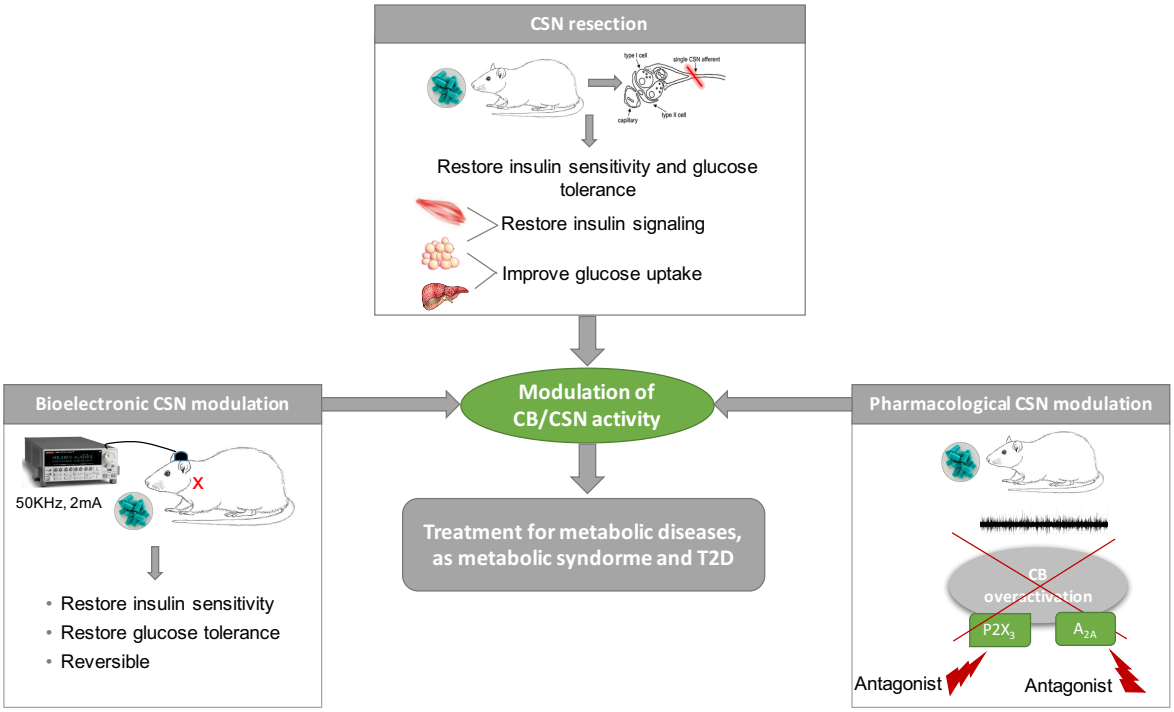


Figure 6.1 - Main achievements of this thesis. Carotid sinus nerve (CSN) resection restored insulin sensitivity and glucose metabolism, an effect that is mediated by an improvement in the insulin signalling pathways in liver and visceral adipose tissue (chapter III). Since the surgical resection of the CSN is prone in adverse effects, it was tested a bioelectronic application (chapter IV) and the antagonism of adenosine/ATP receptors (chapter V) to modulate the CSN activity. The bioelectronic modulation of the CSN activity restored the insulin sensitivity and the glucose tolerance in HFHSu animals. The application of $P2X_3$ and A_{2A} receptors antagonists restored the CSN chemosensory activity in the HF animals. The data presented in this thesis contribute to strengthen that the modulation of carotid body (CB)/CSN activity could be a novel therapeutic approach for metabolic diseases, as metabolic syndrome and type 2 diabetes (T2D).

6.1. Carotid body as a target to treat metabolic diseases, as metabolic syndrome and type 2 diabetes

In the last years, the CB has emerged as a promising therapeutic target for treating sympathetic mediated diseases such as, essential hypertension, hypertension associated with obstructive sleep apnea, chronic heart failure and T2D (Paton *et al.*, 2013b; Iturriaga, 2017). This suggestion was supported by the fact that CB activity is increased in these diseases, contributing to their pathogenesis (Paton *et al.*, 2013b; Iturriaga, 2017).

The role of CB in the development of insulin resistance was first described by Ribeiro *et al*. (2013). In this work, we described that the CB activity is increased in animal models of metabolic syndrome and prediabetes and that CSN resection prevented the development of insulin resistance and hypertension induced by the hypercaloric diets. It was also shown for the first time, that the CB possess insulin receptors that phosphorylates in the presence of insulin (Ribeiro *et al.*, 2013), suggesting that is insulin that triggers CB activation, leading to the sympathetic nervous system overactivity that is associated with metabolic disturbances, like T2D (Ribeiro *et al.*, 2013). Confirming these results, we have recently described that prediabetes patients also exhibit increased CB activity, measured by the Dejour test, which correlates with insulin levels and insulin resistance (unpublished data, Conde *et al.*, 2018a).

Besides preventing the development of insulin resistance and hypertension, in the present work it was also observed that CSN resection restores insulin sensitivity, glucose tolerance and normalizes the high blood pressure in animal models of metabolic syndrome and T2D (Fig. 3.2 and 3.5, chapter III; Fig. 4.3, chapter IV). Moreover, in chapter III, we added data to elucidate the mechanisms that are behind the restoration of insulin action and glucose homeostasis. We showed that CSN resection restored the insulin signalling in adipose tissue, which was accompanied by an increase in glucose uptake in perienteric adipose tissue in HF animals (Fig. 3.3 d-h and 3.4d, chapter III) and, we also found that the liver of HF animals with CSN denervation exhibited an increase in GLUT2 levels and an improvement in glucose uptake (Fig. 3.3k and 3.4c, chapter III). Moreover, we also described that CSN resection not only prevented (Ribeiro *et al.*, 2013) but also reversed the increase in sympathetic nervous system activity (Fig. 3.6, chapter III) in animal models of metabolic syndrome and prediabetes.

These results suggest that the positive alterations mediated by CSN resection involve the normalization of sympathetic nervous system activity to afferent insulin-sensitive organs, the liver and adipose tissue and therefore, the restoration of insulin signalling in the liver and in adipose tissue are contributing to normalize insulin sensitivity and glucose homeostasis is these pre-established metabolic diseases animals. All these finds contributed to demonstrate that the CB could be a therapeutic target to treat metabolic diseases, such as metabolic syndrome and T2D.

6.1.1. Modulation of carotid body activity to treat type 2 diabetes

Overactivation of CB in metabolic diseases has been previously described by an increase in CBs weight in HF animals (Ribeiro *et al.*, 2013) and in patients with T2D (Cramer *et al.*, 2014), as well as by the increase in the expression of tyrosine hydroxylase, the limiting enzyme in the synthesis of catecholamines, in HF and HFHSu animal models (Ribeiro *et al.*, 2013; Dos Santos *et al.*, 2018). Moreover, an increase in spontaneous ventilation and in the release of dopamine induced by hypoxia (5% O_2) in HF and Hsu animals (Ribeiro *et al.*, 2013) was also observed. All these data are in agreement with the increased basal CSN chemosensory discharge found in HF animals previously by Ribeiro *et al.* (2017) and in the context of the present work (Fig. 5.3, chapter V). Our group have postulated in the last years that the major factor contributing to the heightened CSN activity observed in the prediabetes and T2D animal models is hyperinsulinemia (Conde *et al.*, 2014; Conde *et al.*, 2017b; Conde *et al.*, 2018b). We have showed that insulin receptors are present in the CB, and that when activated they are able to initiate a neurosecretory response leading to hyperventilatory responses (Ribeiro *et al.*, 2013) and to increased sympathetic activity (unpublished data, Panarese *et al.*, 2016; Cracchiolo *et al.*, 2018). These data is in line with the findings by Limberg *et al*. (2014), where they observed that hyperoxia, used to desensitize CB chemoreceptors, reduced muscle sympathetic nerve activity in hyperinsulinaemic conditions, suggesting that the CB mediates insulin-dependent sympatho-excitation in humans. Finally our group recently shown that CB chemosensitivity, assessed by the Dejour test, is increased in prediabetes patients, an effect that is directly correlated with insulin resistance and insulin levels (unpublished data, Conde *et al.*, 2018a).

All together, the effect of CSN denervation on the restoration of glucose metabolism and insulin action together with the fact that CB overactivity is contributing

to the development of insulin resistance and glucose intolerance, pointed out that the modulation of the CB/CSN activity might be a new therapeutic approach to treat metabolic diseases, as T2D.

6.1.1.1. Carotid sinus nerve resection: the consequences

Several animal and human studies have suggested the use of unilateral or bilateral CB ablation for the treatment of essential hypertension (Abdala *et al.*, 2012; Fudim *et al.*, 2015; Narkiewicz *et al.*, 2016) and heart failure (Del Rio *et al.*, 2013; Niewinski *et al.*, 2013; Marcus *et al.*, 2014; Niewinski *et al.*, 2017). Moreover, since T2D is also associated with cardiovascular disease, it was also suggested that CB ablation could have beneficial effects in the control of plasma glucose levels (Paton *et al.*, 2013b). However, the CB mediates several physiological responses, like the responses to hypoxia, hypercapnia and adaptation to exercise (Hodges & Forster, 2012), apart from its involvement in glucose homeostasis. Also, the CSN conveys not only the information related with CB chemoreceptors but also baroreceptor information that come from carotid baroreceptors, in close proximity with the CB (Gonzalez *et al.*, 2014). In agreement with these physiological functions, the surgical resection of CSN or CB ablation induce the loss of peripheral hypoxic response (Wade *et al.*, 1970), with decreased sensitivity to CO_2 (Honda, 1985; Timmers *et al.*, 2003; Dahan *et al.*, 2007), impaired response to exercise (Forster *et al.*, 1983; Honda, 1985; Dempsey & Smith, 1994) and fluctuations in blood pressure (Nakayama, 1961; Fudim *et al.*, 2015).

Aiming to overcome the appearance of these adverse effects, Pijacka *et al*. (2018) described a new surgical approach for selective ablation of the CB that was able to reduce the blood pressure in hypertensive rats without affecting the baroreflex. Although these results showed the potential of the surgical approach for humans' purpose, the surgical ablation of CB chemoreflex is still a challenge. Additionally, Pijacka *et al*. (2018) also demonstrated the importance of CB in the haemodynamic response to hypoxia, hypercapnia and exercise in conditions of hypertension. The increase in blood pressure that was observed during hypoxia, hypercapnia and exercise were abolished, increased and unaffected, respectively, after selective ablation of the CB (Pijacka *et al.*, 2018). These data demonstrated that CB ablation may trigger potential cardiovascular risks during hypoxia and hypercapnia in spontaneous

hypertensive rats, suggesting that the modulation of CB activity may be a more effective and safer approach when compared with the CB ablation (Pijacka *et al.*, 2018).

Since the CB ablation or the CSN denervation is prone in side effects, due to alterations in the CB physiological function, it is necessary to discover/develop new approaches to modulate the CB activity. In this thesis, we tested the modulation of the CB activity by a bioelectronic approach and we also investigated the role of ATP and adenosine in the CB overactivation induced by the hypercaloric diets, to understand if it is possible to use the modulation of ATP and/or adenosine signalling in the CB to decrease its activity in metabolic diseases, as T2D.

6.1.1.2. Bioelectronic modulation of carotid sinus nerve as a therapeutic for metabolic diseases: an innovative approach

Biolectronic medicines, are a new type of therapeutic approach aiming to detect and correct aberrant pathological electrical signalling patterns in the peripheral nervous system, by implantation of electrodes and delivery of electrical current through them (Famm *et al.*, 2013; Birmingham *et al.*, 2014). This innovative therapeutic approach might be more effective in the treatment of metabolic diseases, like metabolic syndrome and T2D, since the therapeutic approaches that are currently available for these pathologies do not provide long-term control of the disease. Moreover, even knowing that implantation of electrodes are an invasive procedure with the need of a surgical procedure, it is expected that bioelectronic medicines will have minimal interference with daily activities, providing high adherence and acceptance among patients (Famm *et al.*, 2013; Birmingham *et al.*, 2014; Dorey, 2016).

In the present work, we described that bioelectronic neuromodulation of the CSN with continuous KHFAC was able to restore insulin sensitivity and glucose tolerance in the T2D animal model (Fig. 4.6, chapter IV). Moreover, it was also observed that CSN KHFAC modulation is reversible, since 5 weeks after stopping the electrical current the animals became insulin resistant and glucose intolerant again (Fig. 4.5 and 4.6, chapter IV). All together, these results showed that CSN bioelectronic neuromodulation has significant therapeutic potential for metabolic diseases, namely for T2D.

The potential clinical importance of the electrical block or stimulation of specific nerves has been demonstrated herein in animals but also in human clinical

trials. The implantation of devices that applied KHFAC showed to be effective in the treatment of obesity (Sarr *et al.*, 2012; Shikora *et al.*, 2015; Apovian *et al.*, 2017), postamputation pain (Soin *et al.*, 2015) and chronic back pain (Tiede *et al.*, 2013; Van Buyten *et al.*, 2013). However, herein we have used continuous KHFAC to modulate CB/CSN function and therefore, adverse effects related with the loss of CB physiological functions might appear. Knowing that CSN carries not only information that comes from the CB, but also the baroreceptor information that arises from the carotid sinus (McDonald, 1983), being involved in short-term fluctuations in blood pressure (Marshall, 1994), it would be expected that CSN electrical modulation might come with these adverse effects. In fact, Katayama *et al*. (2018) described that the electrical stimulation (20 seconds) of the carotid sinus, by implantation of a bipolar electrode around the intact carotid sinus encompassing the CSN, activates both the carotid baroreflex and carotid chemoreflex, triggering an increase in ventilation and a decrease in arterial pressure. In the present work, we observed that 9 weeks of CSN neuromodulation normalize blood pressure in T2D animal model. Moreover, we have also observed a normalization of blood pressure in our prediabetes and T2D models 3 and 9 weeks after CSN resection, which could be explained by the long-term adjustments of the arterial baroreflex (Ribeiro *et al.*, 2013; Sacramento *et al.*, 2018a). Therefore, we can suggest that the short-term fluctuations in blood pressure can be compensated in long-term, as recent studies have shown that arterial baroreflex is a long-term regulator of blood pressure (Lohmeier *et al.*, 2002; Zoccal *et al.*, 2009; Lohmeier & Iliescu, 2015). Additionally, since the CB is involved in the response to hypoxia, hypercapnia and adaptation to exercise, the continuous electrical neuromodulation of the CSN may produce exercise intolerance and unsafe hypoxic acclimatization (Wade *et al.*, 1970; Forster *et al.*, 1983; Honda, 1985).

To minimize the off-target effects of the bioelectronic modulation of the CSN, considerable work is needed to find the better way to modulate the CSN activity to treat metabolic diseases, as metabolic syndrome and T2D without affecting CB physiological functions. An alternative to the continuous block of the CSN activity aiming to maintain CB physiological functions might be the use of intermittent electrical block. Intermittent electrical block applied 12h per day (5 minutes of blocking alternating with 5 minutes without blocking) was used to supress vagal activity, and it was shown to be relatively efficacious in promoting weight loss in humans (Camilleri *et al.*, 2008). Another

alternative, and since we are trying to modulate glucose homeostasis, is the use of intermittent blocking postprandially, for example a device that will be only turned-on during the meal and in the next 1-2h after the meal, when insulin levels are higher and can stimulate CB chemoreceptors. This type of intermittent electrical modulation is not new as for the treatment of obstructive sleep apnea it was shown to be efficient the use of an upper-airway stimulated device placed on the hypoglossal nerve to recruit tongue-protrusion function (Maurer *et al.*, 2012; Woodson *et al.*, 2018) that is turned-on by the patient before he goes to sleep and turned-off when the patient wake up (Maurer *et al.*, 2012). However, this approach can lead to low acceptance among patients, since there is the need to turn-on and off the device each time. Nevertheless, in our case for CSN modulation for metabolic diseases and knowing that CB detects insulin levels, we can suggest a device that detects automatically insulin levels, and turn-on when insulin levels are high and turn-off when the insulin levels are restored. Furthermore, our group has been dedicated to the identification and characterization of neuronal circuits that are involved in the CSN overactivity in metabolic diseases (Conde & Guarino, 2018). Our hypothesis is that different fibres within the CSN are involved in the response to different stimuli. Therefore, if proven right, it will be possible to selectively modulate the CSN fibres that mediate metabolic stimuli without affecting O_2 and CO_2 chemosensitivity (Conde & Guarino, 2018).

In the present work, we observed that type 2 and 4 of CSN action potentials, and mainly type 2 action potentials are increased in the HF animals in basal conditions (Fig. 5.3 c and c1, chapter V) but not in response to hypoxia or hypercapnia (Fig. 5.4 b and c, chapter V). These data suggest that probably the selective modulation of these two types or probably only type 2 action potentials (Fig. 5.3d, chapter V) might be used to restore the basal CSN activity in T2D, with minimal effects in CB physiological functions, like the response to hypercapnia and hypoxia.

We can conclude, that bioelectronic modulation of the CSN activity is a novel and effective treatment for T2D and metabolic syndrome however, considerable work needs to be done aiming to develop a device that selectively modulate CSN activity with minimal effects on CB physiological function.

6.1.1.3. Pharmacological modulation of the carotid sinus nerve as a treatment for metabolic diseases

A pharmacological approach to modulate the activity of the CSN is also an attractive possibility, as it is well known that conventional medicines, especially the ones administrated by an oral route are the ones better accepted by patients and with higher adhesion (Bardal *et al.*, 2011). The modulation of purinergic system in the CB has been recently indicated as a potential therapeutic target for diseases involving CB overactivation (Conde *et al.*, 2017a). For example, Pijacka *et al*. (2016) has recently described that $P2X_3$ receptors antagonism decreased blood pressure and basal sympathetic nervous system activity and normalized CB hyperreflexia in spontaneous hypertensive rats, while no effects were observed in rats without hypertension, suggesting that CB $P2X_3$ receptors could be a new target for essential hypertension. Furthermore, our group described that the blockade of adenosine receptors with caffeine is able to inhibit CSN chemosensory activity in chronic intermittent hypoxic rats (Sacramento *et al.*, 2015), which is a characteristic feature of obstructive sleep apnea.

In the present work, we showed that ATP and adenosine are involved in the basal CSN chemosensory activity, mainly via $P2X_3$ and A_{2A} receptors, respectively, in HF animals (Fig. 5.6, chapter V). Moreover, and as previously described by Conde *et al.* (2012a), we also observed that both neurotransmitters contribute to generate CSN in hypoxia, being each contribution dependent on the hypoxic intensity (Fig. 5.7 and 5.8, chapter V). For the first time, herein we described the presence of four types of action potentials in the CSN that were similar between control and HF animals (Fig. 5.2b, chapter V), being all these action potentials involved in the responses elicited by ATP and adenosine in the CSN.

As previously described in chapter V, ATP is the major contributor to the CSN chemosensory activity evoked by intense hypoxia, a condition that is less common and compatible with life. In the top of Everest (8848 m) the inspired PO_2 declined from 150mmHg (at sea level) to 43mmHg, which is an extreme hypoxic environment (West, 2000; Burtscher *et al.*, 2018). Moreover, to live in an extreme hypoxic environment several adaptive biological processes will occur (Burtscher *et al.*, 2018). Intense hypoxias could also appear in very exacerbated pathologies. In chronic obstructive pulmonary disease during exacerbations the PaO_2 could decline to 55mmHg, which induced a marked increase in minute ventilation and a subsequent fall in $PaCO_2$, leading

to the recommendation of long-term oxygen therapy (Kim *et al.*, 2008; Brill & Wedzicha, 2014). In obstructive sleep apnea the oxygen saturation (SaO_2) decreased with severity of the disease and in patients with severe obstructive sleep apnea the minimum SaO_2 was 71.44% (Kuźmińska *et al.*, 2016). Taking all these in consideration we proposed that the modulation of ATP signalling rather than adenosine signalling in the CB to restored the basal CSN chemosensory activity in T2D will have less consequences related with the loss of hypoxic responses. If inhibition of $P2X_3$ receptors in the CB occur aiming the treatment of metabolic diseases, adenosine could keep mediating CSN chemosensory responses to moderate hypoxia and hypercapnia and therefore, the modulation of CB overactivity in T2D without major effects in the CB function can occur.

The clinical use of a $P2X_3$ ATP antagonism is not new as it was recently tested in a clinical trial to treat chronic pathological cough (Abdulqawi *et al.*, 2015). It was described that AF-219, a selective $P2X_3$ antagonist, was effective in decreasing the frequency of cough (Abdulqawi *et al.*, 2015). Besides chronic cough, $P2X_3$ receptors have also been described to be involved in overactive bladder and neuropathic pain (Cockayne *et al.*, 2000; Ford, 2012; Burnstock, 2013; Ford & Undem, 2013), showing its potential as a therapeutic target. Although, its clinical importance should not be underestimated, we cannot ignore the adverse effects that can result from $P2X_3$ ATP antagonism. For example, in the clinical trial for chronic cough treatment, it was observed taste disturbances (Abdulqawi *et al.*, 2015). Therefore, in the future and if $P2X_3$ ATP antagonism is successful in reversing insulin sensitivity and glucose intolerance in T2D, it would be necessary to establish an appropriate dose that could be beneficial with minimal or none adverse effects.

In the CB, the antagonism of $P2X_3$ receptors with AF-353, decreased by 72.36% the chemoreflex sensitivity of the petrosal chemoreceptive neurons in response to chemical hypoxia induced by sodium cyanide (NaCN) in spontaneous hypertensive rats, an effect that was reversible after the washout of the AF-353 (Pijacka *et al.*, 2016). These results suggest however that modulation of $P2X_3$ receptors might affect the responses to hypoxia. Moreover, the *in vivo* application of AF-219, a selective $P2X_3$ receptor antagonist that does not cross the blade brain barrier, not only decreased the blood pressure, but also decrease the heart rate and the respiratory frequency in spontaneous hypertensive rats, without significant changes in non-hypertensive rats (Pijacka *et al.*, 2016). Additionally, in spontaneous hypertensive rats that were

submitted to the CB resection, the infusion of AF-219 still caused a residual decrease in the blood pressure, which suggested an effect of this antagonist in other $P2X_3$ receptors besides the ones that are present in the CB (Pijacka *et al.*, 2016).

Regarding the CB response to hypercapnia, it was not observed any alterations in the ventilatory response to this stimulus in mice deficient in $P2X_2$ and/or $P2X_3$ (Rong *et al.*, 2003). These results contradicts the findings of Prasad *et al.* (2001), that showed a decrease in the petrosal neuron firing evoked by hypercapnia with suramin, a P2 receptor antagonist. Since suramin could be inhibiting the $P2X_2$, $P2X_3$, $P2Y_1$, $P2Y_2$ and $P2Y_{12}$ in the CB, as previously discussed in chapter V, the effect observed by Prasad *et al*. (2001), might not be mediated by the $P2X_3$ receptors as proposed by Rong *et al.* (2003). Therefore, the first next steps would be to prove that the modulation of $P2X_3$ receptors in the CB could be used to treat T2D, while preserving the normal CB function. For that we need to test *in vivo* the effect of the antagonism of $P2X_3$ receptors on the insulin sensitivity and glucose tolerance and of course test the responses to moderate hypoxia (10% O_2) and hypercapnia. Knowing that apart from the peripheral nervous system, ATP also acts centrally, we will use the AF-219, a $P2X_3$ ATP receptor antagonist that do not cross the blood brain barrier (Pijacka *et al.*, 2016).

In conclusion, in chapter V, we propose that probably the modulation of $P2X_3$ ATP receptors in the CB might be a pharmacological therapeutic target for metabolic diseases as T2D. However, more work is needed to confirm and reinforce the potential clinical importance of the antagonism of $P2X_3$ receptors.

Conclusions

7. Conclusions

1. **CB is involved in the genesis and maintenance of insulin resistance and deregulation of glucose homeostasis in prediabetic and early type 2 diabetes animal models through alterations in the insulin signalling pathways in liver and visceral adipose tissue, since:**
    - CSN bilateral resection restored insulin sensitivity and normalized plasma glucose, insulin and C-peptide levels in rat prediabetes and early type 2 diabetes animal models, the HF, HSu and HFHSu rats.
    - CSN bilateral resection restored insulin signalling pathways in skeletal muscle and adipose tissue in both HF and HSu animal models.
    - CSN bilateral resection restored glucose tolerance in HF and HFHSu animals.
    - CSN bilateral resection improved glucose uptake by the liver and perienteric adipose tissue in HF animals.
    - CSN bilateral resection in our animal models also normalized systemic sympathetic nervous system activity, lipid profile, arterial pressure and endothelial function.

2. **Bioelectronic modulation of CSN using KHFAC improved metabolic control in a rat model of T2D, suggesting a novel therapeutic approach for T2D because:**
    - The CSN modulation with KHFAC (50 KHz, 2 mA) during 9 weeks restored insulin sensitivity and glucose tolerance in HFHSu animals, a diet-induced early stage T2D animal model.
    - This approach is reversible and did not damage the CSN, since 5 weeks after to stop the CSN modulation the animals became insulin resistant and glucose intolerant again.

3. **HF diet increased the basal CSN type 2 and 4 action potentials, without changing the CSN chemosensory response to hypoxia and hypercapnia, since:**

- From the four types of action potentials identified in the CSN chemosensory activity, the HF diet increased the number of type 2 and 4 action potentials by 80.36 and 74.28%, respectively.
- HF diet did not modify significantly any of the four types of CSN action potentials in response to intense and moderate hypoxia (0 and 5% O_2) and hypercapnia (10% CO_2).

4. **The modulation of ATP signalling in the CB, via $P2X_3$ receptors, restored the CSN activity in HF animals and decreased intense hypoxia response. Since intense hypoxias are less prone to occur than moderate hypoxia, we suggest that modulation of $P2X_3$ receptors might be a novel therapeutic approach for metabolic diseases, as T2D because:**
   - ATP and adenosine are involved in the CSN overactivity induced by the HF diet, mainly via $P2X_3$ and A_{2A} receptors, respectively, since the application of AF-353 and SCH58261 restored the basal CSN activity in this animal model.
   - ATP has a more relevant role during intense hypoxias, an effect that is mainly mediated by $P2X_3$ receptors.
   - Adenosine has a higher contribution to generate the CSN activity in moderate hypoxias, an effect that is mediated by A_{2A} and A_{2B} receptors in the control animals, and by A_{2A} receptors in the HF animals.
   - The different types of CSN action potentials followed the same profile described above, in intense hypoxia, the action potentials decreased in the presence of ATP receptor antagonists, suramin and AF-353, while in moderate hypoxia the action potentials decreased in the presence of adenosine receptor antagonists, ZM241385 and SCH58261.

In conclusion, the results presented herein clarify the mechanisms by which the CB is involved in the control of glucose homeostasis and reinforce the clinical importance of CB for the treatment of metabolic diseases, like T2D. We showed that CB overactivity in prediabetes and T2D could be modulated by a bioelectronic approach, via the application of KHFAC, and possible by the pharmacologic antagonism of $P2X_3$ ATP receptors.

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