Universidade de Lisboa

Faculdade de Ciências

Departamento de Química e Bioquímica

# *Into the regulation of glucose homeostasis: from periphery to brain*

**Inês Couto Coelho**

Dissertação

Mestrado em Bioquímica

Área de Especialização em Bioquímica Médica

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Orientadores: Professora Doutora Maria Paula Macedo e Professor Doutor Pedro Lima

**2013**

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# Abstract

The main goal of this thesis was to have an integrative view both in peripheral glucose homeostasis and its impact on brain synaptic proteins content.

In our first study we hypothesized that N-acetyl-cysteine (NAC) as a source of the essential amino acid cysteine, critical for glutathione (GSH) synthesis, impacts on peripheral insulin sensitivity. Glucose excursions and insulin sensitivity were assessed together with levels of nitric oxide (NO) in normal and hepatic parasympathetic (HPN) denervated animals. In this study we observed that NAC in the presence of glucose leads to an increase in insulin sensitivity in peripheral tissues in non denervated animals. These results suggest that NAC acting as a source of cysteine for glutathione synthesis is an essential feeding signal increasing insulin sensitivity.

Our second hypothesis states that a high sucrose diet (HSD) impacts on peripheral glucose tolerance resulting in changes in GLUT4 and GLUT12 proteins content and fat accumulation. Moreover, HSD also results in changes in synaptic proteins content in hippocampus and cortex of this animal model. Glucose tolerance, fat and lean mass accumulation were evaluated at 4, 24 and 36 weeks of diet. At these time points animals were sacrificed and hippocampus and cortex removed in order to evaluate synaptic proteins content, namely synapsin-1, rabphilin-3 and synaptophysin as well as those involved in the SNARE complex formation, SNAP-25 and syntaxin. Consumption of a HSD lead to development of glucose intolerance with decreased GLUT4 content and increased GLUT12 in skeletal muscle in a compensatory manner. Fat accumulation was observed in animals submitted to HSD at 24 and 36 weeks of diet simultaneously with alterations in synaptic proteins content implying compromised synaptic function. Within synaptic proteins

The integrity of glucose homeostasis is a key factor that influences not only peripheral insulin sensitivity but also brain synaptic transmission as features of diabetes.

# Resumo

A diabetes *mellitus* é uma doença metabólica bastante comum, sendo considerada atualmente como epidemia. Estima-se que em 2030, cerca de 552 milhões de pessoas tenham desenvolvido diabetes e que cerca de 183 milhões venham a desenvolvam esta doença, não estando diagnosticada, sendo por isso uma doença que constitui grande importância para a saúde pública.

A diabetes *mellitus* pode ser dividida em dois subtipos: diabetes tipo 1 ou tipo 2. A diabetes tipo 1, também denominada por diabetes dependente de insulina, é caracterizada pela falência das células β do pâncreas que deixam de produzir insulina, sendo este tipo mais comum em crianças ou jovens adultos. A diabetes tipo 2 está associada ao estilo de vida que conduz ao desenvolvimento de resistência à insulina, normalmente caracterizada por elevados níveis de glicose (hiperglicemia) e insulina (hiperinsulinémia) no sangue. Antes de a doença ser diagnosticada, os doentes apresentam normalmente um estado de pré-diabetes, que é caracterizado por valores de glicemia elevados no estado pós-prandial. Atualmente existem diversos modelos animais para o estudo de doenças metabólicas, nomeadamente para a diabetes tipo 1 e tipo 2. Nestes modelos a diabetes pode ser induzida química ou nutricionalmente, sendo neste último caso utilizadas dietas enriquecidas em açúcar ou gordura.

A presente tese teve como principal objetivo obter uma visão integrativa da homeostase da glicose a nível periférico assim como o seu impacto ao nível da transmissão sináptica no cérebro, nomeadamente no conteúdo das proteínas sinápticas.

A primeira parte deste trabalho focou-se em avaliar o efeito do aminoácido cisteína (através da administração de N-acetil-cisteína, NAC) na sensibilidade periférica à insulina. Tem sido descrito que os aminoácidos têm um papel importante na sensibilidade à insulina periférica no estado pós-prandial. Além disso, sabe-se que a cisteína é um aminoácido essencial para a síntese de glutationo, que desempenha um papel fundamental na libertação de uma substância denominada HISS (*Hepatic Insulin Sensitizing Substance*), que por sua vez promove um aumento na sensibilidade à insulina nos tecidos periféricos. Assim, na primeira parte desta dissertação, colocou-se a hipótese de que a administração intra-entérica de NAC tem influência na sensibilidade à insulina nos tecidos periféricos. Para a realização deste trabalho foram utilizados ratos Wistar fêmeas com 9 semanas, que foram divididos em 4 diferentes grupos. Ao primeiro grupo foi administrada intra-entericamente glicose (grupo controlo), ao segundo apenas NAC, ao terceiro NAC e glicose e ao quarto grupo foi

administrado NAC e glicose após ser realizada a desnervação hepática dos animais. A desnervação teve como objetivo inibir a ação dos nervos parassimpáticos hepáticos. Para testar a hipótese proposta, foi avaliada a tolerância à glicose através de um teste de tolerância à glucose intra-entérico, em que os resultados são obtidos na forma de área sob a curva dos valores de glicose medidos durante os 120 minutos do teste; a sensibilidade à insulina foi também avaliada, através do RIST (*Rapid Insulin Sensitivity Test*), que é um teste euglicémico em que os níveis de glicose são mantidos perto dos valores basais durante todo o teste. Os níveis de óxido nítrico no plasma e fígado foram determinados através de um método baseado na quantificação de nitratos e nitritos. Observou-se que a administração intra-entérica de NAC na presença de glicose aumenta a sensibilidade à insulina nos tecidos periféricos. Além disso, a desnervação dos animais previne o aumento da sensibilidade à insulina promovida pelas administrações de NAC e glicose, sendo esta sensibilidade semelhante aos animais controlo. Os níveis de óxido nítrico não foram alterados em nenhum dos grupos experimentais referidos, nem no plasma nem no fígado, exceto no grupo de animais desnervados, onde se verificou uma diminuição dos níveis de óxido nítrico tanto no plasma como no fígado.

Os resultados obtidos sugerem que a coadministração de NAC com glicose induz o aumento da sensibilidade à insulina nos tecidos periféricos, atuando como um sinal essencial para o aumento da sensibilidade à insulina. Podemos concluir ainda que são estritamente necessários os nervos parassimpáticos hepáticos para que haja uma maior sensibilidade à insulina nos tecidos periféricos.

A segunda parte deste trabalho teve como objetivo avaliar o efeito de uma dieta rica em sacarose (35%) em ratos Wistar machos durante 4, 24 e 36 semanas, na tolerância à glicose periférica e quais os seus efeitos nas proteínas sinápticas envolvidas na formação de vesículas sinápticas responsáveis pela adequada formação de sinapses intervenientes nos processos cognitivos.

A diabetes *mellitus*, quer tipo 1 quer tipo 2, está associada a diversos problemas devido ao aumento dos níveis de glicose no sangue, que levam à disfunção de diferentes órgãos, principalmente rins e olhos e também de vasos sanguíneos. Nos últimos anos têm vindo a aumentar evidências de que a diabetes afeta também o sistema nervoso central, tendo sido denominadas estas alterações por encefalopatia diabética. Doentes diabéticos tipo 1 e tipo 2 têm demonstrado alterações ao nível estrutural e molecular no cérebro, sendo que foram já observadas alterações ao nível das proteínas sinápticas em modelos animais de diabetes tipo 1, levando a alterações nos processos cognitivos. Tendo em conta os estudos descritos, neste trabalho propôs-se a hipótese de que uma dieta rica em sacarose leva a alterações na

tolerância à glicose periférica, resultando na diminuição dos transportadores de glicose presente no músculo-esquelético, GLUT4 e GLUT12, assim como à acumulação de massa gorda. Além disso, propôs-se também que esta dieta tem impacto no conteúdo das proteínas sinápticas, quer no hipocampo, quer no córtex. Para a realização deste estudo os animais foram divididos em dois grupos, controlo e sacarose. O grupo sacarose teve acesso a uma solução de sacarose (35%) e o controlo apenas a água, além da ração comum aos dois grupos. No final da dieta, após 4, 24 e 36 semanas foi realizado um teste de tolerância à glicose intra-entérico e posteriormente os animais foram sacrificados e foram-lhes removidos os tecidos: músculo-esquelético, hipocampo e córtex, nos quais foi avaliado o conteúdo proteico utilizando a técnica *western blot*. A quantificação de massa gorda e massa magra foi realizada através de ressonância magnética noutro grupo de animais submetidos às mesmas condições. Os resultados obtidos neste estudo revelaram que uma dieta rica em sacarose induz o desenvolvimento de intolerância à glicose com diminuição da expressão de GLUT4 e aumento do GLUT12, numa forma compensatória. A acumulação de massa gorda foi também observada nos animais submetidos à dieta rica em sacarose após 24 e 36 semanas de dieta, simultaneamente com alterações no conteúdo das proteínas sinápticas, o que poderá comprometer a função sináptica.

Este estudo revela que a homeostase da glicose é um processo chave que influencia não apenas a sensibilidade à insulina nos tecidos periféricos mas também a transmissão sináptica ao nível cerebral. O seu adequado funcionamento é essencial para que não se desenvolva um estado de pré-diabetes, que poderá culminar em diabetes tipo 2.

## Keywords

Glucose homeostasis

N-acetyl-cysteine

Insulin sensitivity

Pre diabetes

Synaptic proteins

GLUT4 / GLUT12

Cognitive impairments

## Palavras-chave

Homeostase da glicose

N-acetil-cisteína

Sensibilidade à insulina

Pré diabetes

Proteínas sinápticas

GLUT4 / GLUT12

Problemas cognitivos

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# Abbreviations

| | |
|---|---|
| Ach | Acetylcholine |
| AD | Alzheimer's disease |
| ADA | American Diabetes Association |
| Akt | Serine/threonine specific protein kinase |
| AMPA | α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid |
| ANS | Autonomous nervous system |
| ARC | Arcuate nucleus |
| AUC | Area Under the Curve |
| BBB | Blood-brain barrier |
| BSA | Bovine serum albumin |
| CaMKII | $Ca^{2+}$/calmodulin-dependent protein kinase |
| CNS | Central Nervous System |
| DM1 | Diabetes mellitus type 1 |
| DM2 | Diabetes mellitus type 2 |
| GABA | γ-aminobutyric acid |
| GIP | Glucose-dependent insulinotropic peptide |
| GLP-1 | Glucagon-like peptide-1 |
| GLUT | Glucose transporter-like protein |
| GSH | Glutathione |
| HDIR | Hiss dependent insulin resistance |
| HGP | Hepatic glucose production |
| HISS | Insulin sensitizing substance |
| HPA | Hypothalamic-pituitary-adrenal |
| HPA | Hypothalamic-pituitary-adrenal |
| HPN | Hepatic parasympathetic nerves |
| HSD | High sucrose diet |
| IEGTT | Intra Enteric Glucose Tolerance Test |
| IR | Insulin receptor |
| IRS-1 | Insulin receptor substrate 1 |
| IRS-2 | Insulin receptor substrate 2 |
| IS | Insulin sensitivity |
| LH | Lateral nucleus |
| LTD | Long term depression |
| LTP | LTP Long term potentiation |
| NAC | N-acetyl-cysteine |
| NMDA | N-methyl-D-aspartate |
| NO | Nitric Oxide |
| NOS | Nitric oxide synthase |
| OGTT | Oral Glucose Tolerance Test |
| PI3 | 3-phosphoinositide dependent protein kinase-1 |
| PIP2 | Phosphatidylinositol 4,5-bisphosphate |
| PIP3 | Phosphatidylinositol (3,4,5)-triphosphate |
| PKC | Protein kinase C |

| | |
|---|---|
| PNS | Peripheral nervous system |
| PSD-95 | Postsynaptic density-95 |
| PSNP | Parasympathetic nervous system |
| RIPA | Radioimmunoprecipitation assay |
| ROS | Reactive oxigem species |
| SDS | Sodium dodecyl sulphate |
| SEM | Standard error of the mean |
| SNAP-25 | Synaptosomal-associated protein with 25 kDa |
| SNARE | Synaptosomal-associated protein with 25 kDa |
| SNS | Sympathetic nervous system |
| STZ | Steptozotocin |
| SV | Synaptic vesicle |
| VAMP-2 | Vesicle-associated membrane protein 2 |
| VMN | Ventro-medial nucleus |

# 1. Introduction

## 1.1. Glucose homeostasis

Glucose is a carbohydrate, and is the most important simple sugar in human metabolism.

In plasma, glucose concentration (glycemia) is normally maintained within a range between 90–120 mg/dL (Bano 2013) despite variations in glucose levels after meals and exercise. The normoglycemia is maintained through a complex regulatory and counter-regulatory neurohormonal system (Figure 1.1). The process to maintain glucose in the normal range is called glucose homeostasis. It prevents high postprandial glucose concentrations from accumulating in the interstitial fluid, by directing glucose into stores in skeletal muscle, adipose tissue and liver. Additionally it prevents hypoglycemic events to occur mainly in the fast state. After glucose ingestion the majority is distributed to the skeletal muscle and liver, and the remaining glucose is distributed to adipose tissue as well as to tissues that are insulin independent (Sandoval, Obici, and Seeley 2009).

Plasma glucose derives from intestinal absorption from diet, from glycogenolysis (the breakdown of glycogen in liver) and gluconeogenesis (formation of glucose in liver and kidney from other sources of carbons). During the first hours of fasting, glucose availability is due to glycogenolysis and over long periods of fasting glucose is produced by gluconeogenesis (Aronoff et al. 2004). When removed from plasma, glucose is disposed through different pathways; it may be stored as glycogen in liver and skeletal muscle or may undergo glycolysis (Bano 2013).

Different factors such as nutrient ingestion, stress (mental or physical) or other environmental changes may contribute to changes in glucose homeostasis (Arble and Sandoval 2013).

There are some important factors that regulate glucose homeostasis, namely hormones such as insulin, glucagon, cortisol, growth hormone and catecholamines (Bano 2013) and autonomic nervous system, with both sympathetic and parasympathetic nerves playing an important role (Dicostanzo et al. 2006; Bano 2013; Fernandes et al. 2011)

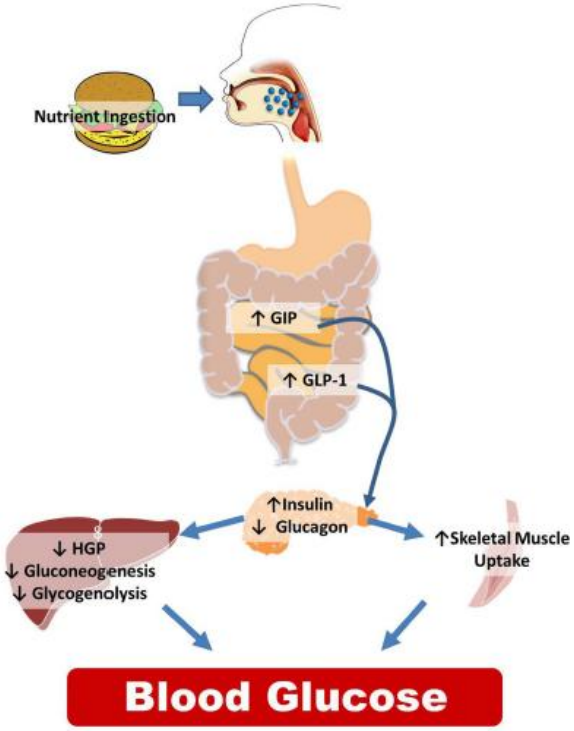


**Figure 1.1:** Peripheral control of glucose homeostasis. After nutrient ingestion glucose absorbed is mainly distributed to liver, skeletal muscle and adipose tissue. Liver has the capacity to store (in glycogen form) and produce glucose through glycogenolysis and gluconeogenesis. Uptake of glucose from skeletal muscle and adipose tissue occur in an insulin dependent pathway, when insulin is released from pancreas. Insulin secretion is promoted largely by raise of plasma glucose levels and is increased through the action of incretins that are released from gut (Adapted from Arble and Sandoval 2013).

### 1.1.1. Hormonal regulation of glucose homeostasis

***Insulin***

Insulin is a small protein composed of two polypeptide chains containing 51 amino acids. Insulin is produced and secreted by the pancreatic β cells. It has a fundamental anabolic role on the response to increased blood glucose and amino acids after meal ingestion (Aronoff et al. 2004). The levels of glucose in blood are detected in the pancreatic β cells through GLUT-2, (Banks, Owen, and Erickson 2012) being a major stimulus for insulin secretion; however, insulin secretion is highly potentiated by gut hormones, called incretins (mainly glucose-dependent insulinotropic peptide (GIP) and glucagon-like peptide-1 (GLP-1)) that are secreted from the intestine in response to meal ingestion (Bano 2013).

Insulin secretion is biphasic: the first phase occurs early after meal ingestion and is thought to suppress hepatic glucose production (HGP). The second phase occurs

within 1–2 hours after the meal and stimulates glucose uptake by insulin sensitive tissues (like skeletal muscle and adipose tissue) (Grayson, Seeley, and Sandoval 2013). Upon secretion from pancreas, insulin acts in its target tissues to promote glucose uptake.

The insulin receptor (IR) consists of two α subunits and two β subunits. Insulin binds to the extracellular α subunit and transduces signals across the plasma membrane, which activates the intracellular tyrosine kinase C terminal domain of the β subunit. Binding of insulin to IR induces a sequence of intramolecular transphosphorylation reactions (Figure 1.2).

IRs are highly expressed in insulin target tissues like muscle, liver and fat, although they exist on the surface of almost all cells. Autophosphorylation of the IR tyrosine residue stimulates the catalytic activity of receptor tyrosine kinase which recruits and phosphorylates insulin receptor substrate (IRS) proteins (IRS-1 and IRS-2). These, in turn, augment the activity of the efector enzyme PI3 kinase that is a target of the IRS1/2 which phosphorylates specific phosphoinositides to form phosphatidylinositol 4,5 bisphosphate (PIP2) to phosphatidylinositol 3,4,5 triphosphate; in turn, this activates ser/thr kinase, phosphoinositide-dependent kinase-1 (PDK1). Activated PDK1 phosphorylates and activates ser/thr kinase Akt/PKB. Akt contains a PH domain that also interacts directly with PIP3. Akt phosphorylation plays an important role in the regulation of GLUT4 to the cell surface to transport glucose into the cell (Figure 1.2) (Bhattacharya, Dey, and Roy 2007).

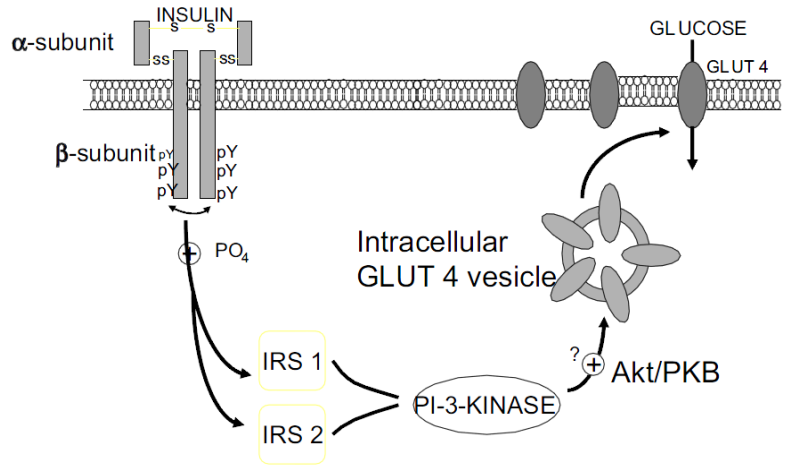


**Figure 1.2:** Insulin signaling. Activation of insulin receptor tyrosine kinase induces phosphorylation of IRS and PI3 kinase. The phosphorylation of these proteins leads to a serie of processes that culminate in the activation of GLUT4. Once activated, GLUT4 is translocated to the plasma membrane and imports glucose into the cell (Adapted from Bhattacharya, Dey, and Roy 2007).

Glucose transport from the bloodstream into cells is the rate-limiting step in whole-body glucose disposal and occurs through a family of glucose transporters with various isoforms (GLUTs) that are differently distributed within tissues (table 1.1) (Waller et al. 2011).

GLUT1 is present in all tissues, being related to basal glucose uptake and it is critical in providing glucose to brain, through blood brain barrier (Leybaert 2005). The GLUT3 has the highest affinity to glucose and for this reason is expressed in situation of high demand of glucose as happens in the brain (Simpson et al. 2008). Other sugars like fructose use GLUT5 to be transported (Douard and Ferraris 2008). In skeletal muscle, GLUT4 is the predominant isoform, and provides the majority of basal and insulin-stimulated glucose uptake (Waller et al. 2011). GLUT12 is highly expressed in skeletal muscle and fat suggesting that it is a second insulin regulated transporter in these tissues (Suzanne Rogers et al. 2013; Purcell et al. 2011; C. A. Stuart et al. 2009), although its function is unraveled.

**Table 1.1:** Tissue distribution and function of glucose transporters. Adapted from Mueckler 1994; Stuart et al. 2009; Doblado and Moley 2009; S. Rogers et al. 2002; Wood and Trayhurn 2003)

| Name | Tissue expression | Proposed function |
|---|---|---|
| GLUT1 | Ubiquitous; adipose, muscle, liver, especially brain and erythrocytes | Basal glucose metabolism; transport across blood-brain barrier |
| GLUT 2 | Hepatocytes, pancreatic β-cells, intestine, kidneys | Glucose sensing; high-capacity low-affinity transport; transepithelial transport |
| GLUT 3 | Especially important in brain | High affinity for glucose; basal transport; uptake from cerebral fluid into brain parenchimal cells |
| GLUT 4 | Skeletal muscle, heart, adipocytes | Insulin-stimulated glucose uptake; especially important in the postprandial state |
| GLUT 5 | Small intestine, testes, adipose, muscle, brain, and renal tissues | Intestinal absorption of fructose |
| GLUT 6 | Brain, spleen, leucocytes | Glucose transport |
| GLUT 7 | Hepatocytes and gluconeogenic tissues | Mediates flux across endoplasmic reticulum membrane |
| GLUT 8 | Testes, brain, and other tissues | Glucose transport |
| GLUT 9 | Liver, kidneys | Hexose and uric acid transporter |
| GLUT 10 | Liver, pancreas | Non determined |
| GLUT 11 | Heart, skeletal muscle, liver, lung | Fructose and glucose transport |
| GLUT 12 | Heart, prostate, muscle, small intestine, adipose tissue | Non determined |

Several tissues, specifically skeletal muscle and adipose tissue respond to insulin and can use either glucose or ketones and free fatty acids as their primary metabolic fuel. The binding of insulin to its cell surface insulin receptors determines the type of energy that cell uses. Therefore, in the presence of large amounts of insulin, the cell preferentially uses glucose, by metabolizing it or storing it as glycogen in the muscle or as fat in the adipose tissue (Figure 1.3).

### *Other hormones*

Glucagon is the major counter-regulatory hormone to insulin (Bano 2013). It is secreted from the α-cells of pancreas and its secretion is stimulated by hypoglycaemia and inhibited by hyperglycemia. This hormone plays an important role during fasting

condition in the maintenance of glucose levels. It acts through binding of its receptors in the liver, stimulating the hepatic glucose production (glycogenolysis) increasing plasma glucose levels (Figure 1.3) (Bano 2013; Aronoff et al. 2004).

Another class of hormones implicated in glucose homeostasis are incretins, secreted by intestinal mucosa after nutrient ingestion, and stimulating pancreas to release insulin. The principal incretins involved in glucose homeostasis are glucose-dependent insulinotropic peptide (GIP) and glucagon-like peptide-1 (GLP-1) (Bano 2013).

GIP stimulates insulin secretion and regulates fat metabolism but does not inhibit glucagon secretion or gastric emptying (Drucker 2007). In contrast, GLP-1 inhibits glucagon secretion and slows gastric emptying, stimulating insulin secretion (Bano 2013).

Other hormones can directly or indirectly impact on glucose homeostasis however this thesis does not pretend to have a detailed review of them.

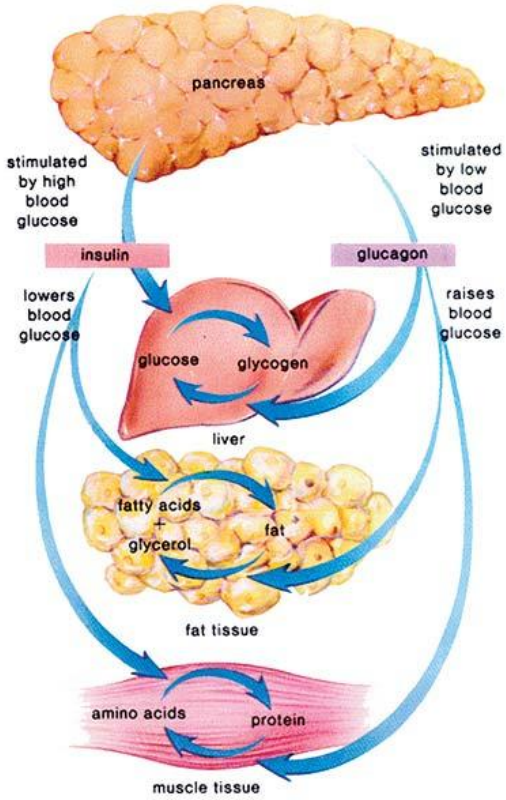


**Figure 1.3:** Hormonal regulation of glucose homeostasis by insulin and glucagon. Rise in glucose values lead to the release of insulin by pancreas that will reduce glucose production by liver. High glucose levels lead to an uptake by insulin sensitive tissues like skeletal muscle and adipose tissue. Glucagon exerts the opposite effect of insulin, its action occurs when low levels of glucose are sensed leading to the production of glucose by the liver. Available on http://thestayingunstuckproject.com

### 1.1.2. Neural regulation of glucose homeostasis

Nervous system is divided in central and peripheral based on their functions. Central nervous system (CNS) comprises the brain and spinal cord (Figure 1.4) (Purves et al. 2004). The peripheral nervous system (PNS) includes the sensory neurons that link sensory receptors with relevant processing circuits in the central nervous system (Figure 1.4). The axons that connect the brain and spinal cord to skeletal muscles constitute the somatic motor division of the PNS, whereas the cells and axons that innervate smooth muscles, cardiac muscle, and glands make up the visceral or autonomic motor division (Purves et al. 2004).

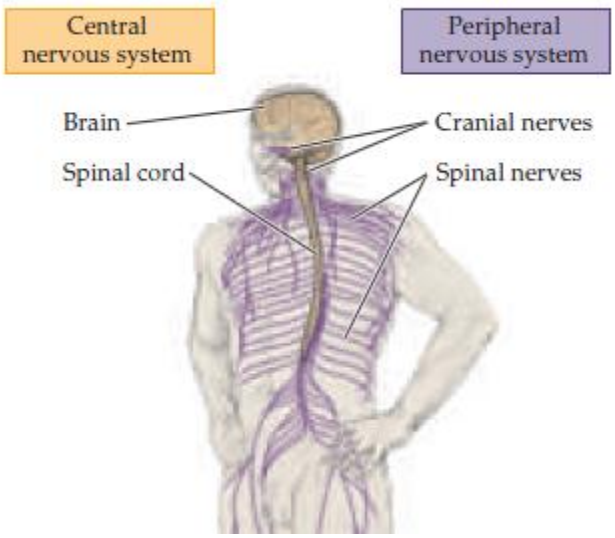


**Figure 1.4:** The major components of nervous system. It is divided in central nervous system composed by brain and spinal cord and peripheral nervous system, composed mainly by cranial nerves and spinal nerves (Adapted from Purves et al. 2004).

The CNS and PNS play a central role in the regulation of glucose homeostasis, sensing and integrating information from neural, hormonal and nutrient signals and thus generating responses that regulate glucose output by the liver and glucose uptake by peripheral tissues (Sandoval, Obici, and Seeley 2009).

#### 1.1.2.1. Role of central nervous system in glucose homeostasis

Neurons in the hypothalamus have a fundamental role in regulating many homeostatic processes (Blouet and Schwartz 2010).
Certain subsets of hypothalamic neurons are responsive to glucose, fatty acids and amino acids. Here, nutrients act as signaling molecules to produce neurochemical and neurophysiological responses, regulating energy intake.

Some nutrient-sensing hypothalamic neurons have been identified in arcuate (ARC), ventro-medial (VMN) and lateral (LH) nucleus of the hypothalamus. These, in turn, project to second order neurons that release peptides that in the end will project to the hindbrain and the periphery, thereby providing a communication between the hypothalamus and the periphery. Finally, this mechanism leads to autonomic, neurohumoral and somatomotor responses that mediate satiety and long-term energy homeostasis (Blouet and Schwartz 2010).

#### 1.1.2.2. Role of peripheral nervous system in glucose homeostasis

Autonomous nervous system is part of PNS and comprises parasympathetic (PSNS) and sympathetic (SNS) nerve systems. The presence of both PSNS and SNS nerve terminals within the liver, as well as direct connections between hypothalamic centers and the liver have already been established (Dicostanzo et al. 2006) (Figure 1.5).
PSNS and SNS play an antagonistic effect in liver, regulating glucose metabolism, primarily by regulating insulin and glucagon (Grayson, Seeley, and Sandoval 2013). While PSNS acts in peripheral uptake of glucose, SNS raises glucose levels due to a reduction in glucose disposal and stimulating hepatic glucose production (Fernandes et al. 2011; Lautt et al. 1998; Matsuhisa et al. 2000).

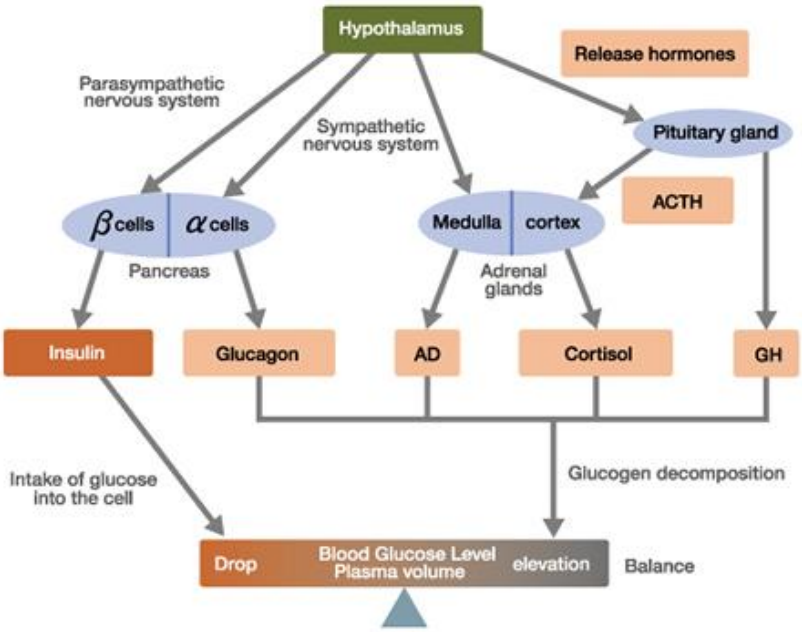


**Figure 1.5**: Effect of autonomic nervous system and endocrine system on glucose regulation. Adapted from "A Comprehensive Approach of Life Science".

The SNS mediates its physiological responses to external stimuli by the activation of the splanchnic nerve, which releases noradrenaline from nerve terminals and adrenaline from adrenal medulla (Teff 2008), leading to the activation of their receptors in the target organs (Dicostanzo et al. 2006). Adrenaline acts by stimulating glycogen breakdown and inhibiting skeletal muscle glucose uptake, which is very important to maintain blood glucose levels in periods of stress (Sandoval, Obici, and Seeley 2009). These hormones are also known as stress hormones, once hypoglycemia or stress conditions lead to activation of SNS ending up on the stimulation of glycogenolysis and gluconeogenesis (Dicostanzo et al. 2006).

PSNS acts in response to stimulus like meal ingestion, through the stimulation of the vagus nerve. The vagus plays an important role in the regulation of blood glucose levels as it innervates liver and pancreas. It was also reported that parasympathetic nerves have an important effect in glucose homeostasis, being synergistic with insulin action and antagonistic of glucagon (Teff 2008). Previous studies demonstrated that ablation of hepatic parasympathetic nerves (HPN) decreases peripheral insulin sensibility, which could be reverted by acetylcholine (Ach) administration (Xie and Lautt 1996) showed to impact only in the postprandial state (Lautt et al. 2011; Fernandes et al. 2011).

The involvement of HPN has also been related to the release of the hepatic insulin sensitizing substance (HISS). This substance is known as a hormone that enters the

bloodstream and stimulates glucose uptake in peripheral tissues (Latour and Lautt 2002; Fernandes et al. 2011) and that will be best described later in this chapter.

Hypothalamic-pituitary-adrenal (HPA) axis also contributes to glucose homeostasis by stimulation of hormones such as growth hormone, from the pituitary and cortisol from adrenal cortex (Sandoval, Obici, and Seeley 2009). They act by reducing the release of glucose, stimulating glucose uptake and inhibiting lipolysis (Bano 2013) (Figure 1.5).

## 1.2. Insulin resistance and how it impinges on Diabetes

Diabetes mellitus is a metabolic disease characterized by hyperglycemia resulting from defective insulin secretion and/or resistance to insulin action. Symptoms of marked hyperglycemia include polyuria, polydipsia, weight loss and fatigue.
A non-control of diabetes leads to long-term complications including retinopathy, nephropathy, neuropathy and many other associated problems (ADA 2013).
Diabetes mellitus may be divided in two different types, type 1 diabetes (DM1), caused by an autoimmune destruction of the pancreatic islets, and type 2 diabetes (DM2) which is normally caused by a combination of resistance to insulin action and an inadequate compensatory secretion of insulin (ADA 2013).

***Type 1 Diabetes***

DM1 formerly also called insulin dependent diabetes, seems to be caused by an autoimmune-mediated destruction of the pancreatic β islets resulting in insulin deficiency. The autoimmune destruction of β cell has multiple genetic predispositions and is also related to environmental factors that are still poorly defined (ADA 2013).
Auto-antibodies against insulin or constituents of pancreatic islet cells can be detected in 85–90% of patients at the time of diagnosis (ADA 2013).

This type of diabetes accounts for only 5-10% of people with diabetes and is more common in childhood and adolescence; however, it can occur at any age (ADA 2013).
Loss of pancreatic β cells results in deficient insulin secretion and failure of blood glucose control (Banks, Owen, and Erickson 2012). People with this type of diabetes have to take exogenous insulin for survival and to prevent development of ketoacidosis (which consists in high concentration of ketone bodies due to breakdown of fatty acids) (Bhattacharya, Dey, and Roy 2007).

***Type 2 Diabetes***

DM2 is often associated with obesity (Bhattacharya 2007). Its main feature is the severe insulin resistance that precedes β cell failure for several years; in that period, insulin resistance is accompanied by compensatory hyperinsulinemia that ensures blood glucose homeostasis. When β cell function reaches its limit, blood

glucose levels rise (Banks, Owen, and Erickson 2012) (Figure 1.6). Globally, more than 90–95% of cases of diabetes are of this kind (Bhattacharya 2007). In addition, over 300 million people suffer from the preclinical stages of diabetes (pre diabetes), characterized by either increased fasting glucose concentration (with value above normal range but below the cutoff for the diagnosis of diabetes), impaired glucose tolerance, or both (Waller et al. 2011).

In the pre diabetes state, characterized by insulin resistance, elevated blood glucose stimulates the pancreatic β cell to release more insulin until either glucose levels return to normal or pancreatic secretion reaches its maximum, in order to compensate the impairment in insulin action (Banks, Owen, and Erickson 2012). This decline in insulin production can eventually culminate in pancreatic β cell failure (Olokoba, Obateru, and Olokoba 2012) (Figure 1.6).

Undiagnosed glucose intolerance, with gradual increased levels of postprandial glycemia, as seen in pre diabetes, is often not severe enough for the patient to notice any of the classic symptoms and is becoming a dramatic health problem (ADA, 2013).

Treatment aims primarily to reduce insulin resistance, which can be achieved with diet, exercise or drug therapy and to increase endogenous insulin secretion, however insulin resistance rarely returns to normal values (ADA 2013). Ultimately, exogenous insulin therapy can be also required.

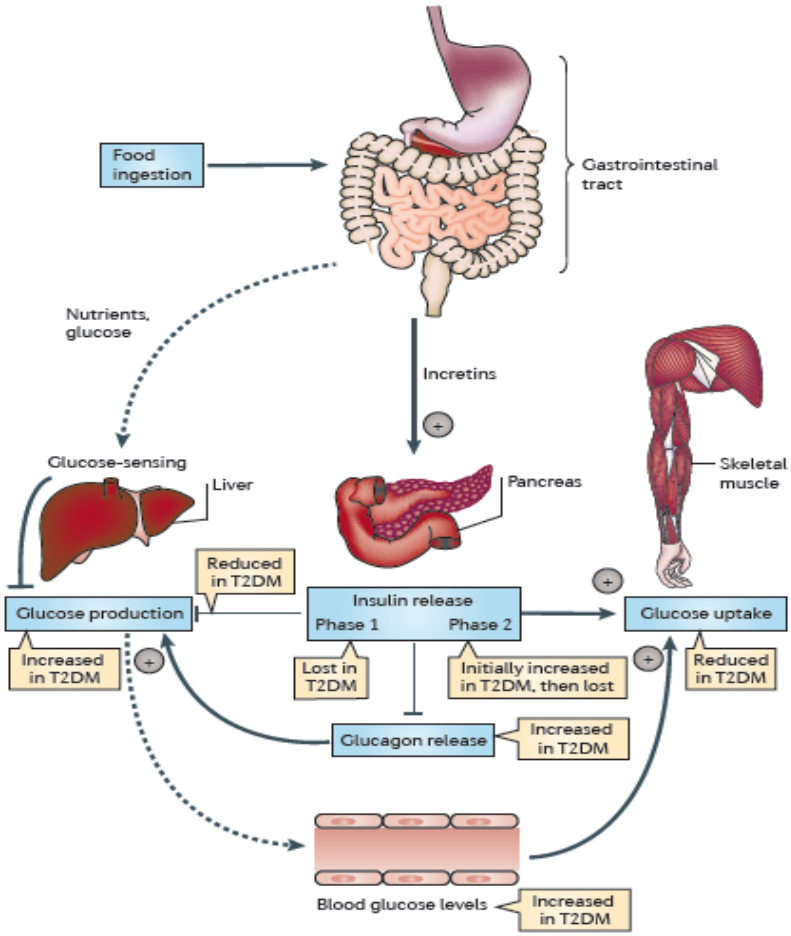


**Figure 1.6**: Glucose homeostasis in DM2. DM2 results from a combination of insulin resistance and inadequate insulin secretion. In the early stages of the disease the first phase insulin response is lost and the second raises to compensate this loss. Moreover, suppression of hepatic glucose production (HGP) by insulin action is reduced. Later, pancreatic β cell loses its ability to compensate by increasing insulin release which leads to an imbalance between insulin and glucagon levels that culminate in a chronic maintenance of high basal and postprandial glucose levels (Adapted from Grayson, Seeley, and Sandoval 2013).

### *High sucrose diet – an animal model of insulin resistance*

The study of animal models that recreate human DM2 became crucial. From these studies the rat allowed several discoveries and advances in the field. These lower organisms show great interest as some biological aspects that are highly conserved and similar to humans (McMurray and Cox 2011). Mice and rat are widely used as animal models for DM2 although rat shows the advantage of being larger than mice, making physiological procedures easier. In contrast the mice became a more feasible model for genetic manipulation (McMurray and Cox 2011).

Prevalence of DM2 is related with lifestyle and food intake being intimately associated with western diets, mainly composed by sugar and fat foods (Shafrir, Ziv, and Kalman 2006). Nutritionally induced animal models of DM2 and insulin resistance show special interest for experimental studies as they reflect the impact of modern lifestyle and the increased caloric intake that occurs in humans (Shafrir, Ziv, and Kalman 2006).

It is accepted that high sucrose diet (HSD) leads to development of hyperinsulinemia and insulin resistance, hypertriglyceridemia as well as a defect in glucose disposal promoted by insulin (Wright, Reaven, and Hansen 1983), depending on the amount of sucrose and duration of diet (Brenner et al. 2003). However, basal levels of plasma glucose do not increase in this model. The HSD animal model is a good model for insulin resistance study, since reproduces metabolic abnormalities as the ones seen in type 2 diabetic patients, being useful to provide new insights of this disease (Wright, Reaven, and Hansen 1983).

### *Meal induced sensitization and HISS hypothesis*

Meal induced sensitization (MIS) refers to the increase of insulin action from the fasted to fed state in order to induce glucose uptake by peripheral tissues (Sadri et al. 2007). MIS occurs through the release of the hepatic insulin sensitizing substance (HISS) from the liver after meal ingestion (Lautt et al. 2011).

The HISS hypothesis proposes that after meal ingestion a hepatic parasympathetic nerve (HPN) reflex occurs that leads to release of acethylcholine (Ach) from the liver. Ach binds to muscarinic receptors M1 in the liver, leading to activation of nitric oxide synthase (NOS) that raises nitric oxide (NO) synthesis from liver (W Wayne Lautt et al. 2011; Sadri et al. 2007). Hepatic glutathione (GSH) levels are also necessary for the appropriated release of HISS (Guarino and Macedo 2006), that enters into the bloodstream and enhances glucose uptake in skeletal muscle, heart and kidney (Fernandes et al. 2011). As insulin also promotes glucose uptake in skeletal muscle, these two hormones are thought to have a synergistic effect on glucose disposal (Figure 1.7).

The HISS dependent contribution to overall postprandial insulin action can be calculated from the difference in postprandial insulin sensitivity (IS) before and after the inhibition of the HISS pathway. This inhibition can be achieved by fasting, parasympathetic denervation, blockade with atropine (antagonist of muscarinic receptors), hepatic NO synthase inhibition and blockade of GSH synthesis (Guarino et al. 2003).

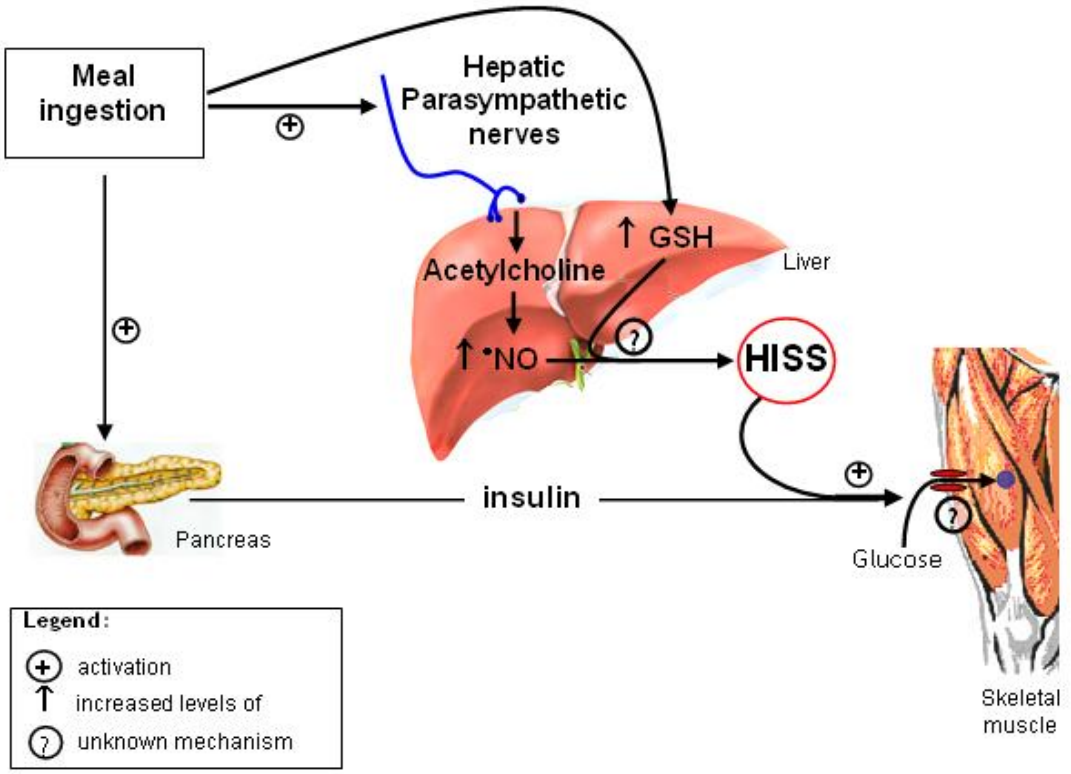


**Figure 1.7**: HISS action. In the postprandial state, HPN reflex is triggered leading to the release of acetylcholine that activates the muscarinic receptors M1 leading to NO production. This process leads to the release of HISS into bloodstream which acts in skeletal muscle inducing glucose uptake.

Sadri, 2007 and co-workers demonstrated that a liquid meal is necessary to trigger MIS and that glucose and sucrose alone do not have this capacity. Besides, recent data from our laboratory described that glucose given with amino acids is capable of induce MIS (Afonso et al. 2011).

Glutathione (GSH) is an endogenous antioxidant (Gibson et al. 2011) and plays also an important role in the regulation of NO homeostasis, with the formation of nitrosothiols (Hogg 2002). Its synthesis depends on amino acids ingestion, since its precursor, cysteine, is a semi essential amino acid. Furthermore, one of the effective precursors of cysteine is its synthetic derivative, N-acetyl-cysteine (NAC) (Aitio 2006).

It has been reported that administration of cysteine as a supplement in high sucrose fed animal model improves postprandial glucose through a mechanism that involves GSH synthesis (Blouet et al. 2007). Besides, studies of Lautt's group and ours observed that administration of N-acetyl-cysteine (as a precursor of cysteine) together with bethanechol (which mimics the activation of the parasympathetic nerves), lead to a complete restoration of postprandial insulin sensitivity in an animal model of insulin resistance.

Previous data have also shown that NAC is able to normalize plasma insulin levels, reduce plasma triglycerides and increase insulin sensitivity in fructose fed rats (Song, Hutchings, and Pang 2005).

The idea that amino acids play an important role in glucose homeostasis, lowering its blood levels, is growing (Bernard et al. 2012). The hypothesis that mechanisms involving greater postprandial insulin response and an increased rate of skeletal muscle glucose uptake by providing precursors of cysteine has been raised in our laboratory.

## 1.3. Central nervous system and diabetic complications

Diabetes is intimately associated with micro and macrovascular complications that led to retinopathy and peripheral neuropathy. However, there is now growing evidence illustrating central nervous system (CNS) complication, also known as “diabetic encephalopathy”, associated to DM1 and DM2.

Diabetes has definitely different etiologies. Recent evidences demonstrate that among diabetics only some patients show cognitive dysfunction, while some are not affected (Kamal et al. 2013). Although both DM1 and DM2 patients show cognitive impairments, the degree and manifestation of cognitive dysfunction seems to be diverse between them (McCrimmon, Ryan, and Frier 2012).

Type 1 diabetic patients show impairments in problem solving, mental and motor speed (Ryan 1988; Artola 2013). On the other side, results of neuropsychological studies of type 2 diabetic patients demonstrate impairments particularly in tasks involving verbal memory or complex information processing and also in the domain of attention, concentration and verbal fluency (Gispen and Biessels 2000; Gold et al. 2007). Cerebral alterations occurring within diabetes include abnormal expression of hypothalamic neuropeptides, hippocampal astrogliosis, decreased hippocampal synaptic plasticity, neurotoxicity and changes in glutamate neurotransmission (Revsin et al. 2005).

Besides its effect on cognition, diabetes seems to be associated to an increased risk of developing Alzheimer’s disease (AD) and dementia. These pathologies emerge from changes in cerebral metabolism at neurochemical, structural and electrophysiological level (Biessels, Bravenboer, and Gispen 2004). In addition, vascular activity and increased oxidative stress are also evident (Coleman et al.). Several co morbid disorders such as hypertension and obesity (McCrimmon, Ryan, and Frier 2012) and elevated body mass index (Grillo et al. 2011) may also contribute to an increased risk of cognitive impairment and dementia.

### 1.3.1. The central nervous system and Diabetes

***Hippocampus***

The hippocampus plays an important role in memory, being considered the primarily region involved in memory formation. It is composed by different subregions, the Cornu Ammonis (CA1, CA2 and CA3 fields), the dentate gyrus (DG) and parahippocampal regions and the entorhinal cortex (EC) (Figure 1.8) (Deshmukh, Johnson, and Knierim 2012).

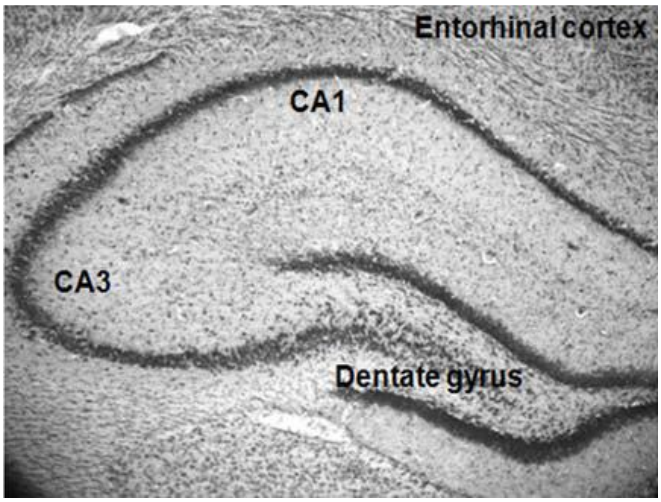


**Figure 1.8**: Hippocampal subregions. Histological cross-section of a rat hippocampus showing the different subregions: *Cornu Ammon* (CA1 and CA3) and dentate gyrus; 5x Magnification

***Cortex***

The mammalian cerebral cortex consists in a continuous piece of tissue with the thickness varying between 1 and 5 mm and composed by a detailed regional pattern (Ragsdale and Grove 2001). It is separated in different anatomic types of cortex, such as five-cell-layered neocortex and one-cell-layered archicortex. Within these regions, the complex functions of the cortex are distributed among many anatomically distinct areas (Figure 1.9) (Ragsdale and Grove 2001).

The cerebral cortex is organized into a complex network of circuits and long-range fiber pathways. This complex network forms the structural substrate for distributed interactions between specialized brain systems (Hagmann et al. 2008).

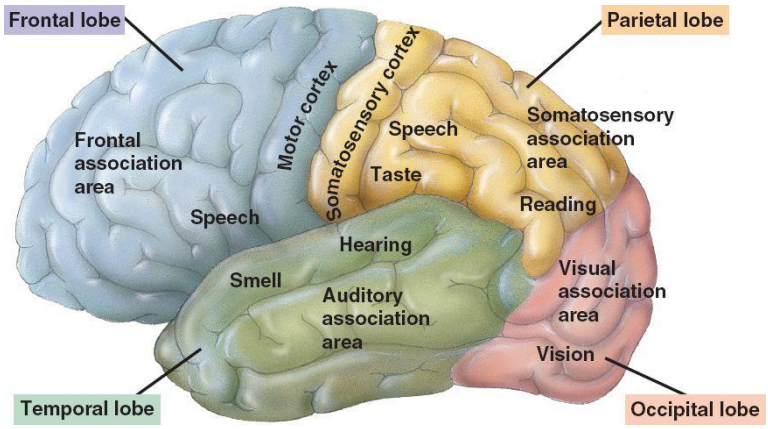


**Figure 1.9**: Different areas of the human cerebral cortex. Cortex is composed by four different lobes, frontal, temporal, parietal and occipital lobes, being each one responsible for different functions including movement, taste, speech, reading, vision, hearing and smell. Available on "http://bio1152.nicerweb.com/Locked/media/ch48/cerebral.html".

### *Synaptic Transmission*

The communication between neurons or between a neuron and non-neural cells such as muscle fibers is mediated through chemical synapses. The crucial process of synaptic transmission occurs by a pre-synaptic membrane potential depolarization, typically caused by the arrival of an action potential (Hennig 2013) that evokes to the opening of pre-synaptic voltage-gated $Ca^{2+}$ channels. The consequent rise in $Ca^{2+}$ at the pre-synaptic terminal leads to the release of neurotransmitter, which binds to post-synaptic receptors, generating a response in the postsynaptic neuron (Hennig 2013).

It is generally accepted that memories are stored as long term alterations in the strength of synaptic transmission. The magnitude and sign of synapse strength is denominated synaptic plasticity, and the most common forms of synaptic plasticity are long-term potentiation (LTP) and depression (LTD) (Hennig 2013; Purves et al. 2004).
LTP is induced by a high-frequency stimulation that will cause a prolonged depolarization in the post-synaptic neuron. The release of glutamate leads to the opening of N-methyl-D-aspartate (NMDA) channel. The sustained depolarization brought up by repetitive stimulation results in $Mg^{2+}$ to be expelled from the NMDA channel/receptor. The activation by glutamate and the removal of $Mg^{2+}$ allows channeling opening which, in turn, allows $Ca^{2+}$ to enter the postsynaptic neurons (together with $Na^{+}$) and this increase in $Ca^{2+}$ concentration within the postsynaptic cell seems to be a fundamental factor for LTP (Figure 1.10A). $Ca^{2+}$ induces LTP by activating signal transduction cascades that include protein kinases in the post-synaptic

neuron such as $Ca^{2+}$/calmodulin-dependent protein kinase (CaMKII) and protein kinase C (PKC) (Figure 1.10A).

On the other hand, LTD is stimulated at a low frequency for long periods and requires post-synaptic depolarization showing dependency on NMDA activation. A low-amplitude rise in $Ca^{2+}$ concentration in post-synaptic neurons occurs, leading to the internalization of post-synaptic α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors, thus decreasing the sensitivity to glutamate released from the pre-synaptic terminals (Figure 1.10B) (Purves et al. 2004).

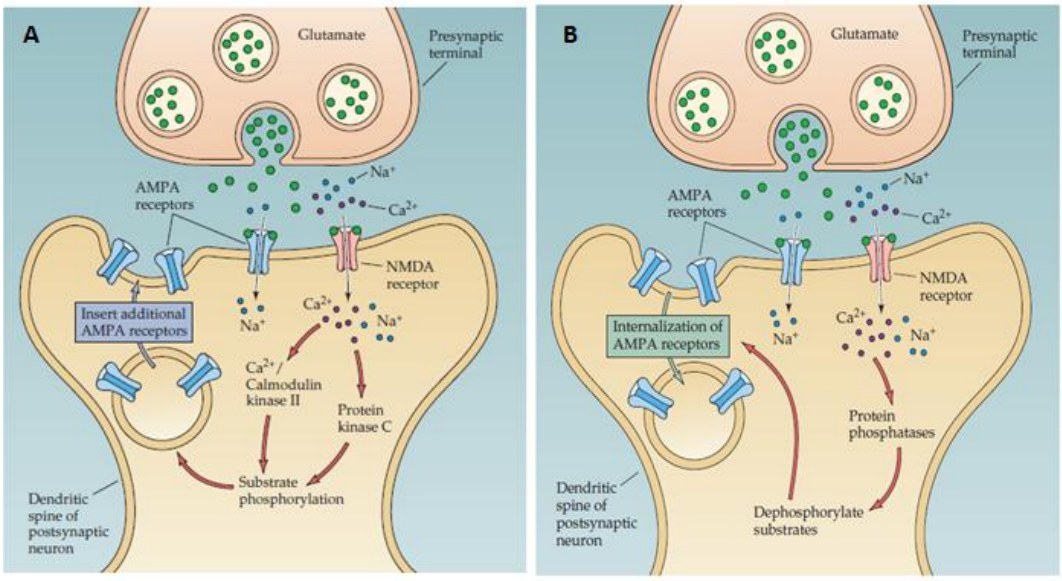


**Figure 1.10:** Mechanisms underlying LTP and LTD. In LTP release of glutamate leads to the open of NMDA channels. $Ca^{2+}$ enters the post-synaptic cell activating several protein kinases. These kinases will act in order to insert new AMPA receptors into the post-synaptic neuron, increasing sensitivity to glutamate (**A**). LTD mechanism occurs due to a low amplitude increase in $Ca^{2+}$ concentration in the post-synaptic cell that lead to activation of protein phosphatases. These will lead to an internalization of post-synaptic AMPA receptors, thus decreasing sensitivity to glutamate (**B**) (Adapted from Purves et al. 2004).

### *Exocytosis*

Exocytosis is a highly regulated process divided in several steps shown in figure 1.11: incorporation of neurotransmitters into synaptic vesicles (SVs) (A), trafficking of the synaptic vesicles to pre-synaptic membrane (B), docking (C), priming (D), fusion of SVs with membrane (E) and after fusion, endocytosis (F) in order to recycle SVs.

The first step of the exocytotic process is the formation of SVs in the nerve terminal, which incorporates neurotransmitters. After that, SVs exocytosis involves two procedures: docking and fusion. SVs docking consists in the migration of vesicles

containing neurotransmitters to close to pre-synaptic plasma membrane where they will be attached at a specialized region known as the active zone (Benarroch 2013). The docked vesicles go through priming, which refers to the maturation of the SVs to become fusion-competent (Xiong and Chen 2010). Fusion competent state of SVs is achieved through molecular rearrangements and interactions between synaptic proteins such as rabphilin and Munc-13 (Kavalali 2002).

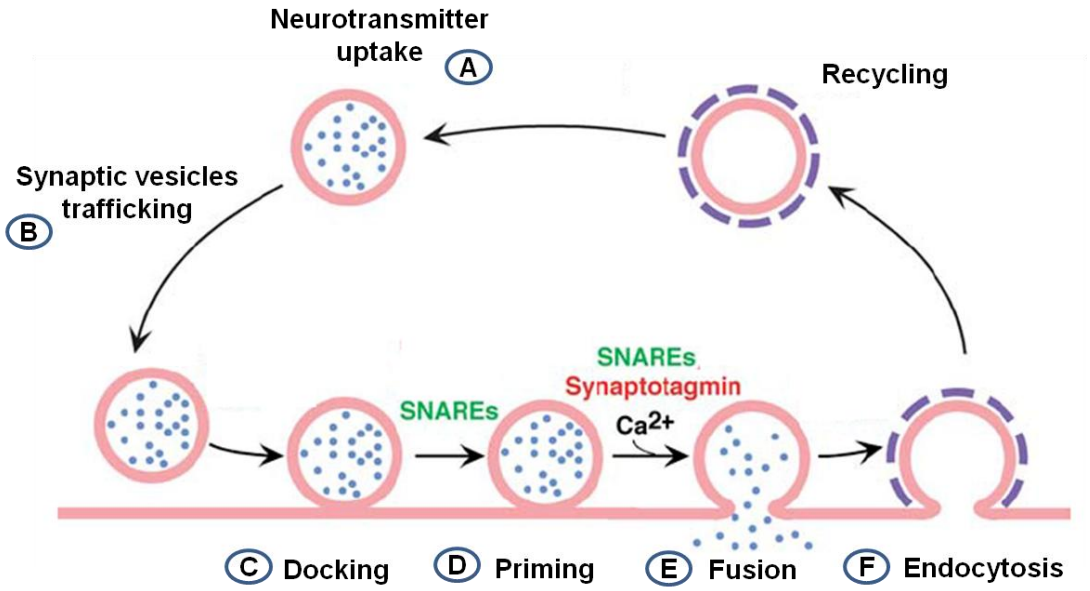


**Figure 1.11:** Steps of synaptic vesicles exocytosis. The SVs exocytosis involves docking, priming, fusion and recycling, occurring this last through endocytosis. SNARE complex is essential for priming and fusion as well as synaptotagmin and $Ca^{2+}$ to induce fusion of SVs and membrane with subsequent release of SVs (Adapted and modified from Li and Chin 2003).

Synaptic vesicles can be divided into docked vesicle pool, which are close to the plasma membrane, or reserve pool, referred to those vesicles more distant to the membrane (Fdez and Hilfiker, 2006).

Synapsins are an important class of proteins for the formation of SVs. In mammals, these proteins are encoded by three different genes, synapsin I, II, and III. These synaptic proteins regulate the releasable pool of synaptic vesicles (SVs), forming a cluster of vesicles that are ready to be released during high synaptic activity (Vasileva et al. 2012). Synapsins phosphorylation/dephosphorylation state regulates the association of SVs with actin allowing the migration through nerve terminals (Easley-Neal et al. 2013)

SVs exocytosis involves the interaction between several proteins (Li and Chin 2003) and the priming and fusion of SVs is dependent on the formation of the SNARE complex. This complex is comprised by vesicle-associated membrane protein 2 (VAMP-2), a synaptic vesicle protein; synaptossomal-associated protein with 25kDa (SNAP-25) and syntaxin proteins (Figure 1.12), which are cell membrane proteins.

Syntaxin can adopt two configurations, either open or closed. When adopting the open form, syntaxin can associate with SNAP-25 and VAMP (figure 1.12B and 1.12C), forming the SNARE complex (Figure 1.12D) (Li and Chin 2003). Munc-13 and Munc-18 proteins are also necessary for an appropriate assembly of SNARE complex. Munc-13 protein binds the core fusion machinery and mediates $Ca^{2+}$ membrane recruitment. Munc-18 has a high-affinity to bind to syntaxin-1 (Benarroch 2013).

Synaptic vesicles contain a family of GTP-binding proteins called Rab3A, B, C, and D that also contribute to SNARE formation. Rabphilin-3 A is a cytosolic protein that plays a role in synaptic function, more specifically in the $Ca^{2+}$-regulated exocytotic and endocytotic processes, being an efector of Rab proteins (Deak et al. 2006). The interaction of Rab and its effectors induces a conformational change in Munc-18 that destabilizes the link between syntaxin-1 and Munc-18, allowing SNARE complex formation (Lin and Scheller 2000).

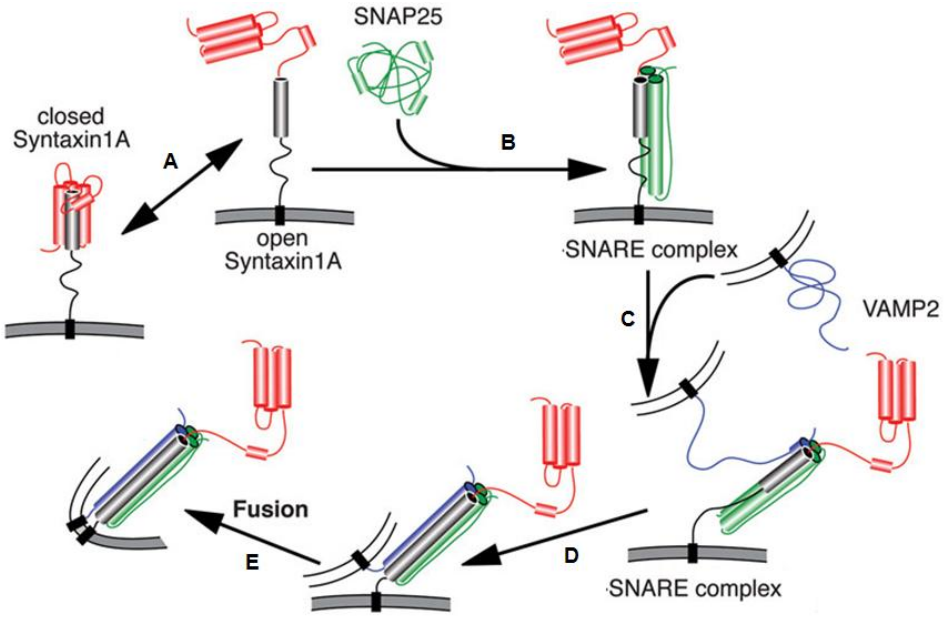


**Figure 1.12:** Formation of SNARE complex. Closed syntaxin-1A turns into it open form (**A**) allowing its binding to SNAP-25 (**B**). This two proteins complex can then bind to VAMP-2 (**C**), leading to the complete formation of SNARE complex (**D**). Several regulatory proteins interact with SNAREs to help the fusion event (**E**) (Adapted and modified from "Madame Curie Bioscience Database").

The assembly of fusion complex is also regulated by synaptophysin. This protein is exclusively located in SVs being widely used as a marker for pre-synaptic terminals (Kwon and Chapman 2011). Synaptophysin mediates VAMP-2 involvement in the SNARE complex (Gordon, Leube, and Cousin 2011). The binding of synaptophysin to VAMP-2 leads to an inability of VAMP-2 to link to SNAP-25, inhibiting SNARE complex formation (Becher et al. 1999). The interaction between synaptophysin and VAMP-2 is proposed to provide a pool of VAMP-2 for exocytosis in periods of high synaptic activity. In this case, these two proteins dissociate and VAMP-2 can integrate SNARE complex, allowing the normal exocytotic process (Xiong and Chen 2010).

The process of fusion finishes when an action potential-induced $Ca^{2+}$ influx occurs through voltage gated $Ca^{2+}$ channels and then primed vesicles undergo exocytotic fusion to release neurotransmitters (Benarroch 2013). The $Ca^{2+}$ influx requires a group of proteins named synaptotagmins. Synaptotagmins are attached to SVs and act as the primary $Ca^{2+}$ sensor for exocytosis. In the presence of $Ca^{2+}$, synaptotagmin binds to the plasma membrane and to syntaxin in the SNARE complex (Figure 1.13A).
The $Ca^{2+}$ influx and binding to synaptotagmin triggers a change in it conformation that warrants its binding with the SNARE complex, allowing membrane fusion (Figure 1.13C) (Benarroch 2013).

Following exocytosis, SVs protein constituents are retrieved from the plasma membrane by endocytosis and recycled for future rounds of exocytosis (Li and Chin 2003).

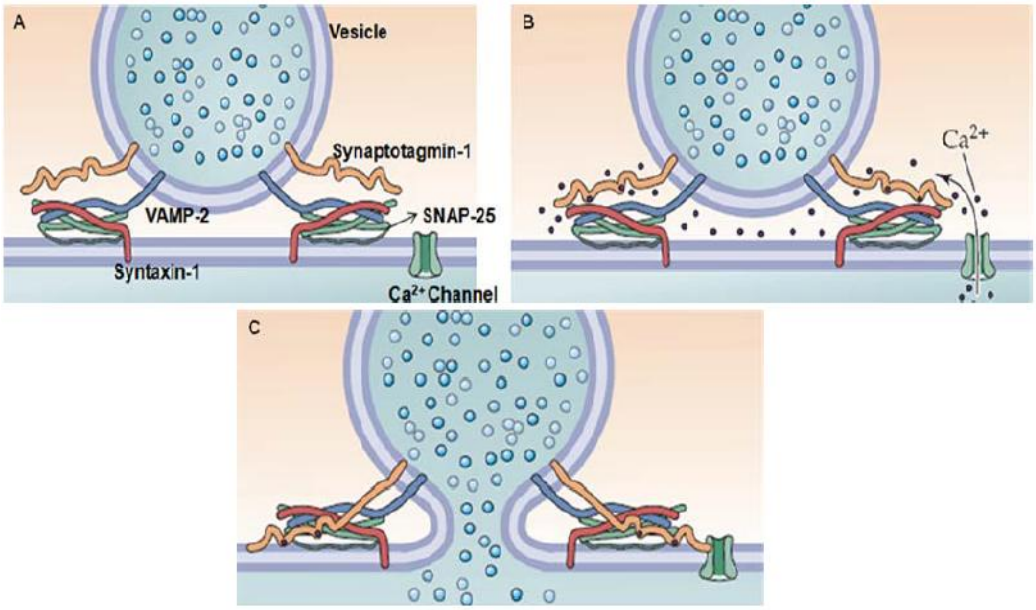


**Figure 1.13**: Mechanism of vesicle fusion triggered by $Ca^{2+}$. In priming, SNARE proteins at the SV and plasma membrane form a complex that brings together the two membranes (A). $Ca^{2+}$ enters in pre-synaptic terminal and then binds to synaptotagmin-1 (B). $Ca^{2+}$ induces the alteration of synaptotagmin-1 conformation and the cytoplasmic region of this protein is inserted into the plasma membrane. Synaptotagmin-1 binds to SNAREs and catalyzes membrane fusion (C) (Adapted and modified from Purves et al. 2004).

### 1.3.2. Alterations of Diabetes and CNS

Many different factors may contribute to diabetes induced cognitive impairments. As illustrated by figure 1.14 diabetes pathophysiological characteristics may include decreased insulin secretion or action, deregulation of glucose homeostasis impairment in the HPA axis, increased risk of developing AD, neuronal atrophy, insulin resistance and oxidative stress, that culminate in cognitive impairments.

Clinical evidence support that insulin is an important contributor and regulator of cognitive function once insulin receptors (IRs) can be found in the hippocampus and they are also essential for neuronal survival, synaptic and dendritic plasticity and learning and memory (Banks, Owen, and Erickson 2012). IRs in brain are ample distributed, but its highest concentration is in the olfactory bulb, cerebral cortex, hypothalamus, hippocampus, and cerebellum (Banks, Owen, and Erickson 2012).

Insulin exerts direct and indirect effects on neurotransmitter systems like NMDA, AMPA and γ-aminobutyric acid (GABA) (Wrighten et al. 2009), which are critical for long-term depression (LTD) process in rat hippocampus. These findings support the role of insulin signaling in recruiting necessary machinery for both excitatory and inhibitory

neurotransmission (Banks, Owen, and Erickson 2012). These facts suggest that impairment of insulin signaling may be an important factor for the development of cognitive impairments in diabetes.

Another contributor to brain dysfunction in diabetes function is hyperglycemia. Hyperglycemia triggers various processes that culminate in cell dysfunction and eventually cell death, leading to slowly progressive functional and structural abnormalities in the brain (Wrighten et al. 2009; Won et al. 2009). Memory and learning deficits accompanied by defects in synaptic plasticity due to elevated glucose levels have been reported in an animal model of diabetes (Kamal et al. 2013). Moreover, studies of (Duarte et al. 2009) show an increase in glucose levels in hippocampus of diabetic animals.

Hypoglycemic episodes may also contribute to diabetic encephalopathy. Prolonged and severe hypoglycemia may lead to brain damage that affects cognition, mood and conscious level (Won et al. 2009). Moderate hypoglycemia has also been associated with neuronal death in the cerebral cortex (Haces, Montiel, and Massieu, 2010; Won et al. 2009).

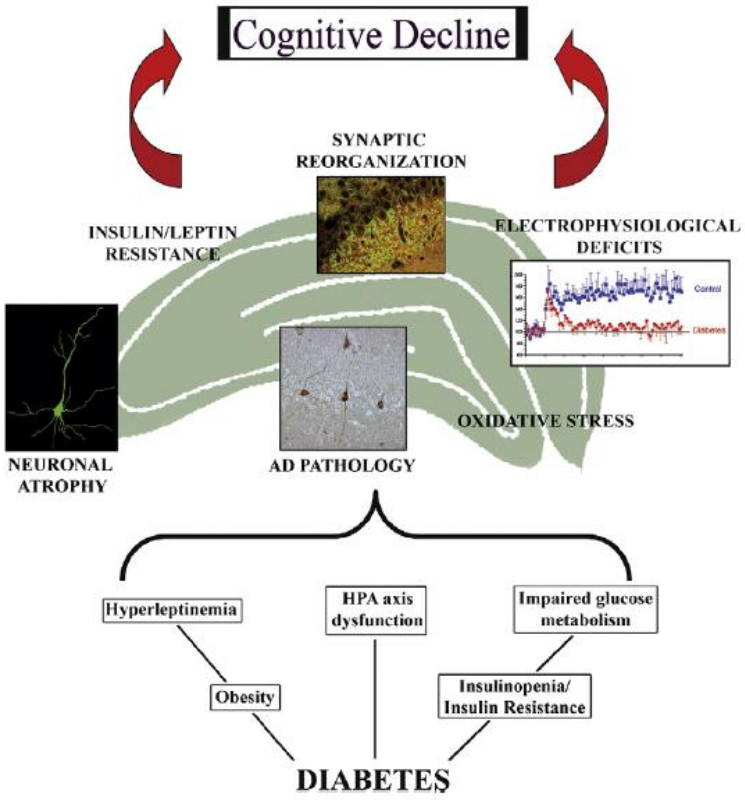


**Figure 1.14**: Possible altered mechanisms underlying diabetes that lead to cognitive impairment. Pathophysiological characteristics of diabetes include decrease in insulin activity, impaired glucose homeostasis and deregulation of HPA axis function. This alterations lead to consequences in brain such as dendritic atrophy in hippocampus, changes in synaptic formation and in electrophysiology as well as an increased risk for developing AD (Adapted from Wrighten et al. 2009).

### *Alterations in the diabetic*

Changes in several brain areas contribute to cognitive deficits associated with diabetes. Learning and memory deficits have been associated with structural and functional deficits in certain brain regions such as the hippocampus and cerebral cortex which in most of the cases seem to be irreversible (Wrighten et al. 2009; Ye, Wang, and Yang 2011). However, hippocampal-dependent tasks seem to be particularly sensitive to this disease.

## Structural changes

Atrophy of hippocampus is evident early in the course of the disease and around 10–15% loss in hippocampal volume has been reported in elderly people with diabetes (Jurdak and Kanarek 2009). Brain atrophy and histological studies of steptozotocin (STZ) diabetic rats (animal model of DM1) have been reported (Ferguson et al. 2005; Manschot et al. 2006) exhibiting morphological changes in the hippocampus, including dendritic atrophy in the CA3 pyramidal neurons (Wrighten et al. 2009). Cortical and subcortical atrophy have also been detected in type 2 diabetic patients by brain magnetic resonance imaging (Manschot et al. 2006).

## Molecular changes

Diabetes also induces changes in neurotransmitters release in different brain regions (Lackovic et al. 1990) although neurotransmitters and brain appear to be differently affected, depending on duration and severity of diabetes.

Changes in synaptic transmission may occur due to alterations at both pre and/ or post-synaptic sites. Pre-synaptic changes have been reported in the hippocampus of diabetic animals, such as alterations in the content of proteins involved in exocytosis (Gaspar, Baptista, et al. 2010; Trudeau, Gagnon, and Massicotte 2004) and dispersion and depletion of synaptic vesicles in mossy fiber terminals (Grillo et al. 2005).

At the post-synaptic side, the increase in post-synaptic density-95 (PSD-95) protein expression and changes in its distribution may contribute to changes in the content and functional properties of glutamate receptors in the hippocampus of diabetic rats (Grillo et al. 2005) leading to changes in synaptic plasticity. In addition, impairments in the NMDA receptor subunit composition at the post-synaptic level has been also reported (Trudeau et al., 2004) as well as a diminished capacity of modulation of AMPA receptor by phospholipase A2 and $Ca^{2+}$, which is an essential step for LTP, explaining the defect of synaptic plasticity observed in diabetes (Trudeau, Gagnon, and Massicotte 2004; Chabot et al. 1997).

Imbalances between excitatory and inhibitory neurotransmission are a triggering factor for neurodegeneration and consequently to changes in cognitive processes. Studies with diabetic animals demonstrate a downregulation of GABA receptors gene expression (Sherin et al. 2012). These alterations in GABA receptor contribute to changes in inhibitory function that could disrupt memory.

## 1.4. Integrative view of peripheral and central effects of insulin resistance

The phenomenon of meal-induced insulin sensitization (MIS) is known as the body response after meal ingestion that doubles glucose uptake in skeletal muscle due to insulin action (Latour and Lautt 2002; Sadri et al. 2007). The MIS is intimately related with HISS release, that acts stimulating peripheral glucose uptake and it has been shown that compromised HISS release after meal ingestion indicates a state of HISS dependent insulin resistance (HDIR). Impaired HISS action is associated with pre diabetic state in which occurs hyperglycemia and a compensatory hyperinsulinemia as well as hyperlipidemia and increased oxidative stress (Lautt 2004). Moreover, Lautt 2003 and co-workers suggest that type 2 diabetes may be due to the loss of HISS action. Insulin resistance has been associated to a decrease in HISS synthesis and release in an animal model drinking high sucrose diet (Ribeiro, Duarte-ramos, and Macedo 2001).

Consumption of high sucrose and high fat diets, also known as western diets, so common in the present society have demonstrated to be associated with memory problems (Kosari et al. 2012). Besides, in the last years the idea that both DM1 and DM2 affect cognition (Biessels et al. 2002; Hernández-Fonseca et al. 2009; Sima 2010), particularly learning and memory is emerging (Gispen and Biessels 2000; Gold et al. 2007).

This thesis aims to give a contribution to improve the knowledge of DM2, not only by understanding its pathophysiological pathways but also by unraveling some of its associated problems, namely mechanisms underlying cognitive impairments.

## 1.5. Hypothesis and Objectives

This thesis comprises two main hypotheses.

It is known that amino acids play an important role in peripheral insulin sensitivity in the postprandial state. As so, in the first part of this thesis we hypothesized that being N-acetyl-cysteine (NAC) a source of the essential amino acid cysteine, crucial for the synthesis of glutathione (GSH), intra enteric administration of NAC acts as a feeding signal to promote an increase in insulin sensitivity in peripheral tissues.
Hence, we aim to assess glucose excursions and insulin sensitivity as well as liver and plasma nitric oxide (NO) levels in both control and denervated animals where NAC was intra enterically administrated, either alone or in addition to glucose.

Type 2 diabetes is associated with overnutrition where sugars play an important role. Consequently, we tested the hypothesis that high sucrose diet (HSD) induces glucose intolerance which is mediated by a decrease in GLUT4 and GLUT12 expression with fat accumulation and leads to alterations in synaptic proteins expression in the hippocampus and cortex.
We intend to evaluate glucose intolerance by performing an intra-enteric glucose tolerance test and GLUT4 and GLUT12 skeletal muscle expression. Content of synaptic proteins, specifically synapsin-1, raphilin-3a, synaptophysin and those involved in SNARE complex formation, SNAP-25 and syntaxin, responsible for an adequate synaptic transmission, was also assessed in two different areas of the brain: the hippocampus and the cortex.

# 2. Materials and Methods

## 2.1. Reagents

All reagents were of the highest degree of purity and were purchased to Sigma Aldrich, Portugal. Heparin and saline (NaCl 0,9%) were purchased from BBraun, Portugal. Human insulin (Insuman) was obtained from Sanofi Aventis, France. Sodium pentoparbital (Eutasil) was obtained from Ceva Sante Animale. Biological glue (Histoacryl) was obtained from BBraun, Portugal and sucrose from Panreac, Barcelona, Spain.

## 2.2. Surgical procedures

**Pre surgical procedure and anesthesia**

Female and male Wistar rats with 9 and 4 weeks-old, respectively, were obtained from our animal facilities (Biotério, Faculdade de Ciências Médicas, UNL) and randomly assigned in different experimental groups. Animals were maintained in climate-controlled conditions and a 12 h light–dark cycle (07.00–19.00 hours). During the conditioning period, rats were fed ad libitum with a standard laboratory chow (Special Diets Services, Witham, UK), and they had free access to tap water. On the day before the experiment rats were fasted for a 24 hour period. The animals were weighed and then anesthetized with an intraperitoneal injection of sodium pentobarbital (65mg/kg). Sodium pentobarbital was the selected anesthetic since it did not affect blood pressure significantly, nor insulin action.

During the experiment the anesthesia was maintained by a continuous intravenous (iv) perfusion of sodium pentobarbital at a rate of 10mg/h/kg with an automatic perfusion pump (B-Braun, Portugal) and through a catheter of polyethylene (PE 50 Intramedic, Becton Dickinson, EUA). At the end of the protocols animals were euthanized with a lethal injection of sodium pentobarbital in accordance with the EU guidelines. The temperature was maintained at 37.0±0.5 ºC using a heating pad (Homeothermic Blanhet Control Unit 50-7061; Harvard Apparatus, Holliston, MA, USA).

All animals were handled according with the EU guidelines for the use of experimental animals (86/609/EEC).

**Surgical procedure**

Surgical protocol was done as previously described (Lautt *et al*., 1998). After anesthesia, tracheotomy was performed in all animals, in order to allow spontaneous breathe during all the experiment. Laparotomy was performed by cannulating intestine in order to administrate a liquid meal intra-enterically. A femoral arterial-venous loop was also performed in order to administrate drugs and collect blood samples, and to allow measurement of mean arterial and venous pressure.

**Plasma glucose quantification**

Quantification of arterial glycemia (mg/dL) was done using a glucose analyzer (1500 YSI Sport Sidekick) by the glucose oxidase (GOx) enzymatic method.

## 2.3. Peripheral glucose experiments

### 2.3.1. Experimental groups

Herein, female Wistar rats were used. Animals were divided in four experimental groups: control, NAC, NAC+Glucose and NAC+Glucose denervated. In the control animals we administrated water intra-enterically (IE) at a rate of 15mL/h and then glucose was also administrated IE to perform an IEGTT which was assessed over 120 minutes and then a rapid insulin sensitivity test (RIST) was performed. In the NAC group, NAC was administered (1mmol/kg of body weight) at 15mL/h and after 2 hours a RIST was started. In the NAC+Glucose group, NAC (1mmol/Kg of body weight, at 15mL/h) and then glucose (1,73 g/kg body weight at 60mL/h) were IE administered. In NAC+Glucose denervated group the experimental protocol was similar to NAC+Glucose, but a hepatic parasympathetic denervation was performed prior to compounds administration.

### 2.3.2. Hepatic parasympathetic denervation

The hepatic parasympathetic denervation was performed by isolation of the nerves at the anterior hepatic plexus and transected at the junction of the celiac and common hepatic arteries. In the sham-operated rats the same procedure was performed without ablation of the nerves.

### 2.3.3. Insulin sensitivity assessment (RIST)

RIST is used to evaluate the animal's hypoglycemic response to the exogenous insulin administration and glycemia is kept constant by intravenous (iv) variable infusion of glucose. After determination of basal glycemia (calculated by three stable values of glucose values), administration of insulin (50 mU/kg/min, 6 mL/h, iv, during 5 minutes) is initiated, using an infusion pump. The beginning of the insulin infusion is considered time zero (t=0 min). After 1 minute of insulin infusion, the first glucose sample is determined, and glucose infusion (100 mg/mL, iv) is started at the rate of 2.5 mg/kg/min with an infusion pump. Arterial glucose levels are sampled at 2min intervals throughout the test period with glucose infusion rates adjusted to maintain animal's glycemia near to the baseline value established before starting the RIST. The RIST is considered finished when the blood glucose levels remain close to the baseline without any further glucose infusion.

The total amount of glucose infused during the RIST quantifies insulin sensitivity and is referred to as the RIST Index and corresponds to the area under the curve of total glucose infusion. The RIST Index is the parameter used to evaluate insulin sensitivity.

### 2.3.4. Nitric oxide measurements

Liver and plasma NO levels were assessed by chemiluminescence-based quantification of nitrate ($NO_3^-$) and nitrite ($NO_2^-$) concentrations, as described previously (Afonso et al. 2006). This method is based on the Vanadium III- induced reduction of $NO_2^-$ and $NO_3^-$ to NO, at high temperature (90 ºC) using a Silvers 280 NO Analyzer (Sievers Instruments, Boulder, CO, USA).

## 2.4. Central nervous experiments

### 2.4.1. Animals

In this set of experiments male Wistar rats were used. Animals were randomly assigned to control or sucrose diet group. Glucose intolerance was induced by a solution of 35% sucrose in drinking water ad libitum (pre diabetes model), starting at 4 weeks of age until the end of the experiment. For the control animals plain water was provided. Animals were sacrificed at 4, 24 and 36 after the beginning of the treatment with sucrose.

### 2.4.2. Assessment of fat mass by resonance

In a separate group of animals body lean and fat mass were measured using in vivo NMR analysis (Whole Body Composition Analyzer; EchoMRI, Houston, TX) after 4, 20 and 36 weeks of high sucrose diet in both control and sucrose groups. Rats were placed in a constraint tube which was then inserted into the EchoMRI-700 for a period of approximately 2 minutes. During that time, total fat and lean mass were evaluated with depletion of water signal, based on the chemical shift differences between the fat and lean mass resonances.

### 2.4.3. Oral Glucose Tolerance Test (OGTT)

The oral glucose tolerance test (OGTT) was used to evaluate the progression of peripheral glucose intolerance; rats were fasted for 24 h and then fed with D-glucose (2 g/kg body weight) by intragastric gavage. Blood was collected from a small cut at the tip of the tail immediately before and at 15, 30, 45, 60, 90 and 120 minutes after glucose feeding, to measure glucose levels using a glucose analyzer. The total amount of glucose in plasma after glucose bolus was calculated from area under the curve (AUC) of every individual animal for both groups.

### 2.4.4. Intra Enteric Glucose Tolerance Test (IEGTT)

The intra-enteric glucose tolerance test (IEGTT) was performed to assess glucose tolerance in animals, at the day of sacrifice. After 24h fasted, a bolus of glucose (1.73g/10mL, 60mL/h) was administered intra-enterically to animals at a rate of 15mL/h and glycemia was measured in response to the administration of glucose over a period of 120 minutes. Arterial blood samples were collected after 2, 5, 10, 15, 20, 25, 30, 45, 60, 75, 90, 110 and 120 minutes of glucose administration for glycemia determination. The total amount of glucose in plasma after glucose bolus was calculated from AUC of every individual animal for both groups.

***Comparison of OGTT and IEGTT as glucose tolerance methods***

Figure 2.1 illustrates two different methods used to assess glucose tolerance. Figure 2.1A shows the curve of an intra-enteric glucose tolerance test (IEGGT) obtained by measuring blood glucose levels at several time points. This test is performed with animals anesthetized and consists in the administration of a bolus of glucose directly in the gut. Blood glucose levels rise reaching a maximum point normally between 20 and 30 minutes after glucose infusion. Glucose levels start to decrease after they reach their maximum, however, blood glucose values do not normally return to the baseline value as it is evident by the curve of the figure 2.1A. Maintenance of high glucose levels during 120 minutes of the test may be explained due to different factors. Anesthesia reduces intestinal motility which leads to a delay in glucose absorption. Moreover, once glucose is given in the gut there is a lack in the signals given by the sense of a meal in the mouth that contribute to a faster disappearance of glucose in the bloodstream.

Figure 2.1B demonstrates the curve obtained from an oral glucose tolerance test (OGTT). This method also evaluates glucose tolerance but it is performed in wake and conscious animals. Glucose is given by gavage directly in the stomach so it can be assumed that the dose of glucose is totally given. Blood glucose levels are measured during 120 minutes and the curve obtained is presented in figure 2.1B. Glucose levels reach their maximum between 15 and 20 minutes after glucose bolus and values usually return to those in the baseline. Once glucose is given in the stomach its absorption starts there, explaining its maximum value earlier than in IEGTT. Further, as animal is in its conscious state the subsequent absorption in the gut is also faster than in IEGGT.

Glycemia is normally higher in OGTT than in IEGTT which may be due to the fact that animals are submitted to an extreme condition of stress that contribute to raise blood glucose levels (B. Nowotny, 2010).

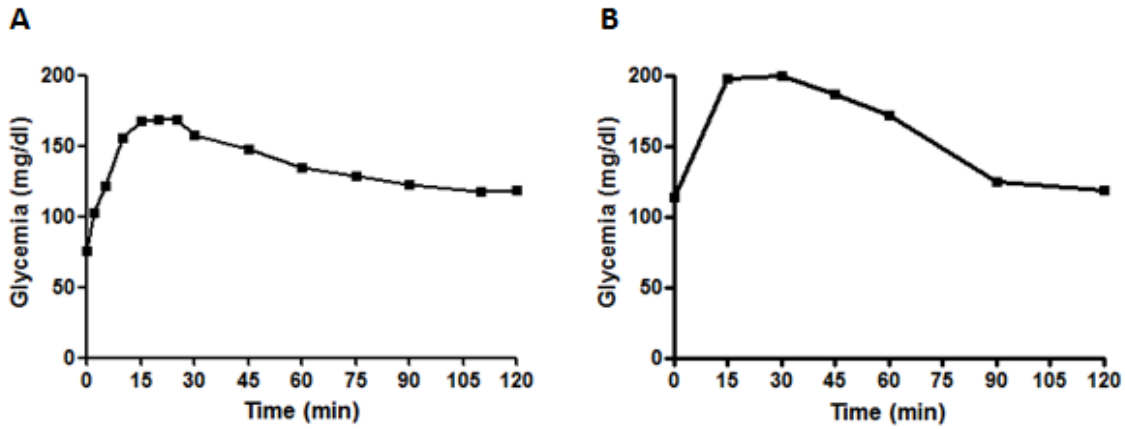


**Figure 2.1:** Glucose levels determined during 120 minutes. In IEGTT glucose levels reach their maximum between 20 and 30 minutes after the intra-enteric administration of glucose and after 120 minutes glycemia is still higher than baseline (**A**). Glucose levels in OGTT reach their maximum value approximately between 15 and 20 minutes after glucose bolus given by gavage directly in stomach and after 120 minutes return to values similar to those in the baseline (**B**).

### 2.4.5. Preparation of skeletal muscle total extracts

After dissection, skeletal muscle was homogenized in lysis buffer [20mM Tris, pH 7.4, 5mM EDTA pH 8.0, 10mM $Na_4P_2O_7$, 100mM NaF, 2mM $Na_3VO_4$, 1% NP-40, PMSF 1mM supplemented with complete miniprotease inhibitor cocktail tablets (Roche, Basel, Switzerland)]. After the homogenization the lysates were keep in ice for 1h and every 10min were performed vortex, and then centrifuged at 14000 x *g* for 20min at 4ºC. The supernatant were stored at -80ºC until .use.

### 2.4.6. Preparation of hippocampal synaptosomal extracts

Percoll purified synaptosomes were isolated as previously described (Duarte et al., 2006), with minor changes. From each animal, one hippocampus (the other hippocampus was used for the preparation of total extracts) was dissected and homogenized in a sucrose-HEPES solution [0.32 M sucrose, 1 mM EDTA, 10 mM HEPES, 1 mg/mL bovine serum albumin (BSA), pH 7.4]. The homogenate was centrifuged at 3,000 x *g* for 10 min at 4ºC. The supernatant was collected and centrifuged at 14,000 x *g* for 12 min at 4ºC. The resulting pellet was resuspended in

45% (v/v) Percoll solution prepared in Krebs–Henseleit Ringer (KHR) solution (in mM: 140 NaCl, 1 EDTA, 10 HEPES, 3 KCl, 5 glucose, pH 7.4). After centrifugation at 16,100 x *g* for 2 min at 4ºC the top layer was removed (synaptosomal fraction), and then washed in 1 mL KHR solution and resuspended in lysis buffer [RIPA: 150 mM NaCl, 50 mM Tris, 5 mM EGTA, 1% Triton X-100, 0.5% sodium deoxycholate (DOC), 0.1% sodium dodecyl sulfate (SDS), supplemented with complete miniprotease inhibitor cocktail tablets (Roche, Basel, Switzerland) and 1 mM dithiothreitol (DTT)]. The samples were stored at -80ºC until use.

### 2.4.7. Preparation of total hippocampal and cortex extracts

After dissection, cortex and one hippocampus from each rat were homogenized in RIPA buffer (150 mM NaCl, 50 mM Tris, 5 mM EGTA, 1% Triton X-100, 0.5% sodium deoxycholate (DOC), 0.1% sodium dodecyl sulfate (SDS)], supplemented with complete miniprotease inhibitor cocktail tablets (Roche, Basel, Switzerland), 1 mM dithiothreitol (DTT), 10 mM NaF, and 1 mM sodium orthovanadate. The resulting homogenate was sonicated (4 pulses, 2 seconds each) and then centrifuged at 16,100 x *g* for 10 min. All procedure was done at 4 °C. The supernatant was stored at -80 °C until use.

### 2.4.8. Western blot analysis

The protein concentration of each sample was determined by the bicinchoninic acid (BCA) protein assay (BIORAD). The samples were denaturated by adding 1x concentrated sample buffer (biorad supplemented with β-mercaptoetanol) and heating for 5 min at 95ºC. Equal amounts of protein were loaded into the gel and proteins were separated by sodium dodecyl sulphatepolyacrylamide gel electrophoresis (SDS-PAGE), using 8%-12% gels. Then, proteins were transferred electrophoretically to PVDF membranes (Millipore, USA). The membranes were blocked for 1 h at room temperature, in Tris-buffered saline (137 mM NaCl, 20 mM Tris-HCl, pH 7.6) containing 0.1% Tween-20 (TBS-T) and 5% low-fat milk. The membranes were incubated with the primary antibody directed against the respective protein (listed in Table 2.1) overnight at 4ºC. After washing for 1 h in TBS-T with 0.5% low-fat milk, the membranes were incubated for 1 h at room temperature with the respective secondary antibody (1:2,000; santa cruz biotechnology), prepared in TBS-T with 1% low-fat milk. The membranes

were processed for protein detection using the ChemiDoc XRS (Biorad). Digital quantification of band intensity was performed using ImageJ 1.46 software. The membranes were then reprobed and tested for β-actin immunoreactivity (1:2,000) to prove that similar amounts of protein were applied in the gels.

**Table 2.1:** List of primary antibodies

| Primary Antibody | Sample | Dilution | Protein (µg) | Source |
|---|---|---|---|---|
| Mouse anti-SNAP25 | Synaptosomes | 1:40,000 | 10 | Synaptic Systems |
| | Total Extracts | 1:5,000 | 20 | |
| Mouse anti-syntaxin-1 | Synaptosomes | 1:40,000 | 10 | Synaptic Systems |
| | Total Extracts | 1:5,000 | 20 | |
| Mouse anti-synapsin-1 | Synaptosomes | 1:40,000 | 10 | Synaptic Systems |
| | Total Extracts | 1:10,000 | 10 | |
| Mouse anti-synaptohpsin | Synaptosomes | 1:40,000 | 10 | Sigma |
| | Total Extracts | 1:10,000 | 10 | |
| Rabbit anti-rabfilin-3a | Synaptosomes | 1:40,000 | 10 | Synaptic Systems |
| | Total Extracts | 1:2,000 | 20 | |

## 2.5. Statistical analysis

Results are presented as mean ± SEM. Statistical analysis was performed using GraphPad Prism version 5.0. The significance of the difference between mean values was calculated through Student's *t* tests or ANOVA test. Differences were considered significant for $P \leq 0.05$.

# 3. Results

## 3.1. Peripheral glucose homeostasis

### 3.1.1. Characterization of glucose homeostasis at periphery in a control model

Body weight (g) and fast glycemia levels (mg/dL) of control animals (without any specific treatment) during time are illustrated in table 3.1. In normal animals weight increases during time, as it was expected. However basal glycemia remains similar with ageing. Intra-enteric glucose tolerance test (IEGTT) typical curve is illustrated by figure 3.1. It is evident an increase in glucose levels during the first minutes as it was expected, due to the bolus of glucose given and values start do decrease until de end of the experiment. However, at the 120 minutes of the test glycemia did not reach yet the same values as seen in the baseline.

**Table 3.1**: Body weights (g) and fast glycemia (mg/dL) of control animals with ageing

| | **Weeks** | **Weight (g)** | **Fast glycemia (mg/dL)** |
|---|---|---|---|
| **Animals** | 4 (n=8) | 241.5 ± 12.68 | 72.57 ± 3.007 |
| | 24 (n=7) | 463.7 ± 8.251 | 73.11 ± 3.482 |
| | 36 (n=6) | 550.0 ± 15.32 | 80.44 ± 4.729 |

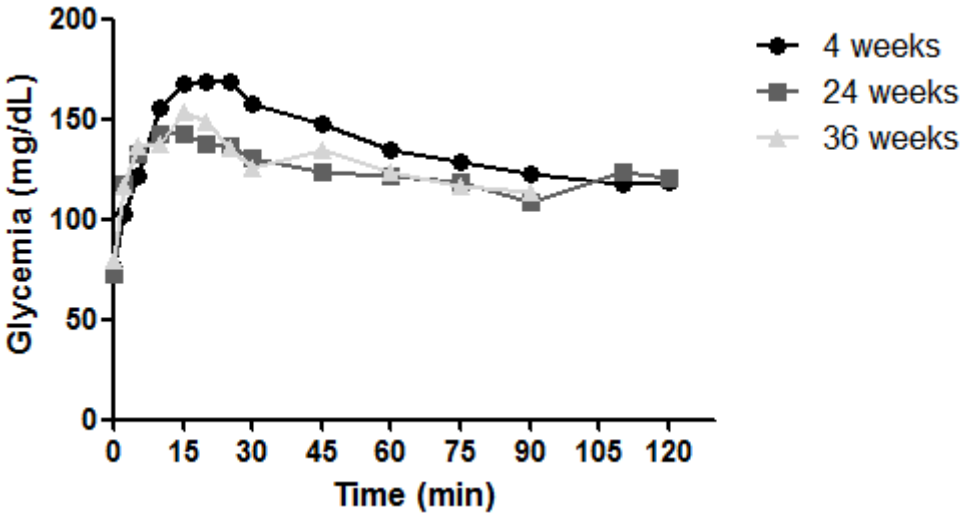


**Figure 3.1:** Standard curves obtained from plasma glucose levels during 120 minutes of an IEGTT of control animals at 4, 24 and 36 weeks of age.

Glucose uptake in skeletal muscle occurs through insulin dependent glucose transporters, namely GLUT4 and GLUT12. Evaluation of these two transporters in the control group, with age, shows that GLUT4 is not altered during time but there is a tendency for an increase in GLUT12 (figure 3.2).

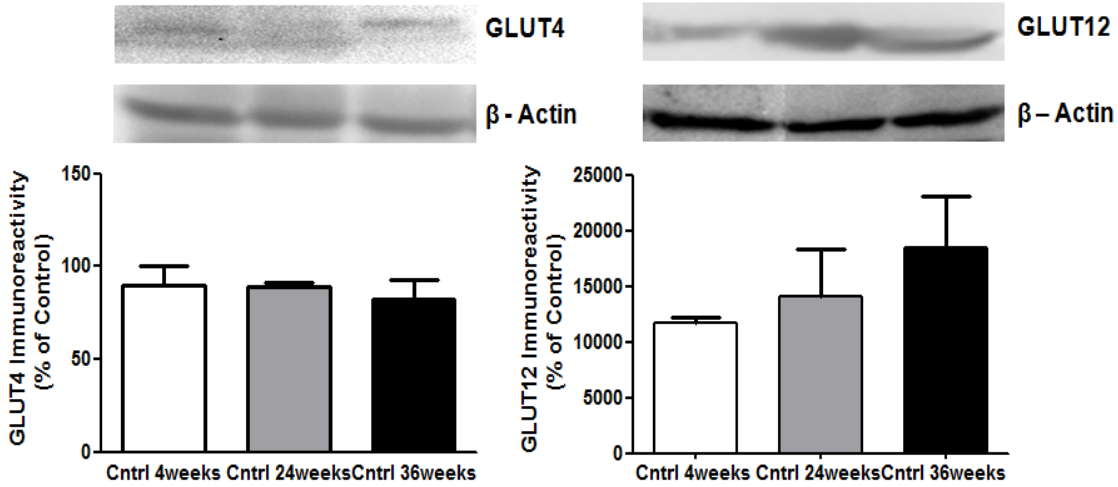


**Figure 3.2:** The total amount of GLUT12 tends to increase in skeletal muscle. Glucose tolerance was evaluated with in intra-enteric glucose tolerance test, and no differences were detected along the time. The total amount of GLUT4 (**A**) and GLUT12 (**B**) were evaluated by western blotting in total extracts from skeletal muscle. In GLUT4 at least, n=3 at 4 weeks, n=4 at 24 weeks and n=5 at 36 weeks. In GLUT12 at least n=6 at 4 weeks, n=2 at 24 weeks and n=5 at 36 weeks Results are presented as mean ± SEM. * p≤ 0.05 comparing comparing to cntrl 4 weeks, used as control, using Student's *t* test.

### 3.1.2. Effect of NAC (N-acetyl-cysteine) in insulin sensitivity

#### 3.1.2.1. Plasma glucose excursions during a glucose tolerance test after NAC administration

To evaluate the effect of NAC on glucose excursions it was performed an IEGTT. We observed that intra-enterically administration of NAC alone had no effect on plasma glucose levels, as expected (AUC 75.596±42.384 mg/dL/min, n=5, figure 3.3). Although there is a significant difference between control and NAC groups, this is explained once NAC administration does not alter glucose levels remaining these at the baseline, yet when glucose is administrated there is a typical increase of glucose levels. Co-administration of NAC and glucose (906,188±66.553 mg/dL/min, n=8) as well as denervation (938.071±133.380, n=7) did not altered glucose tolerance during IEGTT, being AUC glucose levels similar to control (glucose AUC: 710.685±42.384 mg/dL/min, n=7, figure 3.3).

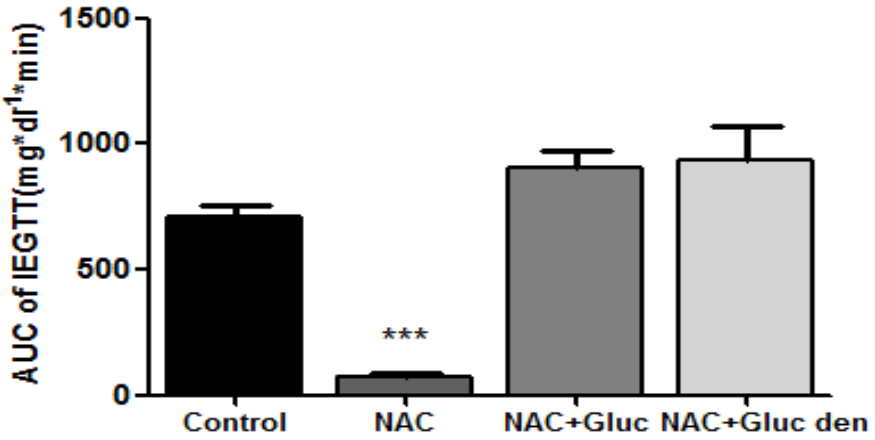


**Figure 3.3:** Effect of NAC in the regulation of plasma glucose excursions. AUC representing the glucose excursions during the 120 minutes of the IEGTT. Administration of NAC *per se* does not promote any glucose excursion, with glucose values similar to basal state all over the 120 minutes of IEGTT. Results are presented as mean ± SEM. *** p≤ 0.0001 comparing to control using Dunnett's post-hoc test.

### 3.1.2.2. Impact of NAC on insulin sensitivity

In order to evaluate insulin sensitivity in the presence of NAC an insulin sensitivity test, RIST, was performed. As it is shown is figure 3.4 administration of glucose (117.5±12.2 mg/kg, n=10) and NAC (105.8±3.4 mg/kg, n=6) alone did not alter insulin sensitivity compared to the fast state (128.6±11.2 mg/kg, n=10). However, administration of NAC in the presence of glucose (227.3±17.8 mg/kg, n=8) double increased insulin sensitivity compared to fast state (figure 3.4).

To assess if the effect in insulin sensitivity is dependent on the hepatic vagus nervous system, a surgical ablation of hepatic parasympathetic nerves was performed. Ablation of these nerves did not result in an increase in insulin sensitivity as previously observed when NAC and glucose were co-administrated (129.3±14.7 mg/kg, n=6) (figure 3.4).

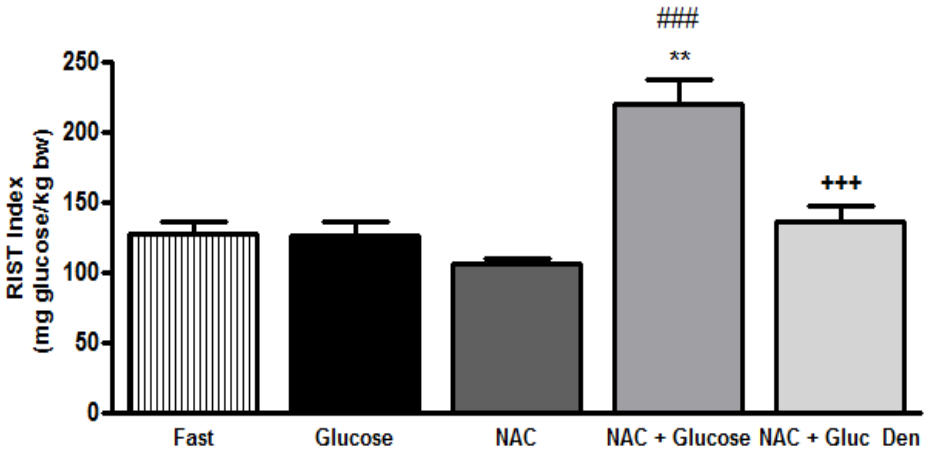


**Figure 3.4**: NAC+glucose increase insulin sensitivity. Insulin sensitivity was evaluated by RIST test. Co-administration of NAC with glucose increases insulin sensitivity. Glucose and NAC administration alone have no effect on insulin sensitivity, a similar effect to the fast state. Denervation does not increase insulin sensitivity. Results are presented as mean ± SEM. ** p≤ 0.01 comparing to fast; ### p≤ 0.001 comparing to glucose; +++ p≤ 0.001 comparing to NAC+glucose, using Dunnett's post-hoc.

### 3.1.2.3. Effects of NAC and glucose on liver and plasma NO levels

NO levels in plasma and liver were assessed using chemiluminescence-based quantification of nitrate ($NO_3^-$) and nitrite ($NO_2^-$) concentrations. As it is demonstrated by figure 3.5 there was no difference in NO levels either in plasma or liver when NAC was given alone (323.5±33.9 µM, n=4; 103.8±26.7 µM, n=4, plasma and liver, respectively) or co-administrated with glucose (412.3±112.6 µM, n=4; 88.4±7.9 µM, n=7, plasma and liver, respectively) compared to those animals that only received glucose alone (434.1±79.9 µM, n=5, 74.3±4.5 µM plasma and liver, n=7, respectively). However, after denervation it was evident a significant decrease in NO levels both in plasma (91.9±11.1 µM, n=6) and liver (52.7±5.5 µM, n=6) compared to aged-matched controls, consistent with loss of activation of cholinergic nerve terminals and consequent NO release (figure 3.5).

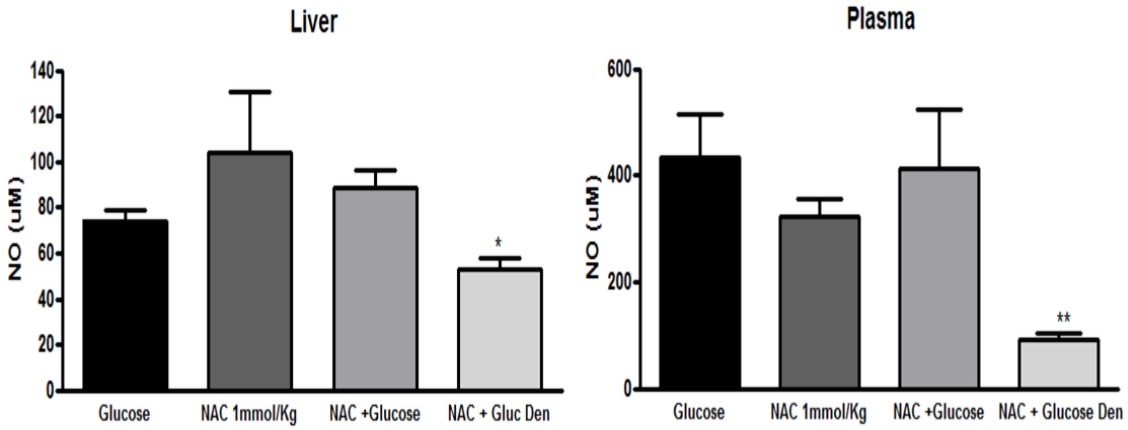


**Figure 3.5:** Effect of NAC in liver and plasma levels of NO. Liver (A) and plasma (B) NO levels were measured using chemiluminescence-based quantification of nitrate ($NO_3^-$) and nitrite ($NO_2^-$) concentrations. Results are presented as mean ± SEM. * p≤ 0.05; ** p≤ 0.01 comparing to control using Dunnett's post-hoc test.

### 3.1.3. An animal model of pre diabetes – high sucrose diet

#### 3.1.3.1. Animals weights and glycemia

Body weight (g) and fast glycemia (mg/dL) of both control and sucrose groups during high sucrose diet (HSD) are illustrated in table 3.2. Before sacrifice and after 4, 24 or 36 weeks of sucrose diet animals were weighted. Body weights between control and sucrose groups were similar at all time points analyzed. Fast glycemia was also similar between both groups for all time points.

**Table 3.2:** Average body weight and fast blood glucose levels of control and sucrose diet animals

| | Sucrose diet duration | Weight (g) | Fast Glycemia (mg/dL) |
|---|---|---|---|
| ***Control*** | 4 weeks | 241.5 ± 12.68 | 72.57 ± 3.007 |
| ***Sucrose*** | | 214.0 ± 10.89 | 71.83 ± 3.877 |
| ***Control*** | 24 weeks | 463.7 ± 8.251 | 73.11 ± 3.482 |
| ***Sucrose*** | | 449.0 ± 11.87 | 76.11 ± 1.989 |
| ***Control*** | 36 weeks | 550.0 ± 15.32 | 80.44 ± 4.729 |
| ***Sucrose*** | | 510.0 ± 18.04 | 77.83 ± 4.222 |

#### 3.1.3.2. Body composition of high sucrose diet animal model

Fat and lean mass were assessed by resonance at 4, 20 and 36 weeks, after sucrose consumption. Besides accumulation of fat mass during time in all controls as it was expected due to aging factor, it was evident a significant increase of fat mass in sucrose animals compared to aged-matched controls at all time points measured (figure 3.6A). At 4 weeks of HSD, animals had 46.91±6.08g (n=5) and aged-matched controls had 29.67±3.9g (n=5) of fat mass; at 20 weeks sucrose animals showed 127.0±21.74g (n=5) of fat mass compared to aged-matched control animals that had 63.69±9.729g (n=5), and 36 weeks after sucrose consumption, sucrose group of

animals had 206.3 ± 30.94g (n=5) of fat mass compared to 90.37±9.225g (n=5) of controls.

Concerning lean mass, it is shown in figure 3.6B a decrease in lean mass at 4 weeks diet duration in sucrose animals compared aged-matched controls. At 20 and 36 weeks of HSD there was no difference in lean mass between groups.

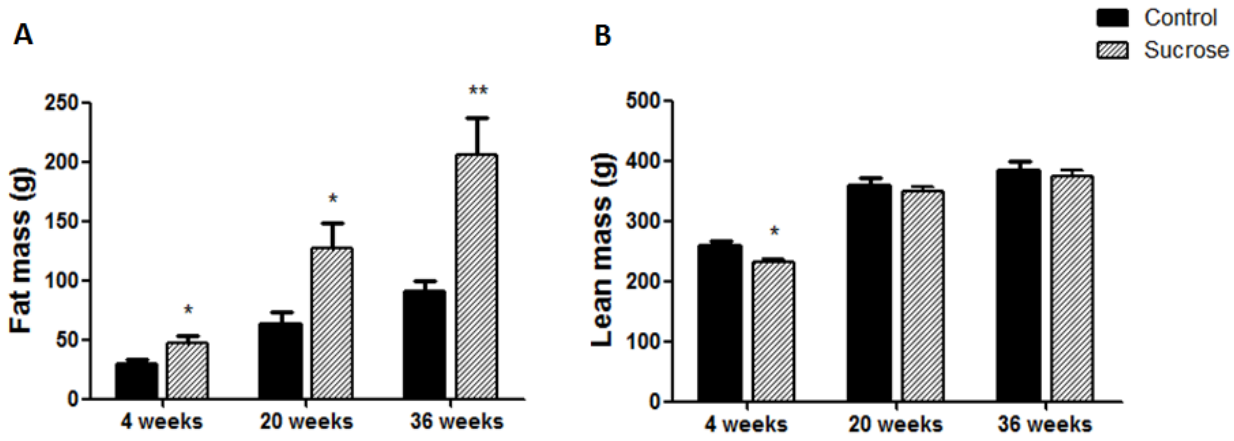


**Figure 3.6**: HSD induces a significant increase in total fat mass. Fat mass measurements revealed statistic increased levels of fat mass in sucrose groups compared to controls, at every time points measured (**A**). Lean mass measurements revealed statistic decreased levels of lean mass in sucrose group compared to controls at 4 weeks of diet duration (**B**). Comparisons are made between control and sucrose group within same time point. Results are presented as mean ± SEM. * p≤ 0.05; ** p≤ 0.01 comparing to aged-matched control using Student's *t* test.

#### 3.1.3.3. Characterization of glucose tolerance in high sucrose diet animal model

To evaluate glucose tolerance during time course of sucrose diet (35%), an OGTT was performed every four weeks in animals drinking sucrose solution and in the aged-matched controls (drinking only water), until the day of sacrifice. Figure 3.7 shows AUC of glucose excursions during OGTT at different time points until 32 weeks of sucrose diet. At 24 and 32 weeks there was a significant increase in glucose excursions ($p<0.05$) between sucrose group aged-matched controls, representing glucose intolerance induced by a HSD (20010±405.1 mg/dL/min, n=9 at 24 weeks and 19595±527 mg/dL/min, n=10 at 32 weeks, compared to aged-matched controls 18320±615.2 mg/dL/min, n=9 and 17990±479.0 mg/dL/min, n=6, 24 and 32 weeks, respectively).

At the other time point no differences were observed between sucrose groups and aged-matched controls.

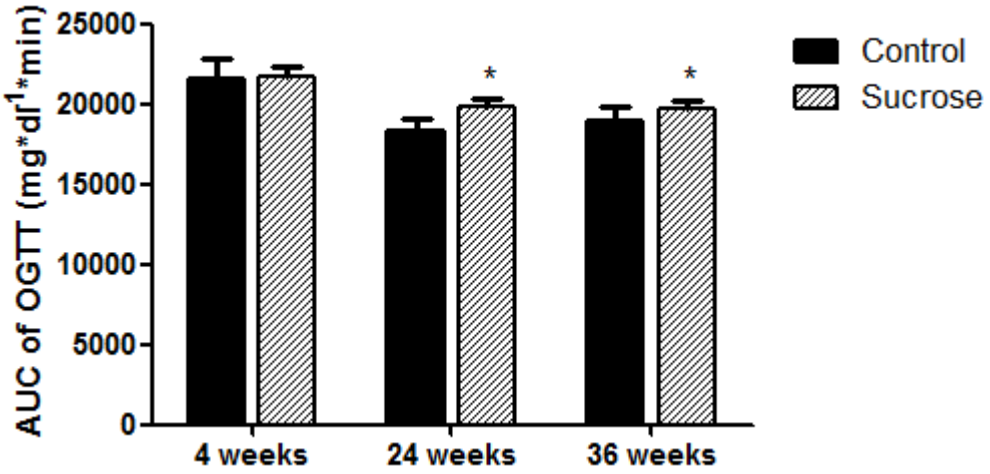


**Figure 3.7**: Glucose excursions levels during oral glucose tolerance test in control and sucrose groups. AUC of blood glucose levels during 120 minutes. AUC glucose levels show statistic differences between control and sucrose groups at 24 weeks and 32 weeks. Comparisons are made between control and sucrose group within same time point. Results are presented as mean ± SEM. * p≤ 0.05 comparing to aged-matched control using Student's *t* test.

At the day of sacrifice it was performed an IEGTT in all animals in order to evaluate the glucose tolerance of the two groups of animals. This measurement was performed with animals anesthetized. Figure 3.8 shows the results of IEGTT performed at three different points, 4, 24 and 36 weeks of sucrose diet. At 4 weeks of HSD no differences between the two groups were observed, even though it is possible to see that in the sucrose group there is a tendency for a higher AUC of glucose compared to controls. We observed that after 24 weeks drinking sucrose the animals had an increase in glucose excursions compared with age-matched control (AUC 1435±108.6 mg/dL/min, n=8 in control and 1772±74.73 mg/dL/min, n=9 in sucrose group). A difference between the two groups was also considered at 36 weeks. Although the difference did not reach statistical significance of p≤0.05, p value is almost equal to 0.05.

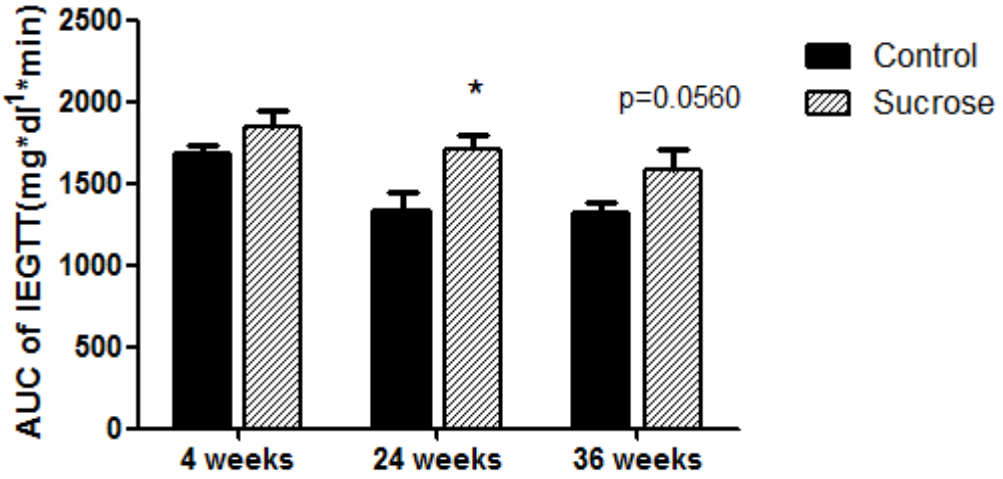


**Figure 3.8**: AUC blood glucose levels during IEGTT. AUC glucose levels show that glucose excursions are higher in sucrose group, being statistically different between control and sucrose groups at 24 weeks of sucrose diet and almost significantly different at 36 weeks with a p value = 0.0560, resulting in a larger area under the curve. Comparisons are made between control and sucrose group within same time point. Results are presented as mean ± SEM. * p≤ 0.05 comparing to aged-matched control using Student's *t* test.

Expression of GLUT4 and GLUT12 was assessed by western blot analysis in skeletal muscle during ageing in animals submitted to HSD (4, 24 and 36 weeks). The results presented in figure 3.9 show that neither GLUT4 nor GLUT12 content changed with ageing in animals drinking high sucrose diet.

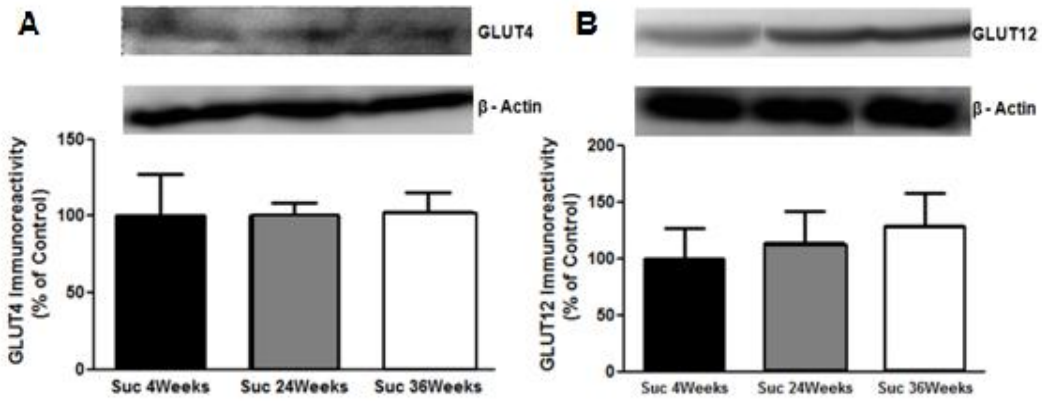


**Figure 3.9:** The total amount of GLUT4 and GLUT12 does not change with age in skeletal muscle of animals drinking a high sucrose diet. The total amount of GLUT4 (**A**) and GLUT12 (**B**) were evaluated by western blotting in total extracts from skeletal muscle. In GLUT4 at least, n=3 at 4 weeks, n=4 at 24 weeks and n=2 at 36 weeks. In GLUT12 at least n=5 at 4 weeks, n3= at 24 weeks and n=5 at 36 weeks. Results are presented as mean ± SEM. * p≤ 0.05 comparing to suc 4 weeks, used as control, using ANOVA test.

Our data showed the protein content of GLUT4 and GLUT12 evaluated by western blot in both groups of animals, sucrose and the aged-matched controls. As shown in figure 3.10 protein levels of GLUT4 remained similar between groups in the early stages of HSD but were decreased at 36 weeks (52.16 ± 21.24% of control) in sucrose group. On the contrary, even though GLUT12 content did not show differences between groups at 4 and 24 weeks of diet, its levels were increased at 36 weeks (123.5±7.04% of control) in the sucrose animals compared to age-matched controls.

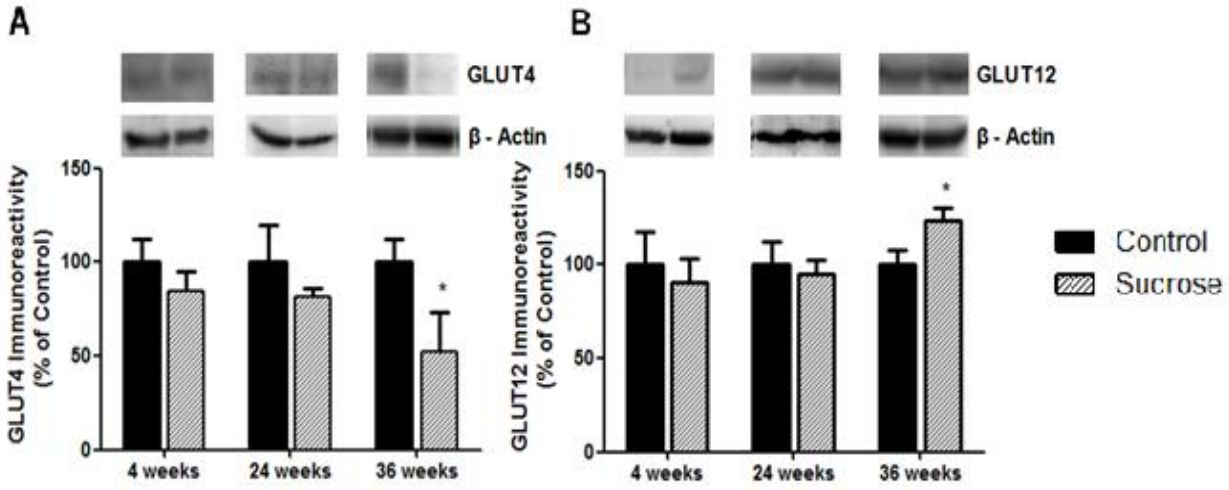


**Figure 3.10**: High sucrose diet changes the total amount of GLUT4 and GLUT12 in skeletal muscle. GLUT4 is decreased at 36 weeks in sucrose animals compared to aged-matched controls (**A**) and GLUT12 is increased at 36 weeks of diet in sucrose animals compared to aged-matched controls (**B**). In GLUT4 at least, n=2 at 4 weeks, n=4 at 24 weeks and n=3 at 36 weeks. In GLUT12 at least n=5 at 4 weeks, n3= at 24 weeks and n=5 at 36 weeks. Comparisons are made between control and sucrose group within same time point. Results are presented as mean ± SEM. * p≤ 0.05 comparing to aged-matched control using Student's *t* test.

## 3.2. Effect of high sucrose diet in synaptic proteins expression

In order to evaluate HSD effect on synaptic proteins content, western blot analysis were performed in hippocampus, in which total extracts and nerve terminals were analyzed and in total extracts from cortex.

### 3.2.1. Content of hippocampal synaptic proteins in HSD animal model

#### 3.2.1.1. Effect of HSD on the content of synapsin-1

Synapsin-1 is involved in plasticity of mature synapses controlling SVs trafficking at pre and post-docking levels. Hippocampal total extracts showed no differences in synapsin-1 levels between control and animals drinking sucrose, however, in hippocampal nerve terminals there was a significant increase of synapsin-1 content after 24 weeks (181.1±12.2% of control) of HSD compared to aged-matched controls (Figure 3.11). At 4 and 36 weeks of diet duration, levels of synapsin-1 remained similar in both groups.

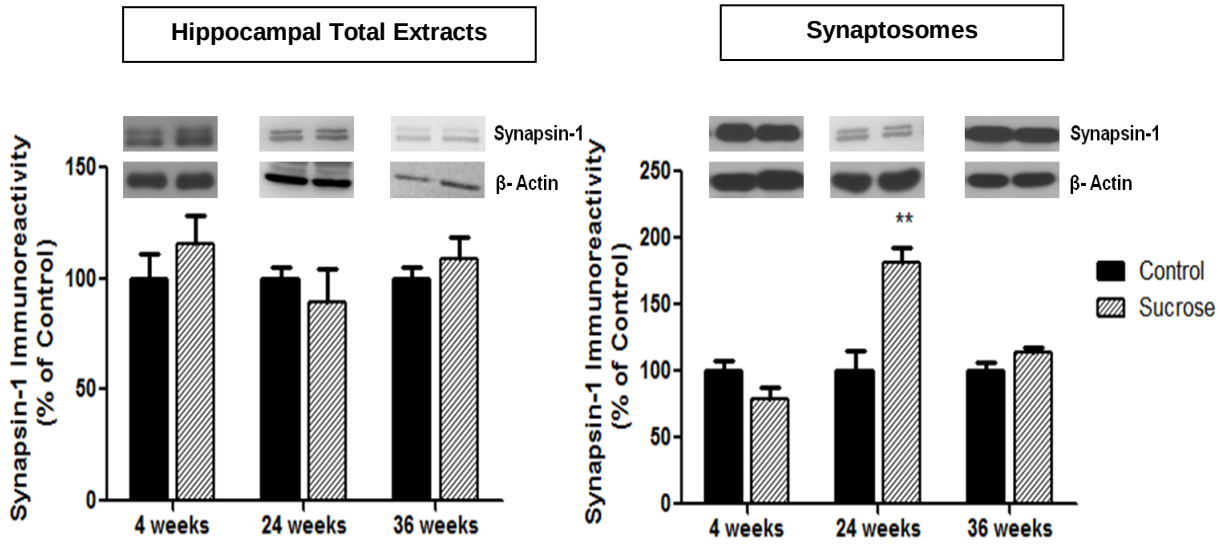


**Figure 3.11**: Sucrose diet induces an increase in the protein content of synapsin-1 at 24 weeks of HSD, in hippocampal synaptosomes. Hippocampal total extracts: 4 weeks n=5; 24 weeks n=8, 36 weeks n =5, at least. Synaptosomes 4 weeks n=7, 24 weeks n=3, 36 weeks n=4, at least.Comparisons are made between control and sucrose group within same time point. Results are presented as mean ± SEM. ** p≤ 0.01 comparing to aged-matched control using Student's *t* test.

### 3.2.1.2. Effect of HSD on rabphilin-3a protein

We evaluated rabphilin-3a, an effector of Rab protein family that plays an important role in vesicle docking and observed that in hippocampal total extracts protein content significantly increased after 36 weeks of sucrose consumption (140.4±8.3%, comparing to the aged-matched control, figure 3.12). No changes were detected for shorter periods of HSD. In the hippocampal nerve terminals there was no difference between the two groups at any time point measured; however at 36 weeks of HSD it is possible to observe a tendency for an increase in the content of rabphilin-3a.

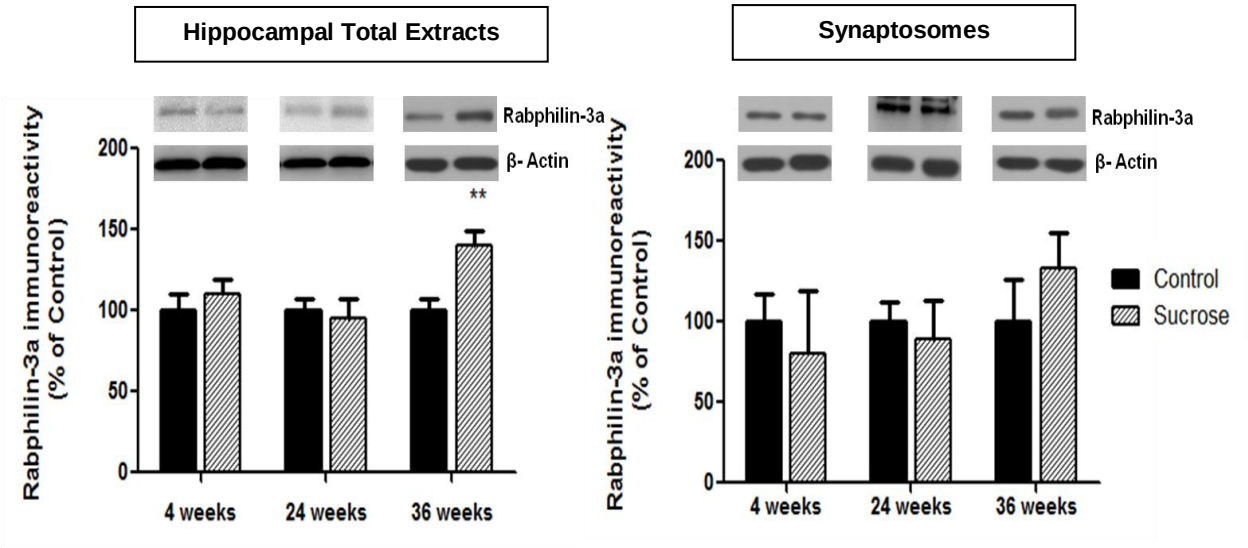


**Figure 3.12:** Sucrose diet induces an increase in rabphilin-3a content at 36 weeks of high sucrose diet, in hippocampal total extracts. Hippocampal total extracts: 4 weeks n=5; 24 weeks n=8, 36 weeks n =5, at least. Synaptosomes 4 weeks n=2, 24 weeks n=3, 36 weeks n=4, at least. Comparisons are made between control and sucrose group within same time point. Comparisons are made between control and sucrose group within same time point. Results are presented as mean ± SEM. * p≤ 0.05; ** p≤ 0.01 comparing to control using Student's *t* test.

### 3.2.1.3. Effect of HSD on the content of SNARE complex proteins

SNARE complex is composed by SNAP-25, syntaxin-1 and VAMP-2, playing a fundamental role in synaptic vesicle exocytosis. Regarding SNAP-25, figure 3.13 shows that in hippocampal total extracts occurred a decrease (79.3±5.3% of the control) in protein content at 36 weeks of HSD but at 4 and 24 weeks no differences were observed between control and experimental groups. At hippocampal nerve terminal, no differences were detected between groups at any time point studied. Yet, it is possible to observe a tendency to a decrease in SNAP-25 content at 36 weeks of diet duration.

Syntaxin-1 did not show alterations either in hippocampal total extracts or nerve terminals as shown in figure 3.14, during all diet duration.

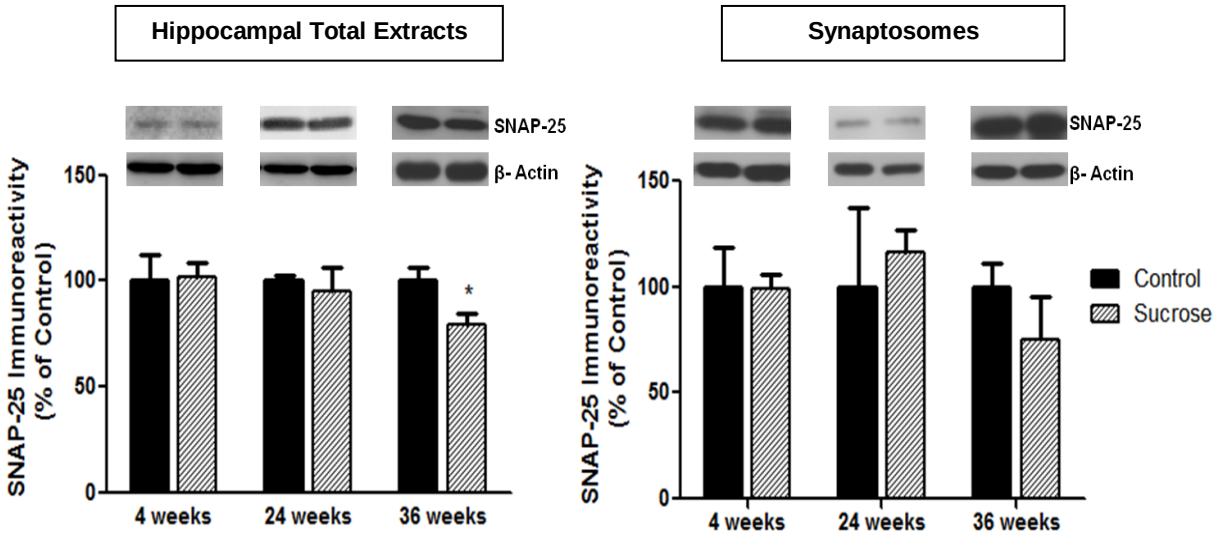


**Figure 3.13:** Sucrose diet induces a decrease in the protein content of SNAP-25 at 36 weeks of sucrose diet in hippocampal total extracts. Hippocampal total extracts: 4 weeks n=5; 24 weeks n=7, 36 weeks n =5, at least. Synaptosomes 4 weeks n=8, 24 weeks n=2, 36 weeks n=5, at least. Comparisons are made between control and sucrose group within same time point. Results are presented as mean ± SEM. * p≤ 0.05 comparing to control using Student's *t* test.

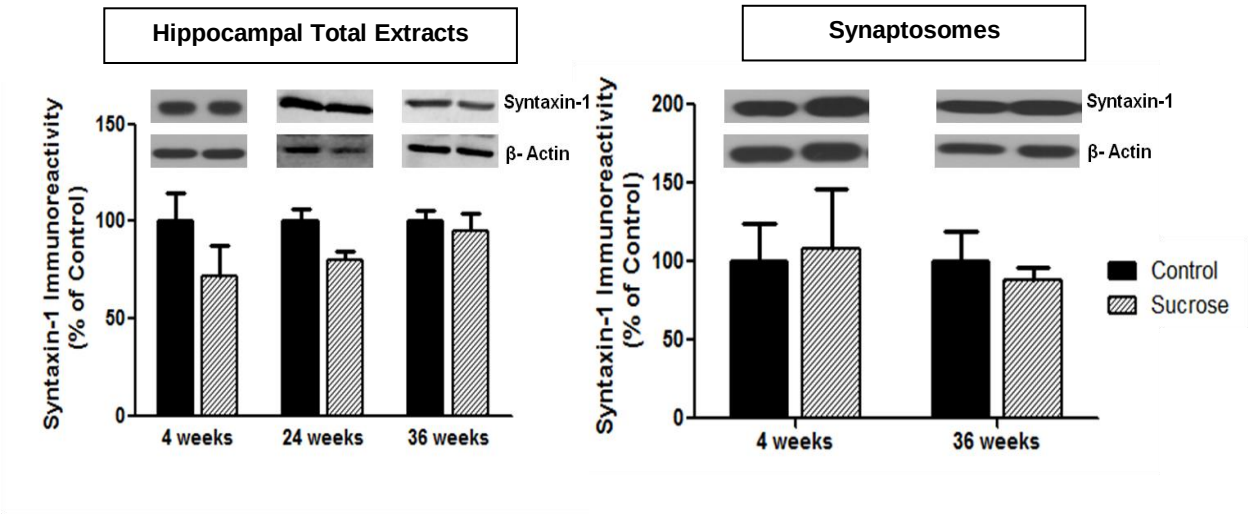


**Figure 3.14**: Sucrose diet does not induce changes in the protein content of Syntaxin-1. Hippocampal total extracts: 4 weeks n=5; 24 weeks n=3, 36 weeks n =5, at least. Synaptosomes 4 weeks n=6, 36 weeks n=4, at least. Comparisons are made between control and sucrose group within same time point. Results are presented as mean ± SEM. * p≤ 0.05 comparing to control using Student's *t* test.

### 3.2.1.4. Effect of HSD on the content of synaptophysin

Synaptophysin is a synaptic protein widely used as a marker for nerve terminals affecting the SNARE complex. In this study, as illustrated in figure 3.15, synaptophysin expression did not show any differences between control and sucrose groups in total hippocampal extracts. However in hippocampal nerve terminals there was a decrease in synaptophysin content (46.82±9.1% of the control) at 4 weeks of HSD duration which did not occur at 24 and 36 weeks.

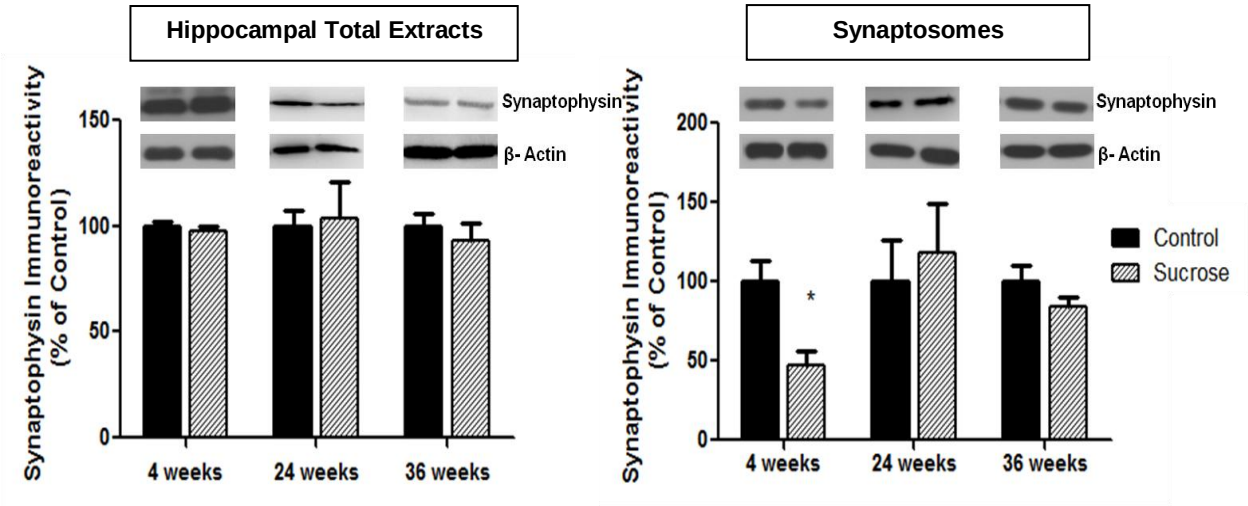


**Figure 3.15**: Sucrose diet induces alterations in synaptophysin at 4 weeks of HSD in Synaptosomes. Hippocampal total extracts: 4 weeks n=3; 24 weeks n=7, 36 weeks n =5, at least. Synaptosomes: 4 weeks n=5, 24 weeks n=2, 36 weeks n=5, at least. Comparisons are made between control and sucrose group within same time point. Results are presented as mean ± SEM. * p≤ 0.05 comparing to control using Student's *t* test.

### 3.2.2. Content of synaptic proteins in cortex of HSD animal model

#### 3.2.2.1. Effect of HSD on synapsin-1 content in cortex

Cortex synapsin-1 levels, as illustrated in figure 3.16, were decreased (69.9±5.3% of the aged-matched control) at 4 weeks of sucrose diet in animals compared to respective control, on the other hand at 24 weeks of HSD it was evident an increase of synapsin-1 (156.5±21.2%) when compared to age-matched control.

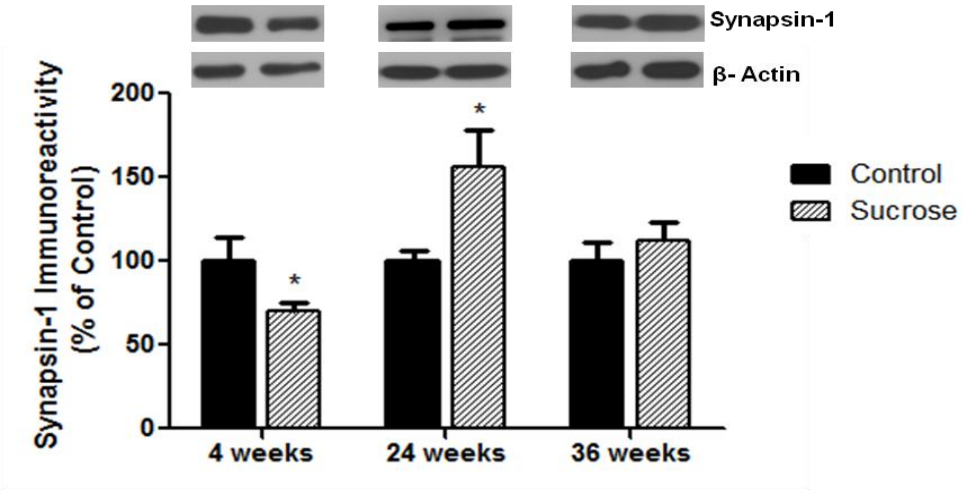


**Figure 3.16:** HSD induces a decrease in synapsin-1 content at 4 weeks diet duration and an increase at 24 weeks diet duration. Cortex total extracts: 4 weeks n=5; 24 weeks n=7, 36 weeks n=6, at least. Comparisons are made between control and sucrose group within same time point. Results are presented as mean ± SEM. * p≤ 0.05 comparing to control using Student's *t* test.

#### 3.2.2.2. Effect of HSD on rabphilin-3a protein content in cortex

HSD had no effect on rabphilin-3a content in cortex as shown by figure 3.17. Nevertheless it is possible to see a tendency for an increase in the content of this protein at 24 weeks of diet but this difference did not reach statistical significance due to discrepancy in values of Western blot quantification.

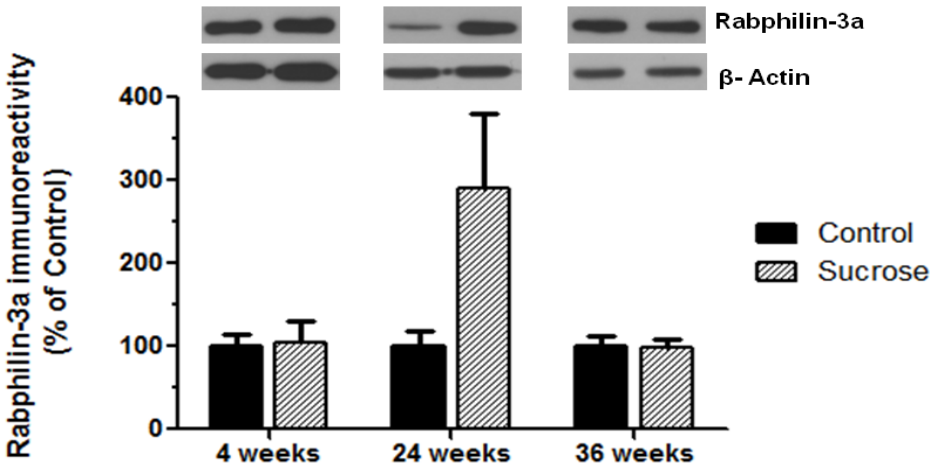


**Figure 3.17:** HSD does not alter Rabphilin-3a content in cortex. Cortex total extracts: 4 weeks n=5; 24 weeks n=8, 36 weeks n=4, at least. Comparisons are made between control and sucrose group within same time point. Results are presented as mean ± SEM. * p≤ 0.05 comparing to control using Student's *t* test.

### 3.2.2.3. Effect of HSD on content of SNARE complex in cortex

SNAP-25 levels in cortex decreased (70.7±2.3% of the control) at 4 weeks of HSD compared to age-matched controls. At 24 and 36 weeks no changes were observed (Figure 3.18). Concerning syntaxin-1, no differences were observed between control and experimental groups at any time points measured (figure 3.19).

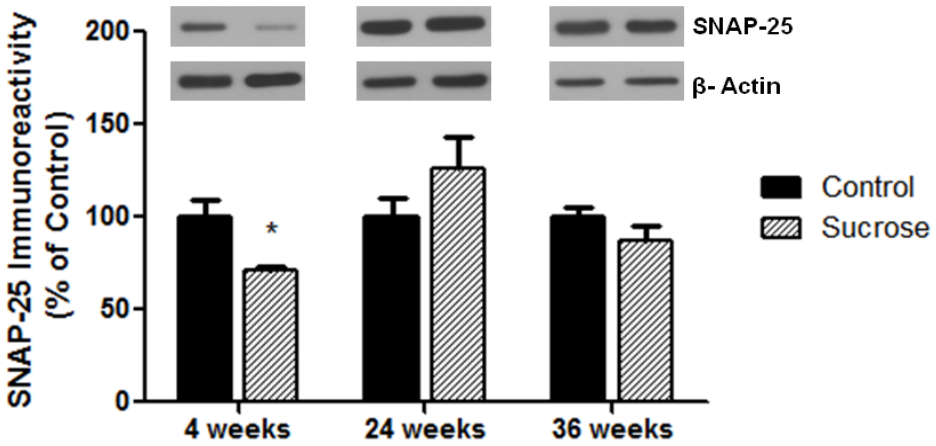


**Figure 3.18:** SNAP-25 proteins levels are decreased at 4 weeks of HSD**.** Cortex total extracts: 4 weeks n=4; 24 weeks n=7, 36 weeks n=5, at least. Comparisons are made between control and sucrose group within same time point. Results are presented as mean ± SEM. * p≤ 0.05 comparing to control using Student's *t* test.

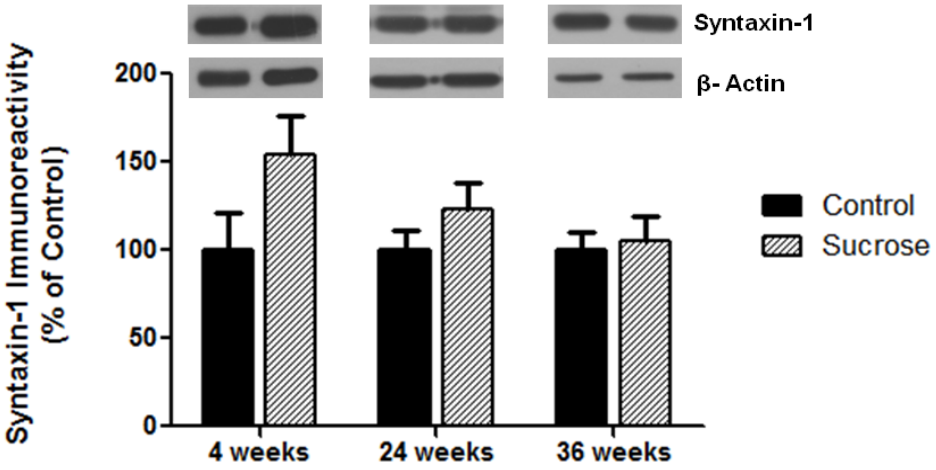


**Figure 3.19**: Syntaxin-1 is not altered by a diet rich in sucrose in cortex. Cortex total extracts: 4 weeks n=7; 24 weeks n=6, 36 weeks n=6, at least. Comparisons are made between control and sucrose group within same time point. Results are presented as mean ± SEM. * p≤ 0.05 comparing to control using Student's *t* test.

### 3.2.2.4. Effect of HSD on content of synaptophysin

Synaptophysin content in cortex did not change during time and there were also no differences between animals drinking sucrose diet and control group (figure 3.20).

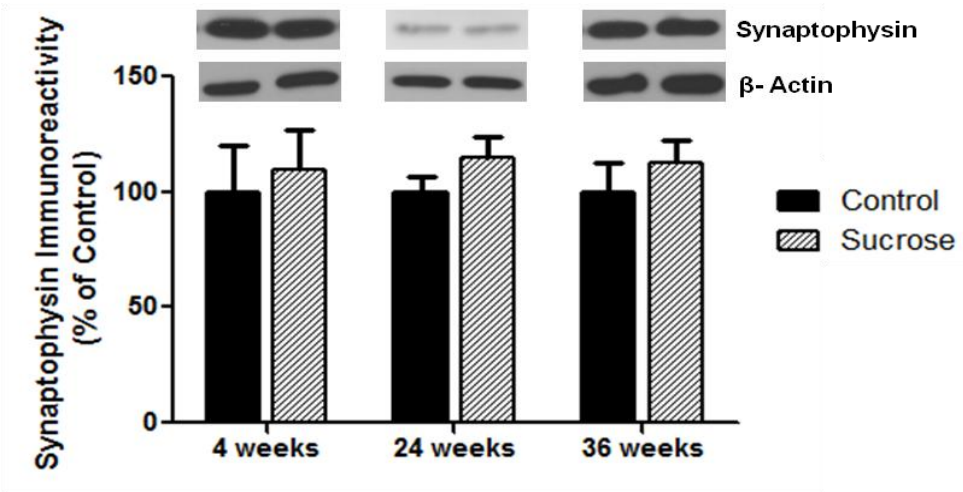


**Figure 3.20:** Synaptophysin is not altered by a diet rich in sucrose. Cortex total extracts: 4 weeks n=5; 24 weeks n=8, 36 weeks n=6, at least. Comparisons are made between control and sucrose group within same time point. Results are presented as mean ± SEM. * p≤ 0.05 comparing to control using Student's *t* test.

# 4. Discussion

The development of this thesis brought new insights related with glucose homeostasis.

In our studies, administration of N-acetyl-cysteine appears as a new compound capable of enhancing postprandial insulin sensitivity in peripheral tissues, in the presence of glucose. Moreover, we also showed that this mechanism is dependent on the hepatic parasympathetic nerves affecting insulin sensitivity at the periphery.

Moreover, high-energy diets, as the high sucrose diet, induced glucose intolerance simultaneously with changes in glucose transporters (GLUT4 and GLUT12) in skeletal muscle and accumulation of fat mass. At a cerebral level, this diet resulted in impairments in synaptic proteins content that may lead to cognitive deficits in these animals.

## 4.1. NAC administration increases insulin sensitivity

The main finding of this work in relation to our first hypothesis is that NAC, as a source of glutathione, together with glucose, function as a feeding signal, increasing peripheral insulin sensitivity.

***Methodological considerations***

In our studies insulin sensitivity was assessed using a rapid insulin sensitivity test (RIST). Several methods can be used to assess insulin sensitivity besides RIST, such as insulin tolerance test (ITT) and hyperinsulinemic euglycemic clamp (HIEC); Comparisons between these three methods have been done (Reid et al. 2002). HIEC, which consists in a euglycemic test where blood glucose levels are maintained during test, failed to be a good method to assess HISS action once it detects the effect of HISS only in the beginning of the test and post-HIEC test induces a blockade of HISS action inducing insulin resistance. In contrast, RIST and ITT emerge as good methods to evaluate HISS action. Although despite its simplicity and easiness of performance, ITT, which consists in a bolus of insulin and subsequent measurement of plasma glucose levels, shows some disadvantages compared to RIST. RIST by maintaining euglycemia does not implicate risk for hypoglycemic episodes with counter-regulatory hormonal responses, which happens in ITT (Reid et al. 2002). For the reasons

mentioned, RIST has shown to be a safer and easier method to interpret than ITT, being our choice to assess insulin sensitivity in these experiments.

### *NAC effects on glucose homeostasis and its rapport to the parasympathetic nerves*

HISS release which is known to be maximal in the postprandial state and inhibited in the fasted state (Lautt et al. 2001), needs both GSH and NO for its appropriate action (Guarino et al. 2003). Cysteine is an essential amino acid that is a precursor of GSH (Dongzhe Song, Hutchings, and Pang 2005) and in this study we used a precursor of cysteine, N-acetyl-cysteine (NAC), to evaluate its effect on insulin sensitivity as a source of GSH. In the present study RIST indexes, as a measure of insulin sensitivity, showed that NAC or glucose alone did not increase peripheral insulin sensitivity. Although, when NAC was co-administrated with glucose it was evident an increase in insulin sensitivity, similar to what is seen in the fed state, reflecting that these two compounds together act as a signal to enhance insulin sensitivity in peripheral tissues. Previous studies from our laboratory (Lautt et al. 2011) also showed that by targeting two feeding signals, NAC (to stimulate GSH release) and bethanecol (mimicking the parasympathetic nerve signal to induce hepatic NO release), HISS action was increased. In these studies, the effect of these two signals was evaluated in an insulin resistance animal model induced by a high sucrose diet, showing that these compounds not only enhanced insulin sensitivity in normal animals as totally restored postprandial insulin sensitivity in diabetic ones. These results also corroborate studies of Sadri et al. 2007 stating that a liquid meal increased insulin sensitivity in the periphery.

Hepatic parasympathetic denervation inhibited the increase in insulin sensitivity observed when nerves were intact, being RIST indexes similar to those seen in the fast state. Hepatic parasympathetic nerves are essential for the synthesis of NO in liver and in our study we showed that NO liver and plasma levels were decreased in denervated animals. These results are in accordance with Guarino's (Guarino et al. 2004) results where it was shown that liver activation of muscarinic receptors leads to an increase in hepatic nitric oxide levels resulting in increased peripheral insulin sensitivity. Moreover, Lautt´s study (Lautt et al. 2011), also highlighted the role of nitric oxide on peripheral insulin sensitivity since administration of bethanecol that acts on muscarinic receptors, together with NAC, enhanced peripheral insulin sensitivity.

Other studies where cysteine effects were evaluated in animal models of insulin resistance showed that this amino acid has benefic effects, restoring insulin sensitivity. Work done by Jain et al. 2009 emphasized the role of L-cysteine where its oral supplementation reduced insulin resistance, glucose levels, oxidative stress and inflammatory markers of type 2 diabetes in a Zucker diabetic fatty rat model. Besides, previous studies with type 2 diabetic patients showed that NAC improves oxidative stress (Masha et al. 2013; Valentino et al. 2008), a feature of diabetes, which is known to be caused by decreased levels of GSH (Samiec et al. 1998).

Taken together these results suggest that NAC affects peripheral insulin sensitivity which might play a role in the regulation of glucose homeostasis in pathological conditions. We suggest that along with glucose, NAC acts as a trigger signal for insulin sensitivity mediated by HISS, resulting in MIS. Moreover, for an appropriate action of this mechanism, the integrity of HPN is necessary.

## 4.2. High sucrose diet impairs glucose homeostasis

The main conclusion of this work was that animals drinking a HSD developed peripheral glucose intolerance associated with decreased levels of GLU4 in skeletal muscle in a long-term diet but not in a short period of time. HSD also induced fat mass accumulation in sucrose animals, although weights between groups remained similar.

A high sucrose diet (35%) was chosen as a nutritionally-induced model of pre diabetes. Different percentages of sucrose can be used, with higher (Brenner et al. 2003; Thresher et al. 2000) or lower (Sheludiakova, Rooney, and Boakes 2012) sucrose concentrations. In our study, a 35% sucrose solution was chosen to reproduce western diets, known to be related with increased ingestion of sucrose and fat (Kosari et al. 2012). During the time of experiment, until the day of sacrifice, animals had free access to chow and sucrose beverages in the experimental group or tap water in the control group. Although no changes in weight were detected between control and high sucrose diet animals, it was possible to see an accumulation of fat in abdominal cavity at the time of sacrifice which was confirmed by the experiments of resonance, in which was evident fat accumulation in sucrose animals during all diet duration. The increased accumulation of fat in sucrose animals was observed even in the early stages of diet, increasing during time, showing that a HSD leads to progressive accumulation of fat

mass. The present results are in agreement with previous studies where it was observed similar values of body weight in animals having a HSD during a short-term period of diet (4 and 8 weeks) (Toida et al. 1996; Pranprawit et al. 2013; Sheludiakova, Rooney, and Boakes 2012; Ribeiro et al. 2005) as well as for a long-term period (55 weeks) (Sumiyoshi, Sakanaka, and Kimura 2006). Despite similar weights between animals it has also been reported an increase in fat mass accumulation in animals drinking sucrose in earlier studies of (Fleur et al. 2011).

Regarding plasma glucose levels, no differences were observed between groups during diet course, in the fasted state, as also stated by previous studies (Santuré et al. 2002; Sheludiakova, Rooney, and Boakes 2012), showing that long-term HSD does not alter glycemia in the fasted state between sucrose and control animals. However, Brenner et al. 2003 and co-workers showed a clear increase in fast glucose levels of sucrose animals after 24 weeks of diets. Nevertheless, the percentage of sucrose given in this study was 60% that contrasts with 35% in the present study.

Higher glucose levels in postprandial state are intimately linked to hyperinsulinemia, indicating a condition of glucose intolerance and insulin resistance (Ribeiro et al. 2005; Santuré et al. 2002; Sheludiakova, Rooney, and Boakes 2012). Evaluation of glucose tolerance by IEGTT and OGTT showed higher glucose excursions in sucrose animals at a long term HSD but not at 4 weeks of diet, suggesting that glucose intolerance is developed in the later stages of diet. These results corroborate previous ones (Pranprawit et al. 2013; Sheludiakova, Rooney, and Boakes 2012), in which animals having HSD developed glucose intolerance, being associated with higher levels of glucose in the postprandial state. Previous studies from our laboratory (R.T. Ribeiro et al. 2005) are in agreement with the present ones showing that alterations in glucose homeostasis as reflected in decreased insulin resistance seem to occur primarily in the postprandial state long before they appear in the fasted state.

Whether the increase in insulin resistance is due to alterations in GLUT transporters expression needed clarification. Our results pointed out a decrease in expression of GLUT4 in sucrose animals in the later stages of diet, which supplements the glucose intolerance/insulin resistance seen, which could be the cause of the raise in glucose plasma levels. On the other hand, GLUT12 expression was increased in these animals, suggesting that it can act as a counter-regulatory mechanism to increase glucose translocation into the cell and minimizing the impaired glucose homeostasis. This mechanism was suggested in studies where overexpression of GLUT12 improved insulin sensitivity in skeletal muscle (Purcell et al. 2011).

Taken together these results suggest that a HSD induces changes in glucose levels, depending on duration of diet and lead to glucose intolerance, associated with decreased expression of GLUT4 with a compensatory increase expression of GLUT12, culminating in insulin resistance and fat accumulation.

## 4.3. High sucrose diet alters synaptic protein content in brain

Evaluation of synaptic proteins demonstrated that a HSD leads to changes in synaptic proteins content both in hippocampal total extracts and hippocampal nerve terminals (synaptosomes) as well as in cortex. Changes in protein content occured both at short (at 4 weeks) and long term (24 and 36 weeks) of diet duration. This is in accordance with previous studies (Gaspar, Baptista, et al. 2010; Grillo et al. 2005; Duarte et al. 2009), although a different animal model of diabetes was used.

### 4.3.1. Synaptic proteins expression changes in hippocampus of a HSD animal model

Synapsin-1 plays an important role in the trafficking of synaptic vesicles to pre-synaptic membrane (Easley-Neal et al. 2013). The content of synapsin-1 was significantly increased in hippocampal nerve terminals at 24 weeks of diet. However it seems that at a short term (4 weeks) HSD did not alter synapsin-1 content. Previous studies have already described decreased levels of synapsin-1 at short term (4 and 8 weeks) diabetes duration (Gaspar, Baptista, et al. 2010) in a type 1 diabetic model; this discrepancy may be related to differences in the DM1 and DM2 onset of cerebral impairments. Synapsins involvement in SVs translocation is achieved through their phosphorylation/dephosporylation. Since we have not measured phosphorylation state it is not possible, at this time, to understand if increased synapsin-1 content was associated or not with the trafficking of SVs to active zones in the plasma membrane.

Rabphilin-3a, a protein responsible for the docking of synaptic vesicles and regulation of exocytosis and endocytosis (Deak et al. 2006) was increased in hippocampal total extracts at 36 weeks of diet duration, indicating that a HSD leads to changes in the content of rabphilin-3a at a long term. Contrary to our finding, previous studies of Gaspar (Gaspar, Castilho, et al. 2010) refer that rabphilin-3a content is not altered in type 1 diabetic animals, reflecting that different types of diabetes might have different dysfunctional SVs trafficking.

Regarding SNARE complex, which is composed by three synaptic proteins, SNAP-25, syntaxin-1 and VAMP-2, we observed a decrease in SNAP-25 content in hippocampal total extracts at 36 weeks diet duration. This decrease indicates an effect of diet at long term and although this decrease is not statistical significant in hippocampal nerve terminals it is possible to observe a tendency for a decrease, which is in agreement with previous studies both in DM1 (Gaspar, Baptista, et al. 2010) and DM2 (Duarte et al. 2012) animal models. Changes in SNAP-25 content suggest that SNARE complex may be disrupted, leading to impairments in neurotransmission and contributing to cognitive problems.

Syntaxin-1 content was not altered either in hippocampal total extracts or nerve terminals, showing that a HSD did not affect hippocampal levels of this protein. Previous research indicate a decrease in syntaxin-1 content in hippocampal nerve terminals of type 1 diabetic animals (Duarte et al. 2009; Gaspar, Baptista, et al. 2010), however these studies refer to different a model of diabetes in which the severity of the disease is pronounced earlier than in a model of HSD, mimicking a pre diabetes condition. Moreover, studies from Gaspar (Gaspar, Castilho, et al. 2010), and co-workers showed no differences in syntaxin-1 content in cultured hippocampal neurons exposed to high levels of glucose, which mimics hyperglycemic condition seen in pre diabetic and type 2 diabetes conditions.

Concerning synaptophysin, which regulates the assembly of the SNARE fusion complex and is also used as a marker for nerve terminals, we showed a decrease in the content of this protein at 4 weeks of high sucrose diet duration at hippocampal nerve terminals, indicating an effect of HSD at a short term. These results corroborate previous findings where synaptophysin content was decreased in hippocampal nerve terminals at short term of DM1 (Duarte et al. 2009) and at a long term in DM2 (Duarte et al. 2012). Changes in synaptophysin content occurred at 4 weeks but its levels returned to those seen in normal animals after 24 and 36 weeks of diet duration, which has already been reported in diabetic animals (Grillo et al. 2005). These results point to a recovery in protein levels at long term diet duration. Changes in the expression and distribution of synaptophysin due to diabetes have also been reported to occur with depletion and clustering of synaptic vesicles in hippocampal mossy fiber terminals (Magariños and McEwen 2000).

Taken together, present data suggest that decrease in SNAP-25 and synaptophysin content in hippocampal nerve terminals may result in synaptic degeneration, which could induce memory impairments associated to deficits in

synapse formation. Moreover, previous studies demonstrated that in spite of changes in synaptic proteins, animal models of DM2 show short and long term spatial memory deficits observed in Y-maze and Morris water maze tasks, showing impaired memory performance (Duarte et al. 2012; Soares et al. 2013).

In the present work, we assessed for the first time protein content of synapsin-1 and rabphilin-3a in an animal model of pre diabetes, induced by HSD. Increased levels of synapsin-1 and rabphilin-3a may indicate that they are acting as a counter-regulatory mechanism to compensate the decreased protein levels (SNAP-25 and synaptophysin) responsible for priming and fusion steps of synaptic vesicle exocytosis. Being synapsin-1 involved in trafficking of SVs to pre-synaptic membrane and rabphilin-3a in docking of SVs, these two mechanisms may be enhanced in animals drinking high sucrose diet in order to translocate more SVs to membrane.

### 4.3.2. HSD affects cortex protein synaptic expression

Cerebral cortex plays an important role in essential functions such as memory, attention, language and perception however, studies analyzing the effect of diabetes on cortex are limited. In this study we aimed to investigate if synaptic proteins content is affected by HSD in total extracts of cortex.

Synapsin-1 content decreased at 4 weeks of sucrose diet and increased at 24 weeks; however at 36 weeks of diet protein content was similar to controls. It seems that an increase in synapsin-1 at short term lead to a compensatory mechanism in which synapsin-1 levels increase, becoming this increase significant at long term. In the end of diet, synpasin-1 content is similar to control animals indicating a total recovery of protein levels in high sucrose animals.

Rabphilin-3a content was not significantly altered in cortex, although it is evident a tendency for increased values in this protein content at 24 weeks of HSD, nevertheless the SEM is too large due to discrepancy in values obtained in quantification, not allowing to see significant differences between groups.

Within synaptic proteins analyzed in SNARE complex, only SNAP-25 content was altered in cortex. SNAP-25 content is decreased at 4 weeks diet duration, suggesting impairment at a short term in this protein that is recovered at long term.

By data obtained in this experiment it is possible to conclude that some synaptic proteins content are altered in cortex, even though, effects seen in these proteins are

more pronounced at a short term of HSD and totally return to levels similar to those seen in normal animals suggesting that at the end of a 36 weeks HSD synaptic protein content do not changes.

As far as we are concerned, this is the first time synaptic proteins content is evaluated in a HSD animal model. Previous studies showed structural alterations in cerebral cortex with swelling of neurons (Hernández-Fonseca et al. 2009) in type 1 diabetic animals and that within cortex atrophy, the most affected area in type 2 diabetic patients is the temporal lobe (Heijer et al. 2003; Brundel et al. 2010), responsible for several functions including visual and storage memories and comprehensive language (Purves et al. 2004). Moreover, evaluation of synaptic density in an animal model of type 2 diabetes evidenced that synapses loss occurs in parallel with cortical atrophy (Ramos-Rodriguez et al. 2013).

It is important to highlight the fact that glucose intolerance in animals drinking HSD at 24 and 36 weeks may not be reproduced in the CNS, being this organ the last to be affected in diabetes. Despite both DM1 and DM2 are characterized by increased levels of plasma glucose (hyperglycemia), they have opposed levels of circulating insulin, that is, in DM1 there is hypoinsulinemia since insulin is less secreted due to pancreatic β cell failure. However, in pre diabetes it is seen a hyperinsulinemic condition in order to compensate from high glucose levels. These facts lead to the question of which mechanism, alterations in glycemia or insulinemia, is responsible for synaptic vesicles dysfunction, being possible that these two types do diabetes differently affect CNS.

# 5. Main conclusions and future directions

This thesis was divided in two main goals. The first goal was to evaluate the effect of NAC as a source of GSH in peripheral insulin sensitivity and the second goal aimed to study the effect of a HSD on cognition, particularly at the synapse level, evaluating content of synaptic proteins in hippocampus and cortex. Regarding effects of NAC in insulin sensitivity, we concluded that NAC when co-administrated with glucose increases insulin sensitivity, by promoting HISS release and action. We propose that this happens due to contribution of NAC to GSH synthesis, which is essential for an adequate action of HISS. Although these results give new insights in understanding mechanisms of glucose homeostasis it would be of great interest to perform complementary studies including evaluation of the effect of other amino acids as well as investigation of cellular and molecular basis of NAC action.

In the second part of this thesis we tested the hypothesis that a HSD induces changes in synaptic proteins, leading to alterations in cognition, which are told to occur in diabetic patients. The data obtained show that a long term HSD changes synaptic proteins content both in hippocampus and cortex, which may be implicated in cognitive problems associated with memory and learning. It was also shown that HSD leads to glucose intolerance in animals, which is thought to be indicative of an insulin resistance condition. However, as insulin was not measured in the present experiments, it would be interesting to measure insulin secretion and peripheral levels in future studies, to confirm that glucose intolerance in these animals is associated with hyperinsulinemia, which is known to happen as a counter-regulatory mechanism for hyperglycemia.
Regarding GLUT4 and GLUT12, as shown in this thesis, increased GLUT12 expression seems to act in order to compensate decreased GLUT4, however the exact molecular mechanism behind this counter-regulatory event remains unknown, being of large concern to be uncovered. Moreover, since these glucose transporters alterations are associated to glucose intolerance and is known that brain is affected by a sucrose diet, it would be appealing to evaluate their expression on brain, namely on hippocampus and cortex.

Structural and functional changes in the brain have been reported to contribute to cognitive problems. However, in the present work we did not evaluate the performance of animals in behavioral tasks such as water-maze and Y-maze task which would be important to perform in future experiments in order to understand if changes in synaptic proteins of a long term HSD are in fact leading to memory and

learning impairments. Besides, since memory and learning underlie mechanisms of synaptic plasticity, electrophysiological experiments could also contribute to understand if alterations in synaptic proteins content are affected at this level.

As mentioned before in this dissertation, alterations in glucose homeostasis at the periphery do not always translate in the same extent to central nervous system, being this one the last system affected in a condition of diabetes. For this reason, future experiments with a longer HSD could be appealing to understand its effects on synaptic proteins content and functionality. Moreover, these experiments could be performed together with behavioral ones to assess the effect of a long HSD in cognition.

About effects of HSD on central nervous system, namely on synaptic proteins content at hippocampus and cortex we unraveled interesting findings. Although previous studies have also revealed alterations in synaptic proteins, the present study is pioneer in evaluating these proteins in a model of pre diabetes induced by a diet rich in sucrose. The results obtained showed that cognitive impairments may be occurring, which could lead to memory and learning problems, being a first step in the understanding of cognitive impairments in type 2 diabetes, a public health concern.
As mentioned previously, it has been reported that temporal lobe is the most affected area in cortex of type 2 diabetic patients; however, it would be important to perform further studies in which synaptic proteins expression would be evaluated in different cortex regions, to understand which of them are mainly affected in a pre diabetic state, relating this alterations with different functions among cortex.

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