DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA

UNIVERSIDADE DE COIMBRA

Sucrose-induced memory deficits

Edna Filipa Pais Soares

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Dissertação apresentada à Faculdade de Ciências e Tecnologia da Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica do Professor Doutor Frederico Pereira (Universidade Coimbra)

Edna Filipa Pais Soares

2012

A duas estrelas que brilham lá no céu

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Abbreviations

AD	Alzheimer disease
AGE	Advanced glycation end products
ALE	Advanced lipooxidation endproduct
AMP	Adenosine monophosphate
AMPA	α-amino-3-hydroxy-5-methylisoxazole-propionate
AMPAR	α-amino-3-hydroxy-5-methylisoxazole-propionate receptor
ANOVA	Analysis of variance
ATP	Adenosine-5'-triphosphate
AUC	Area under the curve
Aβ	amyloid β
AβPP	Amyloid-β protein precursor
BBB	Blood-brain barrier
BSA	Bovine serum albumin
CA	Cornu Ammonis
ChREBP	Carbohydrate regulatory element-binding protein
CNS	Central nervous system
CREB	cAMP response element-binding
DAG	Diacylglycerol
DG	Dentate gyrus
DM	Diabetes mellitus
DNRAGE	Dominant negative RAGE
ECF	enhanced chemifluorescence
ER	endoplasmatic reticulum
ERK	Extracellular signal-regulated kinase
FoxO	Forkhead box O
FPG	Fasting plasma glucose
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
GFAT	Glutamine–fructose-6-phosphate amidotransferase
GluR	AMPA receptor subunit
GLUT	Glucose transporter
GS	Glutamine synthase

GSH	Reduced glutathione
GSK-3	Glycogen synthase kinase 3
GSSG	Oxidized glutathione
GTT	Glucose tolerance test
HbA1c	Glycated hemoglobin
HFa	High Fat
HFCS	High-fructose corn syrup
HFS	High-fat/refined sugar
HPN	Hepatic parasympathetic nerves
HNE	Hydroxynonenal
HOMA-IR	Momeostasis model assessment for insulin resistance
ICV	Intracerebroventricular
IDF	International diabetes federation
IF	Intermediate filament
IFG	Impaired fasting glucose
IGF	Insulin-like growth factor
IGF-1R	Insulin-like growth factor-1 receptor
IGT	Impaired glucose tolerance
IL	Interleukin
IR	Insulin receptor
IRS	Insulin receptor substract
IRS-1 pSer636/639	Insulin receptor substract-1 phosphorylated on serine (636/639) residues
IR-β	Insulin receptor β subunit
ITT	Insulin tolerance test
JNK	c-Jun N-terminal kinase
LTD	Long-term depression
LTP	Long-term potentiation
MAPK	Mitogen activated protein kinase
MEK	MAPK kinase
mTOR	Mammalian target of rapamycin
NADPH	Nicotinamide adenine dinucleotide phosphate in reduced form
NF-κB	Nuclear factor κ-B
NMDA	kainate and N-methyl-D-aspartate

NMDA	kainate and N-methyl-D-aspartate receptor
NR	NMDA receptor subunit
PBS-T	Phosphate buffer saline with 0.1 % Tween-20
PI3K	Phosphoinositide 3-Kinase
PKA	Protein kinase A
PKC	Protein kinase C
PSD-95	Post synaptic density protein
PVDF	Polyvinylidene difluoride
RAGE	Receptor for advanced glycation end products
ROS	Reactive oxygen species
S/T	Serine/threonine
SH2	Src Homology 2
SRAGE	Soluble RAGE
T1DM	Type 1 diabetes *mellitus*
T2DM	Type 2 diabetes *mellitus*
TG	Triglycerides
TNF-α	Tumor necrosis factor-α
UDP	Uridine diphosphate-N-acetylhexosamine
vGLUT	Vesicular glutamate transporter
VLDL	Very low density lipoproteins
WHO	World Health Organization
ZDF	Zucker diabetic fatty

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Publications

Nunes S, **Soares E**, Pereira F, Reis F. (2012). The role of infammation in diabetic cardiomyopathy. *International Journal of Interferon, Cytokin and Medical Research.* 4: 1–15

Soares E, Nunes S, Reis F, Pereira F. (2012). Diabetic encephalopathy: the role of oxidative stress and inflammation in Type 2 diabetes. . *International Journal of Interferon, Cytokin and Medical Research. (In Press).*

Resumo

A pré-diabetes tal como a diabetes tipo 2 - uma doença metabólica heterogénea com uma elevada taxa de prevalência - está associada a um risco acrescido de desenvolver distúrbios do sistema nervoso central. A "encefalopatia diabética" é caracterizada por alterações electrofisiológicas, estruturais, neuroquímicas e degenerativas que poderão originar défices cognitivos, constituindo uma complicação da diabetes relativamente pouco conhecida. O hipocampo, sendo uma estrutura cerebral fundamental nos processos de aprendizagem e memória, apresenta uma elevada susceptibilidade ao ambiente adverso da pré-diabetes/diabetes. O objetivo deste trabalho foi avaliar a memória e a aprendizagem bem como as alterações fenotípicas do hipocampo num modelo animal de resistência à insulina/pré-diabetes (induzido pela adição de sacarose à dieta). Ratos Wistar machos com 17 semanas de idade foram submetidos a uma dieta normal *ad libitum* e divididos em dois grupos: controlo (Cont) (água) e ratos submetidos à ingestão de um elevado teor de sacarose (HSu) (solução de sacarose a 35%). O consumo da solução de sacarose durante um período de 9 semanas resultou em normoglicemia em jejum acompanhada por uma hiperinsulinemia e hipertrigliceridemia num estado não-jejum e intolerância à glicose associada a resistência à insulina, relativamente ao grupo Cont. A sacarose produziu uma diminuição da memória espacial dependente do hipocampo (Y-Maze e Morris Water-Maze). Com o intuito de confirmar esta disfunção cognitiva, foi medida a expressão de várias proteínas por Western Blotting, incluindo marcadores da cascata de sinalização da insulina (IR-β, IRS-1, IRS-1 pSer$^{636/639}$, PI3K), marcadores de plasticidade sináptica (1-glutamatérgicos: GluR1, NR1, PSD-95; 2-maquinaria exocitótica: sinaptofisina) e marcadores de neurotoxicidade (GS, GFAP, RAGE, HNE, TNF-α). Os resultados obtidos demonstraram uma redução na expressão do IR-β nos ratos HSu em relação ao Cont. No entanto, não houve alteração dos níveis basais das restantes proteínas de sinalização (IRS-1 e PI3K). Simultaneamente, o hipocampo dos ratos HSu exibiu um aumento da expressão das subunidades GluR1 e NR1 dos recetores AMPA e NMDA, respetivamente. Isto sugere alterações na plasticidade sináptica induzidas pela sacarose. Finalmente, as alterações de memória não foram acompanhadas por um fenótipo neurotóxico. Em conclusão, neste estudo demonstrou-se que um consumo excessivo de sacarose durante 9 semanas resultou numa condição metabólica sugestiva de um estadio de pré-diabetes, e num comprometimento significativo da memória. Estes défices cognitivos foram acompanhados por alterações na expressão do recetor da insulina e nas sinapses glutamatérgicas.

Abstract

Both prediabetes and type 2 diabetes *mellitus* - a heterogeneous metabolic disorder with high prevalence rates - are associated with an increased risk for central nervous system disorders. “Diabetic encephalopathy” is a relatively unknown diabetes complication, characterized by electrophysiological, structural, neurochemical and degenerative neuronal changes which lead to cognitive functioning limitations. The hippocampus, a relevant brain region for learning and memory processes, presents a high degree of susceptibility to the adverse environment of diabetes. The purpose of this work was to characterize the cognitive function (learning and memory) along with hippocampal dysfunction in a sucrose-induced insulin resistance/prediabetes animal model. 17-weeks-old male Wistar rats were given free access to a standard chow and divided into two groups: control (Cont) (drinking water) and high-sucrose (HSu) (35 % sucrose solution). The consumption of a 35 % sucrose solution for an extended period of time (9 weeks) resulted in fasting normoglycemia accompanied by hyperinsulinemia and hypertriglyceridemia in a fed state, and insulin resistance associated with impaired glucose tolerance. The sucrose effect on rats learning and memory performance was assessed using the Morris Water-Maze and the Y-Maze tests, which convincingly showed that hippocampus-dependent spatial memory was dramatically impaired on the HSu rats. In order to corroborate the hippocampal dysfunction underlying this memory alterations, the expression of several proteins was determined by Western Blotting including insulin signaling pathway markers (IR-β, IRS-1, IRS-1 pSer$^{636/639}$, PI3K), synaptic plasticity markers (1-glutamatergic: GluR1, NR1, PSD-95; 2-exocytotic machinery markers: synaptophysin) and neurotoxicity markers (GS, GFAP, RAGE, HNE, TNF-α) .Herein it was found a reduction in IR-β expression for HSu rats compared to Cont. This was not accompanied by changes in basal levels of further insulin signaling molecules: IRS-1 and PI3K. The results also showed an upregulation of both AMPA and NMDA receptor subunits GluR1 and NR1, respectively, which were not accompanied by a neurotoxic phenotype. Overall, we found that 9 weeks of sucrose consumption resulted in a metabolic condition suggestive of a prediabetic state, which translated into a deficit of rats’ memory performance, accompanied by altered insulin receptor expression as well as altered glutamatergic synapses.

I. Introduction

1. Diabetes *Mellitus*

The World Health Organization (WHO) has defined, in 1985, diabetes *mellitus* (DM) as a complex and heterogeneous metabolic disorder, which is mainly characterized by an abnormal raise in blood glucose levels – hyperglycemia (WHO, 1985). This occurs when 1) pancreatic β-cells fail to secrete sufficient amount of insulin to maintain normoglycemia and/or when 2) there is a resistance to insulin action (Rossetti *et al*., 1990; Saltiel, 2001).

DM is a multifactorial disease in which multiple genetic and environmental factors contribute to its onset and progression. The concomitant increase in life expectancy, obesity, sedentary lifestyle, hypertension and hyperlipidemia might raise the chance of citizens from both developed and developing countries of having glucose intolerance. This suggests that diabetes is certainly one of the most challenging health problems in the 21st century (Tuomi, 2005).

According to the WHO and to the International Diabetes Federation (IDF) there are three main types of diabetes, as follows: type 1 diabetes (T1DM) - also called insulin-dependent or juvenile diabetes it is caused by an auto-immune reaction where the body's defense system attacks the pancreatic β-cells leading to an insulin deficiency state; type 2 diabetes (T2DM) - also referred to as non-insulin dependent or adult-onset diabetes - it is characterized by insulin resistance and relative insulin deficiency; and, gestational diabetes, which consists of high blood glucose levels during pregnancy (WHO, 1998). T1DM represents only 5-10 % of total diabetic people, and appears to be caused by a genetic predisposition, while T2DM affects about 90-95 %, and emerges as a combination of metabolic changes, including a prediabetic state and a metabolic syndrome (Figure 3) (Saltiel, 2001; Tuomi, 2005).

DM is tightly associated with a reduced life expectancy, diminished quality of life and significant morbidity, as a result of its specific complications (WHO, 1998). These complications are commonly divided into: macrovascular such as coronary artery disease, peripheral arterial disease and stroke; and microvascular such as diabetic nephropathy, neuropathy and retinopathy (Stratton *et al*., 2000). There is accumulating evidence identifying the brain as a site of T2DM damage (Gold *et al*., 2007; Bruehl *et al*., 2009). In fact central nervous system (CNS) lesions, partly independent of atherosclerotic disease, can be referred to as "diabetic encephalopathy" (Mijnhout *et al*., 2006; Sima, 2010).

1.1. Prevalence

DM prevalence rates have risen markedly in recent years; therefore, it is reaching pandemic proportions on a global scale (Wild *et al*., 2004; Hossain *et al*., 2007). In fact, IDF has considered this pathology as one of the most common worldwide, affecting about 366 million people at present (Figure 1) and with an estimated increase to 552 million by 2030, thus representing 9.9 % of the adult population (IDF, 2011).

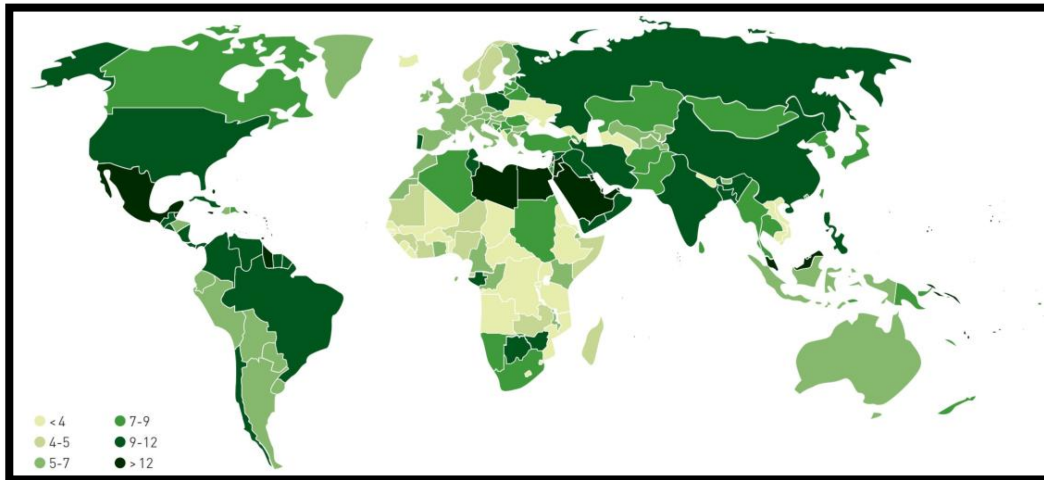


Figure 1: Global prevalence (%) of diabetes *mellitus* in adult population (20-79 years) in 2011, shown by geographic region (Image taken from IDF Diabetes Atlas, 5th Edition (IDF, 2011)).

Recent estimates for 2010 from the Portuguese Society of Diabetology indicate that 12.4% of portuguese adult population is diabetic, from which 5.4 % were undiagnosed (OND, 2012). Additionally, IDF foresees that, globally, half of those who have diabetes are unaware of their condition, mostly on low- and middle-income countries (IDF, 2011).

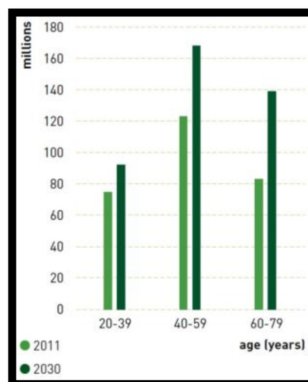


Figure 2: Number of people with impaired glucose tolerance by age group (20-79 years) in 2011 and projections for 2030 (Image taken from IDF Diabetes Atlas, 5th Edition (IDF, 2011)).

The importance of age on the prevalence of diabetes is very clear. In 2011, the greatest number of people with diabetes was in the 40- to 59-year age group, and the projections for 2030 suggest that this pattern will remain (Figure 2) (IDF, 2011). Most of these people live in developing countries, while in developed ones the majority of diabetic subjects are older than 64 years of age (Wild *et al.*, 2004).

Diabetes is one of the leading causes of illness and premature death, with 4.6 million deaths in 2011 (IDF, 2011), and it continues to disproportionately affect the socially disadvantaged (Wild *et al.*, 2004; Hossain *et al.*, 2007).

2. Prediabetes and Type 2 diabetes

2.1. Pathophysiology

Most type 2 diabetic people have shown a multiple set of risk factors that commonly appear together, depicting what is now known as the “Metabolic Syndrome” (Figure 3). Such syndrome includes insulin resistance and raised fasting plasma glucose, abdominal obesity, high cholesterol and high blood pressure. All this features characterize a pre-diabetic state, when people are prone to diabetes. Indeed, people with metabolic syndrome have a fivefold greater risk to develop T2DM (Cefalu, 2006; Eckel, 2007).

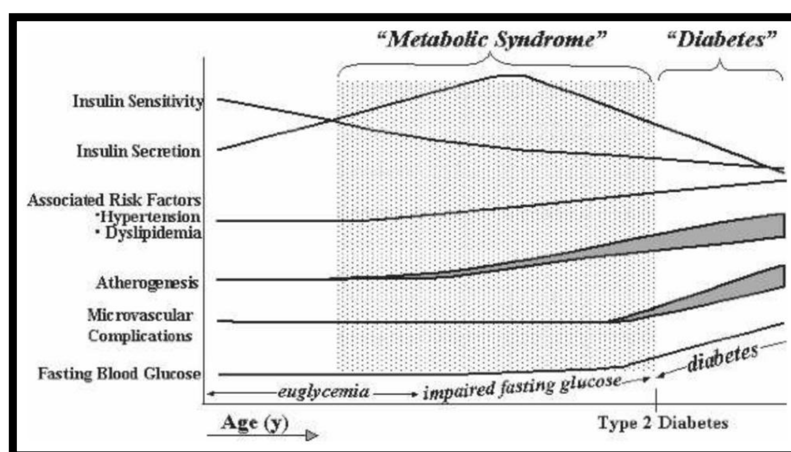


Figure 3: Schematic representation of clinical and laboratory findings in the natural history of type 2 diabetes, reflecting the importance of metabolic syndrome in the genesis of the disease. The shaded area signifies the presence of the metabolic syndrome (Image taken from Cefalu, 2006).

Pancreatic β-cell dysfunctions followed by the resistance of target tissues to insulin, usually associated with abnormal insulin secretion, are the major pathophysiological events

contributing to the development of T2DM. Interplay between genes, aging and metabolic changes produced by obesity could contribute to the onset of T2DM (Saltiel, 2001).

Clinically, insulin resistance implies that higher than normal concentrations of insulin are required to maintain normoglycemia. This can arise from defects in insulin signal transduction, changes in the expression of proteins or genes that are targets of insulin action. A cross talk between other hormonal systems and metabolic abnormalities can also contribute to insulin resistance (Saltiel, 2001). Thus, it is defined as a patophysiological condition where insulin is unable or less effective to promote insulin signaling through insulin receptor (IR), thus compromising downstream multiple metabolic and mitogenic aspects of cellular function (Saini, 2010). This in turn leads to an increased insulin release and consequently inadequate secretion. Elevated fasting glucose levels, as an insulin resistance consequence, can then ultimately lead to T2DM diagnosis. This insulin-resistance phase is now envisioned as a prediabetic state, being mainly characterized by two conditions: impaired fasting glucose (IFG) and/or impaired glucose tolerance (IGT) (Tabák *et al*., 2012). In fact, diagnostic criteria for prediabetes include either IGT with 2 h plasma glucose ≥140 mg/dL (7.8 mmol/L) but <200 mg/dL (11.1 mmol/L) and/or IFG with fasting plasma glucose (FPG) ≥100 mg/dL (5.6 mmol/L) but <126 mg/dL (7.0 mmol/L) (ADA, 2004).

2.2. Central Nervous System

CNS has the ability to gather information about the body's nutritional state and implement appropriate metabolic and behavioral responses to alterations in fuel availability. This feedback signaling mechanism ensures the maintenance of energy homeostasis, body fat content and glucose metabolism (Jordan *et al.*, 2010).

DM has been related to CNS lesions that can be referred to as "diabetic encephalopathy" (Mijnhout *et al*., 2006; Sima, 2010). This relatively unknown DM complication is characterized by structural, electrophysiological, neurochemical and degenerative neuronal changes, and seems to be related with cerebral atrophy, Alzheimer disease (AD) as well as depressive behaviors. Diabetic encephalopathy along with chronic hyperglycemia and dyslipidemia compose the most relevant risk factors for cognitive dysfunction (Biessels *et al*., 2002; Ristow, 2004; Mijnhout *et al*., 2006; Hernández-Fonseca *et al*., 2009). This may be the consequence of the combination between a disruption of insulin activity and a defective glucose metabolism (Ristow, 2004; van der Heide *et al*., 2006).

Indeed, several studies have shown an increased incidence of dementia in T2DM subjects (Gispen and Biessels, 2000; McNay and Recknagel, 2011; Ott *et al*., 1999; Biessels *et*

al., 2006). Furthermore, brain morphological alterations could be related with abnormal synaptic plasticity and cognitive impairments observed in experimental diabetes (Hernández-Fonseca *et al*., 2009).

Neurons and pancreatic β-cells share several common features, such as high metabolic activity and low regeneration rates, being extremely susceptible to environment and genetic effects. Therefore, it is plausible that peripheral and central DM consequences also share patophysiological mechanisms, although temporarily lagged (Ristow, 2004). However it is not clear, from a wide range of DM comorbidities such as hyperglycemia, hyperinsulinemia, impaired insulin signaling and associated innate inflammation, which mechanisms may play the most significant role.

3. Cognitive dysfunction

The relationship between DM and cognitive dysfunction was already proposed in 1922 by Miles and Root (Miles and Root, 1922). There is an increasing body of neuropsychological studies showing brain structural changes and behavior deficits (Strachan *et al*., 1997; Biessels *et al*., 2002; Winocur *et al*., 2005; Reijmer *et al*., 2010), such as cognitive decrements (Gispen and Biessels, 2000; Reijmer *et al*., 2010) as well as learning and memory impairments (Ristow, 2004), therefore confirming this early proposal.

Large population studies detected an association between DM, dementia, and AD (Leibson *et al*., 1997; Ott *et al*., 2002; Peila *et al*., 2002; Anderson *et al*., 2001). Furthermore, Ott *et al*. (1999) found that the risk of dementia is nearly doubled in diabetic subjects, an effect that cannot be accounted only by vascular factor, following a population of over 6000 subjects for up to 6 years. Brain structural changes underlying cognitive deficits are observed especially in older diabetic adults (Stolk *et al*., 2007a; Hayashi *et al*., 2011) who apparently face a greater risk of vascular dementia (Reijmer *et al*., 2010). Along these lines, *in vivo* brain imaging studies (Araki *et al*., 1994; Manschot *et al*., 2006) have reported “accelerated brain ageing” in diabetic patients as well as in animal models (Biessels *et al*., 2002; Kamal *et al*., 2003). However, others have recently shown that cognitive impairments are already present among obese adolescents with T2DM (Bruehl *et al*., 2009). Assuming that prospective studies assessing the neurological effects of diabetes in humans can be difficult to carry out in a controlled setting, the need for animal models is fundamental. However, investigation of the cognitive profile of diabetic animal models is extremely limited. Indeed, behavioral studies in diabetic rodents have produced conflicting results (Li *et al*., 2002; Bélanger *et al*., 2004),

possibly because of the differences in used animal models and in the duration of diabetes (Gispen and Biessels, 2000). Furthermore, the differences in cognitive profile observed for both type 1 and type 2 DM suggest that CNS insulin signaling (see section 5.1) has a significant role in these complications (Brands *et al*., 2004). T1DM animal models are useful for studying the effects of chronic hyperglycemia, but its endocrinological features do not adequately reflect either type 1 or type 2 diabetes (Gispen and Biessels, 2000), suggesting that T2DM and all its pathological features are useful for understanding the origins of cognitive deficits in humans with similar abnormalities (Winocur *et al*., 2005). Interestingly, very recently it was demonstrated that normal adults with newly diagnosed pre-diabetes or T2DM showed insulin resistance associated with reductions in regional cerebral glucose metabolism and subtle cognitive impairments (Baker *et al*., 2011).

3.1. Hippocampus

The hippocampus is located in the medial temporal lobe, underneath the cortical surface. This brain structure has received a great deal of attention due to its essential role for cognitive functions such as learning and memory processes (Zhao *et al*., 1999; Broadbent *et al*., 2004; Neves *et al*., 2008; Sanderson *et al*., 2008). As shown in figure 4, the hippocampus is subdivided into four subregions called Cornu Ammonis (CA) areas, which are the CA4, CA3, CA2 and CA1. Adjacent to the CA lies the dentate gyrus (DG), which is divided into the fascia dentata and the hilus. The neurons in the CA4 region do not have pyramidal morphology like those in the other CA subregions. Thus, many neuroanatomists do not recognize the CA4 as a separate region, but instead consider it as part of the hilus.

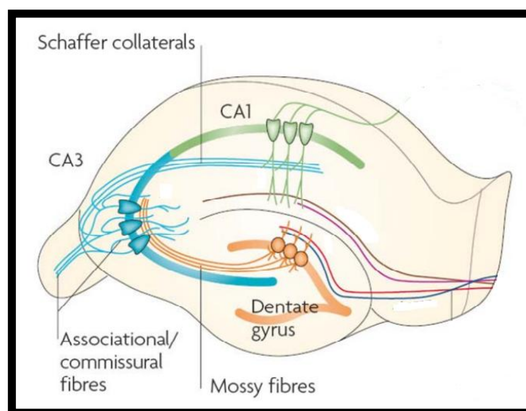


Figure 4: Anatomy of the hippocampus (adapted from Neves *et al*., 2008).

It was recently recognized that this region is insulin-sensitive, and thus presenting a high degree of susceptibility to the adverse environment of diabetes (Zhao *et al*., 2004; Winocur *et al*., 2005; Risk *et al*., 2006; Gold *et al*., 2007; Anarkooli *et al*., 2008; Brhuel *et al*., 2009; McNay and Recknagel, 2011). In fact, memory seems to be the most reliable altered cognitive function in T2DM (Strachan *et al*., 1997; Winocur *et al*., 2005; Gold *et al*., 2007). Furthermore, according to Gold *et al*. (2007), hippocampal damage associated with memory impairments are most probably the early brain complications of this pathology. However, there is little information on the hippocampal function underlying cognitive impairment seen in pre-diabetes in human subjects. Recent studies have found both specific verbal memory impairments and specifically hippocampal volume reductions among individuals with T2DM (den Heijer *et al*., 2003 Gold *et al*., 2007; Bruehl *et al*., 2009; Sima, 2010). Additionally, cognitive deficits related with hippocampal dependent tasks tend to be more prevalent in T2DM subjects and as the disease progresses and/or the patient ages, deficits are also seen in tasks mediated by other brain regions (McNay and Recknagel, 2011). A likely explanation for hippocampal dysfunction/neurodegeneration could be the interplay between defective insulin signaling (see section 5.) and aberrant glucose handling (see section 6.).

4. T2DM and Synaptic plasticity

In T2DM animal models, impairments of spatial learning occur in association with distinct changes in hippocampal synaptic plasticity (Gispen and Biessels, 2000). Neuronal plasticity is the critical capacity to compensate for challenges, involving cellular and molecular mechanisms of synapse formation and function, neurite growth and behavioral adaptation (Molteni *et al*., 2002).

Several reports have demonstrated that DM might impair synaptic structure and function in hippocampus at both presynaptic (Grillo *et al*., 2005; Baptista *et al*., 2010; Gaspar *et al*., 2010) and postsynaptic levels (Biessels *et al*., 1996; Kamal *et al*., 1999), which can somehow underlie the development of cognitive deficits and the increased risk of depression and dementia (Biessels *et al*., 1996; Stranahan *et al*., 2008). Furthermore, changes in the hippocampal presynaptic content of exocytotic proteins (e.g. synaptophysin, SNAP25), and receptors (e.g. adenosine ones) involved in neuromodulation and depletion of glutamatergic synaptic vesicles from hippocampal nerve terminals have been detected in diabetic models (Magarinos and McEwen, 2000; Trudeau *et al*., 2004; Gaspar *et al*., 2010; Duarte *et al*., 2012).

Additionally it was proposed that the glutamate neurotransmission, which is a highly regulated process, is affected by DM. This would negatively impact synaptic transmission and contribute to the development of cognitive impairments (Trudeau *et al*., 2004).

Regulation of glutamate receptor properties can contribute to long-term potentiation (LTP) and long-term depression (LTD) (see sections 4.2 and 4.3) and consequently to learning and memory processes. In fact, there is a growing body of evidence showing that abnormal regulation of glutamatergic receptors appears to play an important role in diabetes-induced impairment in synaptic plasticity and may therefore contribute to the development of cognitive defects in diabetic patients (Valastro *et al*., 2002; Trudeau *et al*., 2004; Santiago *et al*., 2009; Gaspar *et al*., 2010; Duarte *et al*., 2012).

4.1. Glutamatergic synaptic transmission

Glutamate is the major excitatory neurotransmitter in mammalian CNS. After release from the presynaptic button, glutamate crosses the synaptic cleft and can activate both ionotropic and metabotrobic receptors. Only recently it has become clear that glutamate is packed by vesicular glutamate transporter (VGLUT) into vesicles for subsequent release during fast synaptic transmission. To date, 3 different subtypes (VGLUT1, VGLUT2, and VGLUT3) have been molecularly identified and functionally characterized (Bellocchio *et al*. 2000; Fremeau *et al.* 2001, 2002; Takamori *et al*. 2001, 2002; Herzog *et al*. 2004). It has been proposed that VGLUT levels are critical for the balance between excitation and inhibition (Cline 2005; Erickson *et al*. 2006). Specifically, variations in VGLUT1 level critically affect the efficacy of glutamatergic synaptic transmission (Balschun *et al*. 2009). These authors demonstrated that VGLUT1-deficient mice had a reduced ability to express LTP and exhibited a specific deficit in spatial reversal learning in the water maze. However, some authors have shown that early transient changes in the content of vesicular transporters, if anything, translate into a slight increase in the basal release of glutamate after eight weeks of DM (Baptista *et al*., 2010; Gaspar *et al*., 2010). The accumulation of glutamate in the synaptic cleft can lead to excitotoxic neuronal damage due to excessive activation of glutamate receptors (Trudeau *et al*., 2004; Dong *et al*., 2009). Moreover, excessive activation of glutamate receptors is a characteristic feature of brain damage during stroke and ischemia, conditions exacerbated by hyperglycemic states (Li *et al*., 2000). This is of relevance to diabetic encephalopathy.

Glutamate receptors arise as an object of particular attention since their regulation appears to be crucial for controlling synaptic operation during learning and memory (Trudeau et al., 2004). They are divided into two main groups: the fast-acting ligand-gated ionotropic

channels and the slower-acting metabotropic receptors. The ionotropic receptors are cation-specific ion channels, which mediate the flux of Na^{+}, K^{+} and Ca^{2+} ions when activated by glutamate and are subdivided into three groups: α-amino-3-hydroxy-5-methylisoxazole-propionate (AMPA), kainate and N-methyl-D-aspartate (NMDA) receptors (Ozawa *et al*., 1998). AMPA receptors (AMPARs) are responsible for the primary depolarization in glutamate-mediated neurotransmission and along with NMDA receptors (NMDARs) play key roles in synaptic plasticity, including LTP and LTD (Dingledine *et al*., 1999; Santos *et al*., 2008) and disease (Lau and Tymianski, 2010).

AMPAR are activated by a glutamate-induced conformational change such that the ion channel opens allowing the flux of ionic current, and are responsible for the primary depolarization in glutamate-mediated neurotransmission Long-lasting and activity-dependent changes in synaptic strength (LTP or LTD) are associated with changes in the phosphorylation and cellular distribution of AMPAR, and are thought to underlie learning and memory formation (Whitlock *et al*., 2006; Santos *et al*., 2008). If the time of glutamate exposure is prolonged, the persistent reactivation of the receptor results in channel closure known as desensitization.

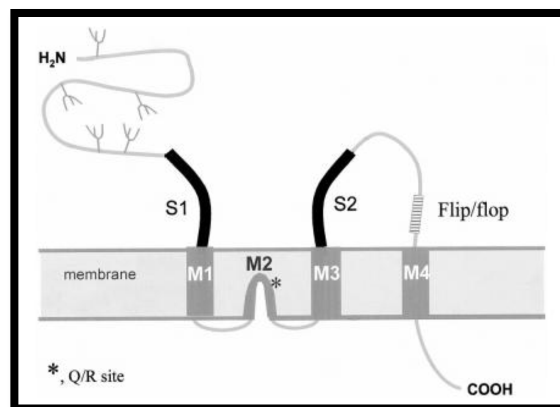


Figure 5: Structure of AMPA receptor subunits. The transmembrane topology is shown along with the flip/flop alternatively spliced exon, and the two ligand-binding domains (S1 and S2). Glycosylation sites are shown as trees in the N-terminal region (Image taken from Dingledine *et al*., 1999).

AMPAR are expressed as a combination of four subunits: GluR1-4. These subunits combine in tetramers in different stoichiometries and directly influence a variety of properties of the ion channel itself (i.e. desensitization/resensitization kinetics and conductance properties) as well as the receptors localization and trafficking to the synapses (Ozawa *et al*., 1998; Dingledine *et al*., 1999; Santos et al., 2008). Several studies reviewed by Santos *et al*. (2008) showed a widespread distribution of AMPARs in the brain, as expected from their key role in excitatory neurotransmission. GluR1–GluR3 subunits are enriched in the outer layers of

the cerebral cortex, hippocampus, olfactory regions, basal ganglia, lateral septum and amygdale and although regarded as neuronal receptors, they have also been detected in glial cells (Santos *et al*., 2008). Specifically, synaptic insertion of GluR1 has two functions: the established role of increasing synaptic strength via its ligand-gated ion channel, and a novel role through the structurally stabilizing effect of its C-terminus that permits an increase in spine size (Kopec *et al*., 2007). Accordingly, hippocampal LTP involves phosphorylation of GluR1 subunit and its delivery to synapse, whereas LTD is the result of dephosphorylation and endocytosis of GluR1 containing AMPAR (Din *et al*., 2010).

These receptors are highly mobile proteins that undergo constitutive and activity-dependent translocation to, and removal from, synapses. There are multiple routes for AMPARs trafficking and their respective synaptic contribution depends on the precise subunit composition and specific signaling cues. Briefly, the pre-synaptic AMPAR localization involves AMPAR mRNA translation on the rough endoplasmic reticulum (ER), subsequent exportation from the ER and trafficking to the Golgi and further packaging in cytosolic vesicles to be translocated to the synaptic terminal region (Dingledine *et al*., 1999). This pre-synaptic localization points to a role of these receptors in the modulation of pre-synaptic function (Schenk and Matteoli, 2004).

AMPAR are directly inserted into the postsynaptic density following its trafficking via the cytoskeleton (Gerges *et al*., 2006). Post-synaptic AMPAR, mediating the most fast excitatory synaptic transmission, are crucial for many aspects of brain function, including learning, memory and cognition. Furthermore, aberrant AMPAR trafficking is implicated in neurodegenerative diseases (Henley et al., 2012). The phosphorylation status of AMPARs is a fundamental determinant of their trafficking and function. In fact, the interplay between phosphorylation and dephosphorylation is determinant for controlling AMPAR surface expression and endocytosis. Although multiple kinases and phosphatases are involved, a working model is that activity-dependent phosphorylation of GluR1 delivers AMPARs to synapses in LTP, whereas GluR1 dephosphorylation is a signal for internalization and LTD (Henley *et al*., 2012).

It was proposed stargazin - a transmembrane AMPAR regulatory protein (TARP) that regulates AMPAR trafficking, surface expression and channel kinetic are able to stabilize synaptic AMPARs through directly interaction with postsynaptic density-95 (PSD-95) protein via a C-terminal PDZ-binding domain (a common structural domain found in the signaling proteins) (Dingledine *et al*., 1999; Santos *et al*., 2008; Henley *et al*., 2012). Additionally, removal of GluR1, GluR2 and GluR3 from the surface eliminates all AMPAR currents, but does not alter NMDAR currents or dendritic morphology (Santos *et al*., 2008; Henley *et al*., 2012).

NMDARs are heterodimers composed of NR1, NR2 and NR3 subunits. The NR2 subunit contains the glutamate-binding domain. All functional NMDARs are heteromultimeric complexes of the NR1 subunit in combination with at least one of the four NR2 subunits (A–D). The surface distribution of NMDA receptors can be classified into three categories: synaptic, perisynaptic and extrasynaptic. The synaptic NMDAR pool is defined by its association with the PSD and it is activated by synaptically released glutamate. Interestingly, a small portion of synaptic NMDAR signaling is dependent on synaptically released glutamate from adjacent synapses in hippocampal pyramidal neurons (Dingledine *et al.*, 1999). Extrasynaptic NMDARs are probably not activated by synaptically released glutamate under physiological conditions but may be activated by glutamate derived from other sources. The results of several reports suggest that the localization of NMDA receptors in the synaptic pool is regulated by NMDAR interaction with PDZ-binding domain, especially PSD-95 (Li *et al.*, 2002; Li *et al.*, 2003; Lim *et al.*, 2003).

Trudeau *et al*. (2004) suggested that an up-regulation of hippocampal NMDARs is associated with early stages of the disease. Indeed, diabetes studies performed in the rat hippocampus have shown that T1DM could induce an up-regulation of glutamate NMDAR and AMPAR, and, therefore, evoke LTP defects (Valastro *et al.*, 2002; Santiago *et al.*, 2009). These mechanisms could underlie the neurological complications within the brain of DM patients. Overactivation of glutamatergic receptors can cause cell damage by increasing intracellular Ca^{2+} concentration in neurons, thereby leading to the generation of free radicals and activation of proteases, phospholipases and endonucleases as well as transcriptional activation of specific cell death programs.

4.2. Long-term potentiation

Several reports have demonstrated the occurrence of LTP, which is an electrophysiological model of synaptic plasticity, *in vivo* following behavioral learning and memory tasks (Martin *et al.*, 2000; Whitlock *et al.*, 2006). The process of LTP is composed of two phases: induction or early LTP and maintenance or late LTP. These phases correspond to behavioral memory, which also has two components: short-term memory and long-term memory (Lynch, 2004).

During LTP induction, glutamate is released from the presynaptic terminal and activates AMPARs and NMDARs on the postsynaptic terminal. Moreover, several signaling pathways are involved in LTP induction including, cAMP-dependent protein kinase A (PKA) (Esteban *et al.*, 2003), protein kinase C (PKC) (Boehm *et al.*, 2006), extracellular signal-regulated kinase 1/2

(ERK 1/2) in the MAPK cascade (Atkins *et al*., 1998), PI3K (Opazo *et al*., 2003) and tyrosine kinase Src (Hayashi and Huganir, 2004).

Increase in synaptic AMPAR function through changes in their number, composition and/or properties result in the LTP of synaptic efficacy. On LTP induction, AMPARs undergo PKA-dependent insertion at perisynaptic sites, are stabilized in the membrane by actin polymerization and are then translocated to the synapse for full expression of LTP (Henley *et al*., 2011). For example, the delivery of AMPAR subunit GluR1 to the synaptic membrane was demonstrated to play a role in LTP. While originally thought to be less dynamic than their AMPAR counterparts, increasing evidence demonstrates that NMDARs themselves can be regulated in an activity-dependent manner, and both LTP and LTD of NMDAR-mediated transmission have been reported in several brain areas, including hippocampus (Hunt and Castillo, 2012).

Numerous studies strongly suggest that dysfunction in hippocampal LTP is related to the regulation of glutamate receptor properties, being associated with diabetic conditions (Di Mario *et al*., 1995; Biessels *et al*., 1996). In fact, and based on numerous electrophysiological experiments, DM-induced brain abnormalities and behaviour alterations could be derived from defects in expression of LTP in hippocampal slices (Biessels *et al*., 2002).

4.3. Long-term depression

The amount and kinetics of calcium influx largely determines the direction of plasticity, with large and rapid influxes contributing to LTP and conversely, smaller prolonged influxes leading to LTD (Sjostrom and Nelson, 2002). Accordingly, LTD is a lasting decrease in synaptic effectiveness that follows some types of electrical stimulation in the hippocampus. Two broad types of LTD may be distinguished: heterosynaptic LTD, occurring at inactive synapses, normally during high-frequency stimulation of a converging synaptic input; and homosynaptic LTD, which occurs at activated synapses, normally at low frequencies (Bear and Abraham, 1996).

Conversely to what happens with LTP, synaptically evoked LTD is accompanied by a loss of surface AMPARs (Henley *et al*., 2012). AMPAR endocytosis is thought to be important in the expression of LTD triggered by NMDAR activation. In fact, Beattie *et al*., (2000) have demonstrated that NMDAR activation can cause endocytosis of AMPARs through signaling mechanisms implicated in NMDAR-dependent LTD, specifically calcium influx and calcineurin activity. However, surprisingly, the actions of insulin on AMPAR trafficking seem to be mediated by an independent signaling mechanism, indicating that AMPAR endocytosis can be

triggered by multiple signaling pathways, only some of which are activated during LTD (Beattie et al., 2000).

While most studies have focused on the role of LTP in behavior, far less is known about the role of LTD. Hippocampal LTD is thought to contribute to memory loss. However, recent studies have found that hippocampal LTD may instead contribute to spatial memory formation, by weakening previously encoded memory traces when new information is learned (Nicholls *et al*., 2008; Ge *et al*., 2010).

It is interesting to note that the facilitation of LTD and the impairment of LTP are common diabetes consequences (Gispen and Biessels, 2000). For example, LTP expression was impaired in the CA1 and the CA3 field of the hippocampus of young adult T1DM rats, whereas LTD expression was enhanced in the CA1 field (Biessels *et al*., 1996; Kamal *et al*., 1999).

5. Insulin: a peripheral hormone operative in the brain

Insulin is one of the most important anabolic hormones identified to date. It is produced by the pancreatic β-cells and its primary biological effect is to maintain the glucose levels in the physiological range; however, it also stimulates lipogenesis, protein synthesis and has been found to promote cell division and growth through its mitogenic characteristics in several cell types. In vertebrates, insulin represents a super-family of structurally related proteins, including insulin-like growth factor-1 (IGF-1), IGF-2 and relaxin (Shabanpoor *et al*., 2009).

The initial and most prevalent phase of T2DM is characterized by an important increase of insulin secretion in order to maintain homeostasis within the organism. Although the brain was described for a long time as an insulin-insensitive organ, early as 1978, Havrankova *et al*. (1978) localized the IR in the CNS by ligand autoradiography. Nowadays it is widely recognized that both insulin and IR are widely distributed in various regions of the developing and adult brain with highest concentrations in the olfactory bulb, hypothalamus, cerebral cortex, cerebellum and hippocampus. Additionally, IRs are expressed by both neurons and astrocytes (Havrankova *et al*., 1978; Plum *et al*., 2005). The co-localization of insulin, IR and glucose transporters 1 and 4 (GLUT1 and GLUT4) in selective cerebral regions, namely in hippocampus, suggested that cerebral glucose metabolism is regulated by insulin signaling pathways (Zhao *et al*., 1999; Shulingkamp *et al*., 2000; MacNay and Recknagel, 2011). Furthermore, insulin has been shown to cause translocation of GLUT4 to the cell surface of hippocampal neurons. Insulin and IGF-1 also play other important and multifaceted roles in the brain including neurotrophic, neuromodulatory and neuroendocrine (Shulingkamp *et al*., 2000; van der Heide

et al., 2006; Cardoso *et al*., 2009; Huang et al., 2010; Sima, 2010; Ketterer *et al*., 2011) as well as in pathological functioning related to neurodegenerative diseases and memory and learning impairments induced by DM (Cardoso *et al*., 2009; de la Monte, 2009; Sima, 2010; McNay and Recknagel, 2011). Specifically, insulin and IGF-1 are involved in neuronal survival and facilitation of synaptic plasticity through the regulation of energy metabolism, oxidative stress, gene regulation of other neurotrophic factors and their receptors, cholinergic gene expression, expression and phosphorylation of neuroskeletal proteins including tau and regulation of β amyloid. Furthermore these peptides have anti-inflammatory and anti-apoptotic effects (see section 9) (Xing *et al*., 2007; Chiu and Cline, 2010).

Brain insulin derives primarily from pancreatic β-cells secretion and it is subsequently transported across the blood-brain barrier (BBB) by a saturable, insulin receptor-mediated transport process (Banks *et al*., 1997). This is confirmed by the fact that there is correspondence between a raise in peripheral insulin levels and cerebrospinal fluid insulin content (Wallum *et al*., 1987). However, it was reported that less than 1 % of the peripherally administered insulin reaches the CNS in dogs, due to the saturable character of its transport through BBB (Woods and Porte, 1977). Although brain insulin's pancreatic origin, a small proportion of insulin might be produced in the brain, specifically in hippocampal pyramidal neurons (Schechter and Abboud, 2001; Kuwabara *et al*., 2011). In fact, previous studies showed the existence of pre-proinsulin I and II mRNA and insulin immunoreactivity in neurons confirming the idea that insulin can be synthesized *de novo* in the brain (Schechter *et al*., 1996; Zhao *et al*., 1999). The evidence for significant synthesis of insulin within the adult mammalian brain is now convincing to the point of being overwhelming (McNay and Recknagel, 2011).

There is clear evidence that systemic insulin resistance is accompanied by central insulin resistance, and that such impaired insulin signaling is the causal mechanism underlying cognitive impairments. Some authors suggest that cognitive deficits observed as a consequence of T2DM are likely due in large part to impaired central insulin modulation of cognitive and metabolic processes in the hippocampus, and that insulin signaling is a critical component of, for example, memory processing (McNay and Recknagel, 2011). Furthermore, impairments in the insulin signaling pathway in the brain have been implicated in Alzheimer's disease and aging besides T2DM induced cognitive deficits (Gispen and Biessels, 2000; van der Heide *et al*., 2006; Cardoso *et al*., 2009). Moreover, recent work has conclusively shown that endogenous intrahippocampal insulin is a key component of memory processes, and that acute blockade of hippocampal insulin signaling produces profound cognitive deficits, supporting the physiological role of insulin as a hippocampal modulator (McNay *et al*., 2010). Since hyperinsulinemia seen in T2DM may result in insulin resistance either by negative

regulation of the insulin receptor or its further desensitization beyond a reduction of the insulin transport into the brain. Both hyperinsulinemia as well as hypoinsulinemia have been related to the decrease in cognitive functions and AD in aging brain (Gispen and Biessels, 2000; Sheen, 2010).

5.1. Insulin/IGF Signaling

Important pathways in the regulation of synaptic plasticity and memory formation overlap with the insulin signaling pathway; therefore, it is not surprising that insulin has profound effects on information storage and synaptic physiology (van der Heide *et al*., 2006; McNay *et al*., 2010; Costello *et al*., 2012). Like other hormones, insulin binds to specific cell receptors in CNS, thereby exerting its effects through the activation of several signaling pathways: phosphatidylinositol-3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) are the most relevant ones involved in learning and memory processes (Figure 8) (Zhao *et al.*, 2004; van der Heide, 2006; Cardoso *et al*., 2009). These intracellular signaling cascades will ultimately lead to changes in glucose transport, glycogen and lipid synthesis and specific gene expression. In addition to insulin, IGFs also modulate neuronal growth, survival, differentiation, migration, metabolism, gene expression, protein synthesis, cytoskeletal assembly, synapse formation and plasticity (D'Ércole *et al*., 1996). Specifically, IGF-1 receptor (IGF-1R) signaling is important in regulating the proliferation of progenitor cells during prenatal development and the survival of mature neural cells postnatally (Liu *et al*., 2009) and it functions as a survival factor in the CNS to inhibit neuronal death during aging (Sun, 2006). Additionally, there is a regulatory function of IGF-1 on synaptic excitability in hippocampal neurons, through MAPK signaling mechanism (Xing *et al*., 2007). A recent paper suggests that IGF-2 may also play an important role in hippocampal memory processes (Chen *et al*., 2011).

5.1.1. Insulin and IGF-1 receptors

The IR are widely expressed throughout the brain, with higher density in cerebral cortex, hypothalamus and hippocampus, where they perform several functions such as on glucose metabolism, food consumption regulation and on learning and memory formation (Shulingkamp *et al*., 2000; van der Heide *et al*., 2006; Huang *et al*., 2010).

The insulin and IGF-1 receptors are structurally similar hormone receptors and belong to the tyrosine kinase superfamily (Shulingkamp *et al*., 2000). As shown in Figure 6, the IR is a tetrameric protein consisting of two α and β subunits linked by disulfide bonds: α subunits (135-kDa) constitute the extracellular domain, where insulin will bind, while β subunits (95-kDa) are transmembrane proteins which have an intracellular tyrosine kinase domain (Chiu and Cline, 2010). While insulin presents higher affinity for IR than IGF-1, the latter presents higher affinity for IGF-1R than insulin (Conejo and Lorenzo, 2001). However, several studies suggest that the activation of this two insulin receptors is the basis of a complex but relevant role in the regulation of brain metabolism, neuronal growth and differentiation and neuromodulation (Gasparini and Xu, 2003; Wada *et al*., 2005).

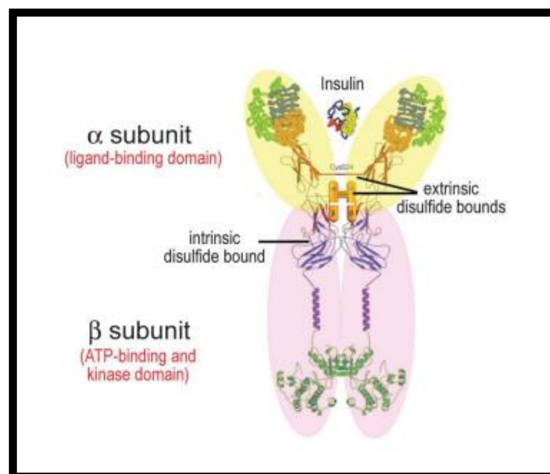


Figure 6: Insulin receptor structure. This receptor is composed of an α (yellow) and β subunit (pink) bridged by an intrinsic disulfide bond, which dimerizes with another insulin receptor monomer through extrinsic disulfide bonds to form a functional receptor (Image taken from Chiu and Cline, 2010).

Upon extracellular insulin binding, the two α-subunits became closer enabling ATP binding to the β-subunits intracellular domain. This activates receptor auto-phosphorylation on specific tyrosine residues, triggering the phosphorylation of cellular substrates, including members of the insulin receptor substrate (IRS) family (IRS-1-4) and the adaptor proteins, Shc, Gab-1 and Cbl (Figure 7). Except for Cbl, these proteins are deeply associated with insulin

signaling pathways related to T2DM-induced cognitive impairments (see section 3.), through phosphorylation on multiple tyrosine residues which creates recognition sites for additional effector molecules containing Src Homology 2 (SH2) domains (Kido *et al.*, 2001; Saltiel, 2001; Fulop *et al.*, 2003; van der heide *et al.*, 2006; Saini, 2010). Accordingly, each scaffold protein can interact with different SH2-containing proteins that direct a pathway of signal transduction.

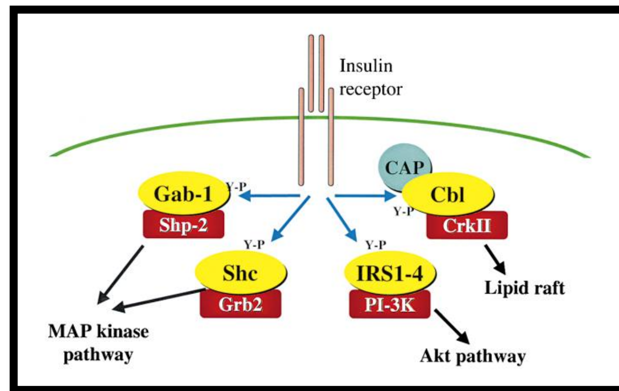


Figure 7: Tyrosine kinase substrates of the insulin receptor. Insulin stimulates the tyrosine kinase activity of its receptor, leading to the phosphorylation of a number of cellular substrates, including Gab-1, Shc, IRS 1–4, and Cbl (via its recruitment by the adaptor protein CAP), which in turn will activate multiple signaling cascades (Image taken from Saltiel, 2001).

Alterations in IR expression, trafficking, ligand binding, auto-phosphorylation, and/or kinase activity have been identified in cases of severe insulin resistance (Saltiel, 2001).

Additionally, IRS proteins seem to present a further important regulatory step in the IR activation. The degree of serine/threonine (S/T) phosphorylation determines either a positive or negative regulation of the signal transmission via IRS proteins. Furthermore, the activation of PI3K needs the tyrosine phosphorylation of IRS. A deregulation in S/T phosphorylation could affect the insulin action, as both increased and decreased phosphorylation could lead to defective IR-mediated tyrosine phosphorylation (Fulop *et al.*, 2003). Moreover, it has been shown that serine phosphorylation of IRS proteins can reduce the ability to attract PI3K, thereby minimizing its activation and probably leading to an accelerated degradation of the IRS-1 protein. The over phosphorylation of S/T could be induced by circulating free fatty acids, tumor necrosis factor-α (TNF-α) or a chronic increase in the insulin level as may occur during T2DM, neurodegenerative disorders and aging (Fulop *et al.*, 2003; Saini, 2010). There are other mechanisms involved in IRS serine phosphorylation including mammalian target of rapamycin (mTOR), JNK and PKC pathways (Saini, 2010).

5.1.2. PI3K/Akt pathway

PI3K is a heterodimer composed of a catalytic subunit (110 kDa) and a regulatory or adapter subunit (85 KDa), being highly expressed in the nervous system, including the hippocampus. Following insulin/IGF-1 stimulation, tyrosine (Saltiel, 2001) phosphorylation of the IRS family proteins mainly induces their binding to the SH2 domains of p85 triggering downstream activation of serine/threonine kinases including protein kinase B (PKB)/Akt and PKC (Figure 7 and 8) (Kido *et al*., 2001; Saltiel, 2001; van der Heide *et al*., 2006; Saini, 2010).

Interestingly, over the past few years it has become evident that the effects of insulin in neuronal survival and synaptic plasticity are mediated by a common signal transduction cascade, which has been identified as ''the PI3K route''. Furthermore, this same route is required for the induction of LTP and LTD (Kamal *et al*., 2000; van der Heide *et al*., 2006; McNay and Recknagel, 2011). In fact, insulin signaling has recently been shown to regulate AMPARs trafficking and NMDAR conductance (van der Heide *et al*., 2006; Cardoso *et al*., 2009; Huang *et al*., 2010).Additionally, increases in insulin receptor protein levels have been found in hippocampal synaptic membrane fractions after short-term memory formation (Zhao *et al*., 1999; Teter et al., 2004). Moreover, it has been shown that alterations in IR density correlate with changes in synaptic strength, learning and memory (van der Heide, 2006). Furthermore, insulin administration can rapidly reverse insulin resistance-induced memory deficits (McNay *et al*., 2010). Once activated, PKB/Akt [which is a member of the AGC (cAMP-dependent, cGMP-dependent and PKC) protein kinases family] mediates cell survival, protecting cells against apoptotic stimuli by inactivating proteins belonging to the apoptotic machinery, protein kinases such as glycogen synthase kinase 3β (GSK-3β) and several transcription factors, including Forkhead box O (FoxO) (Kido *et al.*, 2001; van der Heide *et al.*, 2006). In addition, GSK-3β is also implicated in memory impairment, since it is able to phosphorylate and inhibit the CREB protein, a universal modulator of memory (Cardoso *et al*., 2009)

Insulin also stimulates protein synthesis and cell growth by phosphorylation of mTOR through the activation of the PI3K/Akt pathway. Finally, PI-3K/Akt signaling pathway also promotes the translocation of GLUT-4 from the endossomal pool to the membrane surface, enhancing glucose uptake into the cell (Fulop *et al*., 2003; Cardoso *et al*., 2009; Saini *et al*., 2010).

5.1.3. MAPK/ERK1/2

Insulin also activates the Ras–MAPK pathway that triggers the recruitment of the serine/threonine kinase Raf, which functions as an upstream kinase for MAPK kinase (MEK). MEK phosphorylates MAPK/ERK1/2 on threonine and tyrosine residues, resulting in its activation and leading to the regulation of gene expression (Figure 8; van der Heide *et al*., 2006; Cardoso *et al*., 2009). In the hippocampus, MAPK/ERK1/2 activation is also regulated by a wide variety of neurotransmitter receptors including metabotropic glutamate receptors and NMDAR (Haddad, 2005), pointing it as a downstream intracellular mediator of NMDA receptor-mediated excitotoxic cell death (van der Heide *et al*., 2006).

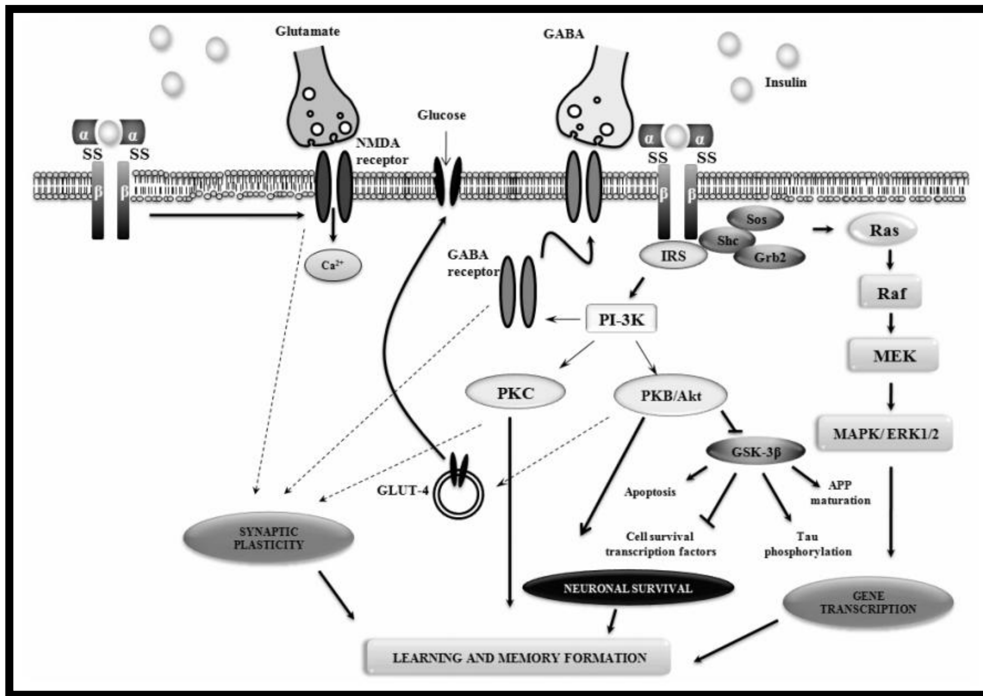


Figure 8: Schematic illustration of the potential molecular mechanisms of insulin signaling in the brain. Insulin binds to the insulin receptor activating the intrinsic tyrosine kinase, which phosphorylates endogenous substrates, such as the insulin receptor substrates (IRS) and Src-homology-2-containing protein (Shc), leading to the activation of two major downstream signaling pathways: 1) the phosphatidylinositol 3-kinase (PI3K) and 2) mitogen-activated protein kinases (MAPK/ERK1/2) pathways. PI-3K mediates the activation of the protein kinase-B (PKB/Akt) favoring neuronal survival. Activated PKB/Akt can interfere with the apoptotic machinery, inactivating the proapoptotic proteins. Furthermore, PI3K/Akt activation inhibits GSK-3β, which is involved in the triggering of the apoptotic cascade, inhibition of the activation of several cell survival transcription factors, tau protein hyperphosphorylation, and amyloid-β protein precursor (AβPP) maturation and processing. PI3K/Akt signaling cascade also induces the translocation of insulin-sensitive glucose transporter 4 (GLUT4) to the membrane surface, enhancing glucose uptake. Insulin-mediated PI3K signaling pathway is implicated in learning and memory, as well as in synaptic plasticity through the regulation of glutamate and GABA (γ-aminobutyric acid) receptors trafficking and channel activity. While the GABAergic receptors mediate the inhibitory synaptic transmission and the glutamatergic receptors mediate the vast majority of the excitatory synaptic transmission, the balance of glutamatergic and GABAergic transmissions is required to maintain normal brain function. Moreover, activation of excitatory glutamatergic synapses induces Ca^{2+} influx at postsynaptic sites, where it acts as a second messenger. Insulin is also able to active the MAPK/ERK1/2 signaling pathway, which is responsible for the activation of several transcription factors that alter protein expression (Image taken from Cardoso *et al*., 2009).

6. Glucose Neurotoxicity

In order to maintain normal function and energy metabolism, the brain uses glucose as the main fuel, primarily by oxidative metabolism (McCall, 2004). Both hypoglycaemia, and chronic hyperglycaemia, even in the absence of DM symptoms, can result in brain damage (Sheen, 2010). Neurons have a constantly high glucose demand and, thus, cannot accommodate episodic glucose uptake under the influence of insulin, but rather depending on glucose extracellular concentration (Tomlinson and Gardiner, 2008).

The normal fate of intracellular glucose is phosphorylation of the number-six-position carbon and entry into glycolysis, but in the presence of abnormally high levels of glucose in the interstitial fluid, it is diverted to metabolic pathways that can result in neurotoxicity (Tomlinson and Gardiner, 2008): polyol, hexosamine and advanced glycation end-product pathways (Figure 9). Thus, DM evoked hyperglycaemia, which can cause up to fourfold increase in neuronal glucose levels (Tomlinson and Gardiner, 2008), is highlighted as one of the main causes of diabetic encephalopathy (McCall, 2004).

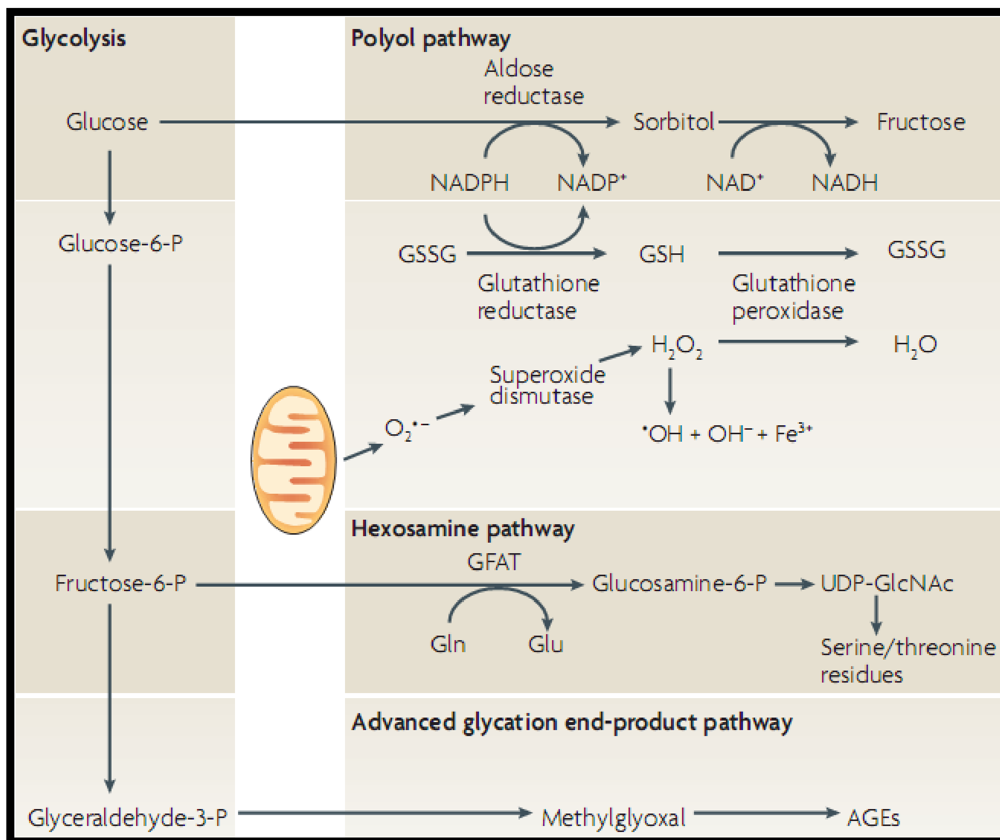


Figure 9: Metabolic pathways favored by raised glucose levels. The normal fate of intracellular glucose is phosphorylation of the number-six-position carbon by hexokinase and entry into glycolysis. However, if high glucose levels saturate hexokinase, glucose is diverted into the polyol pathway, leading to a compromised recycling of glutathione disulphide (GSSG) to glutathione (GSH), which in turn can compromise the conversion of hydrogen peroxide to water, favoring oxidative stress. Further down the glycolytic pathway, fructose-6-phosphate can drive the synthesis of uridine diphosphate-N-acetylhexosamine (UDP-GlcNAc), which can combine with serine and threonine residues on intracellular proteins and compromise the proteins' function (hexosamine pathway). Glyceraldehyde-3-phosphate can be converted to highly reactive methylglyoxal, which forms advanced glycation products on proteins and other macromolecules (advanced glycation end-product (AGE) pathway). GFAT, glutamine–fructose-6-phosphate amidotransferase. (Image taken from Tomlinson and Gardiner, 2008).

6.1. Polyol Pathway

Following saturation of hexokinase, hyperglycemia can trigger an excessive flow of the poliol pathway, where glucose is reduced to sorbitol by the aldose reductase. Subsequently, sorbitol can be oxidized to fructose by sorbitol dehydrogenase. Overall, this pathway can lead to cell damage through an increase in osmotic pressure, fructose accumulation and a decrease in nicotinamide adenine dinucleotide phosphate in reduced form (NADPH) levels that favors oxidative stress (Evans *et al*., 2002Tomlinson and Gardiner, 2008;).

Furthermore, stress-sensitive signaling pathways including p38 MAPK and c-Jun N-terminal kinase (JNK) are strongly activated by sorbitol (Evans *et al*., 2002).

6.2. Hexosamine pathway

An excessive flux of glucose can also lead to the hexosamine biosynthetic pathway activation, where fructose-6-phosphate (derived from glycolysis) gets diverted into a signaling pathway in which an enzyme called GFAT (glutamine: fructose-6 phosphate amidotransferase) converts the fructose-6 phosphate to glucosamine-6 phosphate and finally to UDP (uridine diphosphate) N-acetyl glucosamine. After that, the N-acetyl glucosamine fosters modification of serine and threonine residues of transcription factors, just like the more familiar process of phosphorylation, and over modification by this glucosamine often results in pathologic changes on the expression of several gene products, including leptin (Evans *et al*., 2002; BrownLee, 2004).

The overproduction of glucosamine may initiate endoplasmatic reticulum stress, which can promote a JNK-dependent serine phosphorylation of IRS-1, which in turn results in suppression of insulin-receptor signaling pathway, leading to insulin resistance (Evans *et al*., 2002; Tomlinson and Gardiner, 2008).

6.3. Advanced Glycation End-Product Pathway

Glycation (also called glucoxidation or Maillard reaction) is a multistep nonenzymatic reaction responsible for the irreversible synthesis of AGEs: heterogeneous group of molecules that play an important role in disrupting biological activities of proteins as well as their degradation processes, leading to the loss of their normal function and, ultimately, cell dysfunction and death (Kikuchi *et al*, 2003; Peppa *et al*., 2003; Miranda and Outeiro *et al*, 2010). The initial product of this reaction is called a Schiff base, which is formed by the direct addition of open-chain glucose to lysine groups on proteins. This Schiff base undergoes a slow and spontaneous rearrangement (the Amadori rearrangement) to form a stable AGE, as is the case of the well-known DM pathological hallmark: glycated hemoglobin (HbA1c) (Kikuchi *et al*, 2003; Peppa *et al*., 2003; Tomlinson and Gardiner, 2008). A series of subsequent reactions, including successions of dehydrations, oxidation-reduction reactions, and other arrangements lead to the formation of AGEs. The formation of these highly reactive products is fostered by both T2DM-induced hyperglycemia and oxidative stress (Peppa *et al*., 2003). In fact, the inhibition of oxidant pathways prevents intracellular AGE formation (Giardino *et al*., 1996).

Conversely, AGEs may be also linked to increased generation of reactive oxygen species (ROS) by multiple mechanisms, such as by decreasing activities of superoxide dismutase and catalase, diminishing glutathione stores, and activation of PKC (Ramasay *et al*., 2005). Moreover, glucoxidation products (e.g. Methylglyoxal, Figure 9) can also contribute to the formation of AGEs (Tomlinson and Gardiner, 2008). Finally, the cytotoxicity of glycation results from the following three mechanisms: 1) inhibition of specific functions of proteins; 2) cross-linkage, aggregation, and impaired degradation of proteins; and 3) ROS production (Kikuchi *et al*., 2003).

AGEs are also operative in SNC. It was further suggested that glycation, through proteins degradation processes alteration, will interfere with both axonal transport and intracellular protein traffic in neurons, compromising neuronal survival (Kikuchi *et al*., 2003; Ramasay *et al*., 2005). Kikuchi and colleagues reviewed glycation as a 'sweet tempter for neuronal death' and speculated that the synergism between glycation and oxidative stress are involved in neurotoxicity and consequently neuronal cell death (Kikuchi *et al*., 2003).

Furthermore, it has become clear that AGEs cytotoxicity also affects physiological brain aging (Kikuchi *et al*., 2003; Peppa *et al*., 2003 Ramasay *et al*., 2005; Miranda and Outeiro, 2010). In fact, such mechanism could play a unifying role in both T2DM-related accelerated brain aging and neurodegenerative processes (Ramasay *et al*., 2005).

In the CNS, AGEs, along with other ligands including S100 and amyloid β (Aβ) proteins, are able to interact with a variety of cell-surface AGE-binding receptors (RAGE), leading either to their endocytosis and degradation or to cellular activation and pro-oxidant/inflammatory events (Peppa *et al*., 2003; Ding and Keller, 2005; Ramasay *et al*., 2005; Sparvero *et al*., 2009).

6.3.1. RAGE

RAGE is a multiligand receptor of the immunoglobulin superfamily of cell surface molecules acting as counter-receptor for diverse molecules (Ding and Keller, 2005).

These receptors are expressed at low levels in normal tissues, but become upregulated at sites where its ligands accumulate (Chavakis *et al*, 2004). RAGE effects on neuronal, astrocyte, and microglia populations are based upon RAGE levels and upon the specific isoforms present on the cell surface (Ding and Keller, 2005; Sparvero *et al*., 2009).

There are multiple isoforms of the RAGE receptor. The three dominant isoforms are generated as the result of alternative splicing from a single RAGE gene and can loosely be defined as being the full-length RAGE, dominant negative RAGE ($_{DN}$RAGE), that lacks the intracellular domain, therefore binding RAGE ligands without directly transducing a signal, and

soluble RAGE (sRAGE), which is released into the extracellular space and allowed to interact with RAGE ligands prior to their potential binding to full-length RAGE (Figure 10). Each of these forms of RAGE contains the V-type and C-type domains for ligand binding, but each isoform differs considerably in cellular function and presumably is able to bind RAGE ligands with similar affinity. Together, the presence of these 3 main isoforms of RAGE suggests that the ability of any RAGE ligand to induce RAGE signaling depends on the coordinated effects of the different RAGE receptors. Furthermore, a growing number of studies have highlighted the presence of additional RAGE splice variants in human brain tissue (Figure 10), including astrocytes, and neurons, which appear to be cell type specific, and may be linked with specific pathological conditions, such as inflammation, cancer, neurodegeneration and diabetes (Ding and Keller, 2005; Sparvero *et al.*, 2009). Full-length RAGE has been identified as a direct mediator of physiological as well as pathological responses, including oxidative stress, chemotaxis, angiogenesis, inflammation, neurite outgrowth, apoptosis and proliferation. The intracellular domain of RAGE is necessary for many forms of RAGE signaling, including the activation of nuclear factor k-B (NF-κB), MAPK and JNK (Chavakis *et al.*, 2004; Bierhaus *et al.*, 2005; Ding and Keller, 2005; Ramasamy *et al.*, 2005; Evans, 2002; Sparvero *et al.*, 2009).

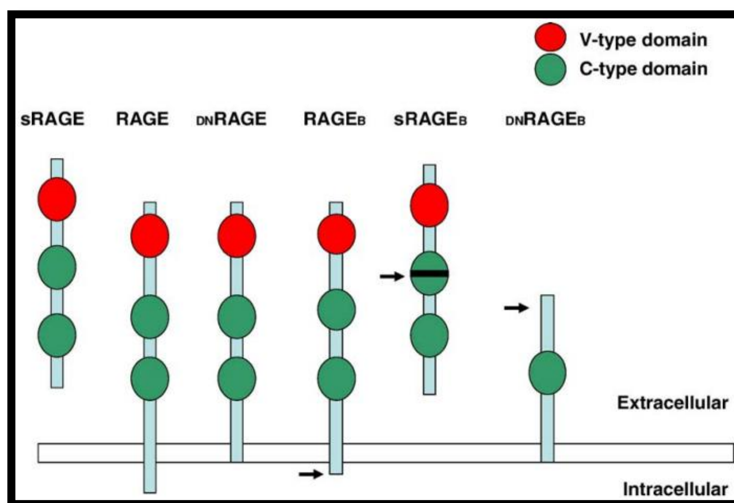


Figure 10: There are multiple isoforms of the RAGE receptor. The 3 dominant isoforms of the RAGE receptor are known as full-length RAGE, soluble RAGE (sRAGE), and dominant negative RAGE (dnRAGE). Each of these receptors contains a V-type domain and 2-C type. At least 3 additional forms of RAGE are found in the human brain (RAGEb, sRAGEb, dnRAGEb), arrows indicate the areas of each brain isoform that is unique (Image taken from Ding and Keller, 2005).

Thus, the ligand-RAGE axis, currently with AGEs, may be a part of quite a tangled web in the aging brain-linked to the complications of aging, diabetes and neurodegenerative disorders (Ramasamy *et al.*, 2005).

7. Oxidative stress

Another contributing mechanism for glucose neurotoxicity is the oxidative stress that stems from enhanced free radical concentration and decreased antioxidant defense system. Glucose auto-oxidation, oxidative metabolism of glucose in the mitochondria, intercellular activation of the polyol pathway and non-enzymatic protein glycation can contribute to hyperglycemia-driven oxidative stress (Mattson, 2000; Evans *et al*., 2002; Tomlinson and Gardiner, 2008).

Permanently, excessive levels of ROS lead to the damage of proteins, lipids, and DNA and also play a significant role in activating stress-sensitive signaling pathways that regulate gene expression resulting in cellular damage (Evans *et al*., 2002). Protein oxidation seems to be highly associated with cognitive decline and cellular dysfunction leading to neurodegenerative disorders (Smith *et al*., 1991; Sohal, 2002; Dalle-Donne *et al*., 2003). For example, animal studies have been performed to testify that increases in oxidative stress in the rat hippocampus are associated with decreases in behavioral performance in hippocampal-dependent learning and memory tasks, such as the Morris water maze (Fukui *et al*., 2001; Kelly *et al*., 2009).

Additionally, as previously mentioned, oxidative stress can promote insulin resistance. Specifically, it leads to the activation of multiple serine kinase cascades that target insulin receptor as well as IRS and Shc proteins (Kyriakis and Avruch). In fact it was demonstrated that increased phosphorylation on discrete serine or threonine sites in such proteins can evoke hampered insulin signaling, including impairment in PI3K and PKB activation, and in glucose transport (Cardoso *et al*., 2009; de la Monte, 2009). Interestingly, PKB is considered to be a central modulator in preventing apoptotic cell death. Indeed, increased PKB activity can promote cell survival during free radical exposure, oxidative stress, Aβ exposure, among others (Paz *et al*., 1997; Qiao *et al*., 1999; Chong *et al*., 2003; Kang *et al*., 2003).

On the other hand, hyperinsulinemia (Facchini *et al*., 2000) and/or insulin/IGF resistance can also lead to increased oxidative stress and, ultimately, to cell death. For example, AD abnormalities are associated with inhibition of insulin/IGF-1 signaling, which negatively regulates GSK-3β via a PI3K/Akt-dependent mechanism (Cardoso *et al*., 2009). As an insulin resistance consequence, GSK-3β and aberrant intra-neuronal hyperphosphorylated tau accumulation can be activated by oxidative stress, contributing to neurodegeneration (Chen *et al*., 2004).

8. Inflammation

Commonly, systemic inflammation exacerbates CNS inflammation and correlates with cognitive decline (Leonard and Myint, 2006). This is of concern to T2DM, since this disease is characterized by low-grade inflammation (Rocha and Libby, 2009). Inflammation and IR, convergent pathogenic processes in both T2DM and AD, occur both in the periphery and in the brain suggesting common mediators (e.g. cytokines) and/ or defective control by homeostatic regulators (e.g. insulin) (Bhat, 2010). Recently, GSK-3 (which is modulated by insulin; see section 5.1.2.) has been implicated as a key regulator of the inflammatory response (Ramirez *et al*., 2010). For example, anti-inflammatory and therefore neuroprotective effects of GSK-3 inhibition have been shown in models of spinal cord injury (Cuzzocrea *et al*., 2006).

Another significant mechanism involved in inflammatory cascade initiation is NF-κB expression. It is noteworthy that impaired insulin signaling and hyperglycemia contribute to the increased expression of this transcription factor. Elevated free fatty-acids, ROS, TNF-α, IL-1β, and other proinflammatory cytokines can further increase NF-κB expression (Barnes and Karin, 1997). Although the mechanisms have not been fully elucidated, TNF-α may stimulate the serine phosphorylation of IRS proteins, leading to decreased tyrosine phosphorylation and PI3K association (Pickup and Crook, 1998; Saltiel, 2001). Furthermore, the AGE-RAGE axis besides playing a role on oxidative stress represents a key mediator of inflammation (Bhat, 2010).

9. The dual role of insulin

It has been highly debated the dual role of insulin in CNS. For example: insulin treatment can be considered ‘’good’’ when insulin is absent or concentrations are low and insulin can be ‘’bad’’ when its levels are continuously high (Gispen and Biessels, 2000van der Heide *et al*., 2006). This dose-dependent dual role suggest that at low doses, it has anti-inflammatory effects during short-term inflammatory provocation and during chronic hyperinsulinemia or inflammation it may exacerbate inflammatory responses and increase oxidative stress markers (Krogh-Madsen *et al*., 2004).

On the other hand, insulin was suggested as a neuroprotective agent against neurodegenerative diseases, where oxidative damage plays a major role (Schmidt *et al*., 2003). However, there are conflicting results for the effect of insulin as a possible antioxidant/neuroprotector and further studies are needed to cast light on this issue (Bender *et al*., 2006). For example, Bélanger and colleagues suggested that the absence of cognitive

and electrophysiological dysfunctions in Zucker Diabetic Fatty (ZDF) rats, a T2DM model, might be due to protective action of hyperinsulinemia (Bélanger *et al*., 2004), others proposed that insulin per se or their signaling pathways can protect neurons against oxidative stress-induced apoptosis, through Akt activation or GSK-3β inhibition (Cardoso *et al*., 2009), thus favoring memory formation. On the contrary, there are studies showing that insulin appears to induce free radicals generation and lipid peroxidation (Ryu *et al*., 1999) and epidemiological studies indicate that long-term hyperinsulinemia is a risk factor for dementia (Ott *et al*., 1999; Biessels *et al*., 2006). These observations might result from direct effects of insulin on the hippocampus. Even though there is emerging evidence concerning to insulin as a cognitive and neuronal modulator (McNay and Recknagel, 2011), other studies have shown that hyperinsulinaemia is associated with accelerated cognitive decline in non-dement and non-diabetic patients (Kalmijn *et al.*, 1995; Vanhanen and Soininen, 1998). Under low insulin levels there may be inadequate trophic support leading to cell death as well as learning impairments due to a desensitization of NMDA-dependent processes (van der Heide, *et al*., 2006). Benedict *et al*. (2011) have largely reviewed intranasal administration of exogenous insulin as a therapeutic option in the treatment of cognitive impairment, and conclude that it may be a useful for central nervous system insulin deficiency and resistance, a pathophysiological core feature of cognitive impairments. Conversely, hyperinsulinemia may desensitize the PI3K pathway resulting in inadequate responses to other trophic factors or facilitate excitotoxicity by potentiating NMDA receptors (van der Heide, *et al*., 2006).

Besides this controversial results, what seems to be clear is that T2DM-induced cognitive deficits are likely due in large part to impaired central insulin modulation of cognitive and metabolic processes in the hippocampus, in agreement with the fact that IRs are highly expressed in this brain structure (McNay and Recknagel, 2011).

10. Glial cells

Glial cells are dynamic signaling components with the potential to modulate neuronal action on a slow timescale. These cells, associated with synapses, integrate neuronal inputs and can release transmitters that modulate synaptic activity (Haydon, 2001). Indeed, evidence shows that glial cells can: 1) respond to neurotransmission, 2) modulate neurotransmission, and 3) instruct the development, maintenance, and recovery of synapses (Auld and Robitaille, 2003).

They are conventionally divided into two types: macroglia, which includes astrocytes and oligodendrocytes; and microglia.

Microglia arises from macrophages outside the nervous system and is unrelated to other cells of the nervous system. Microglia activation is characterized by proliferation, migration and expression of immune-related antigens. The rapid convergence of microglia processes at the site of injury suggests that microglia may provide a physical barrier to protect healthy tissue, as it is capable of rapid extension towards sites of acute CNS damage (Parkhust *et al*, 2010). The main and evident function of oligodendrocytes is the formation of a myelin sheath around most of axons in the CNS (Baumann and Pham-Dinh, 2001). Astrocytes are supposed to be the “good” glia, regarding their functions in regulating cerebral blood flow and maintaining synaptic function. Astrocytes responds particularly to pro-inflammatory cytokines and it is believed that these cytokines participate in astrocyte activation following CNS injury.

The present study is focused on DM-induced cognitive dysfunction and, consequently, on the characterization of astrocytic profile on account of its involvement in synaptic plasticity.

10.1. Astrocytes and DM

Astrocytes are complex, highly differentiated cells that tile the entire CNS in a contiguous fashion and that make numerous essential contributions its normal functioning. Astrocytes are generally characterized by one or more end-feet contacting a basal lamina around blood vessels (Nimmerjahn, 2009). Accumulating evidence suggests that astrocytes provide structural, metabolic and trophic support to neurons, modulating synaptic transmission and plasticity, and being involved in a wide range of CNS pathologies, including trauma, ischemia and neurodegeneration (Vesce *et al*., 1999; Haydon, 2001; Pekny and Nilsson, 2005; Sofroniew, 2009). In particular, astrocytes express a repertoire of neurotransmitter receptors mirroring that of neighbouring synapses. Astrocytes are endowed with the capacity of both uptaking and releasing glutamate, thus exerting an active participation in synaptic transmission and in the processing of information in the brain (Vesce *et al*., 1999). Failure of astrocyte glutamate transporters is associated with elevations in extracellular glutamate and, therefore, excitotoxicity, which has been reported in the brains of DM individuals (see section 4.1).

Astrocytes are involved in a wide range of CNS pathologies, including trauma, ischemia and neurodegeneration. A key component of the astrocytes cytoskeleton, that warrants cell integrity and resilience, is the intermediate filament (IF) network, further associated with transduction of biomechanical and molecular signals. Glial fibrillary acidic protein (GFAP) is the main IF protein in astrocytes, in addition to vimentin, nestin and synemin. Classically, GFAP is a marker for astrocytes, known to be induced upon brain damage or during CNS degeneration

(astrocytic activation), and to be more highly expressed in the aged brain (Teismann et al, 2004; Middeldorp J. *et al*, 2011). In response to essentially any CNS pathology, astrocytes undergo a characteristic change in appearance, i.e., astrocytes exhibit hypertrophic cell bodies and increased processes with augmented density of GFAP, a phenomenon referred to as reactive gliosis (or astrogliosis) (Pekny and Nilsson, 2005; Middeldorp *et al*., 2011). It has been proposed that astrogliosis may be the earliest brain response to hyperglycemia (Lebed *et al*., 2008). Furthermore, Duarte et al. (2012) have provided the first evidence that T2DM mice display a loss of nerve terminal glutamatergic markers and astrogliosis in hippocampus underlying memory impairment. However, others have found decreased astrocyte GFAP levels in the cerebral cortex, hippocampus, and cerebellum of T1DM animal model, without a significant decrease in relative astrocyte numbers or astrocyte glutamate transporters (Coleman *et al*., 2004).

11. Diet-induced insulin resistance [(pre)diabetes] in rodents

Both genetic and environmental factors contribute to the development of metabolic abnormalities. Diet represents one environmental factor that can influence a metabolic disorder. Several experimental studies have demonstrated that the macronutrient composition of a diet is determinant for normal insulin action. Accordingly, high fat (HFa) and/or high sucrose (HSu) diets were shown to contribute to syndromes such as hypertriglyceridemia, hyperinsulinemia, insulin resistance and glucose intolerance in rodents,(Hallfrish, 1979; Kanazawa *et al*., 2003; Ribeiro *et al*., 2005; Cao *et al*., 2007; Stranahan *et al*., 2008; Carvalho *et al*., 2012), even though the mechanisms involved are not fully understood.

Besides saturated fats, simple sugars are believed to be major components of the Western diet that promote obesity and insulin resistance (Gross et al., 2004). In particular, the administration of a sucrose-rich diet to normal rats was shown to be an effective experimental model of insulin resistance and dyslipidaemia that resembles some biochemical and hormonal aspects of the human metabolic syndrome (Ferreira *et al*., 2010). Therefore one can think of this model as an appropriate one to study prediabetic complications.

11.1. What is with high-sucrose diets?

'Sugars' is a common term to describe mono and disaccharides and represent an important part of the total caloric intake. Glucose, fructose and sucrose are the most consumed carbohydrates in diet and the effect on health of a high intake of sugars is still

subject to scientific and public debate (Laville and Nazare, 2008). Coherently, most of diets used in rodent studies are either high-fat hypercaloric, or have grossly elevated contents of fructose or sucrose (up to 70–80 % of total caloric intake) that lead to obesity (Storlien *et al*., 2000). The potential dietary adverse effect of sucrose may appear for HSu intakes (>30 % of caloric intake) (Daly *et al*., 1997; Laville and Nazare, 2008), which is far higher than the average sucrose intake. Others argue that a 40 % sucrose diet is in accordance with the concentrations ingested by a subset of Americans, and can indeed accelerate metabolic syndrome, fatty liver, and T2DM, independent of excess energy intake (Roncal-Jimenez *et al*., 2011).

During absorption, sucrose is hydrolyzed into equal quantities of fructose and glucose and although both share the same chemical formula ($C_6H_{12}O_6$), their bioavailability and metabolism are markedly different: 1) fructose after capture is almost all metabolized by liver whereas the main part of an oral glucose load is metabolized in peripheral tissues; 2) fructose is metabolized in liver by a specific pathway whose first enzymatic step is catalysed by fructokinase, whose velocity is far superior than glucokinase or hexokinase; and 3) fructose metabolism is mainly insulin-independent where glucose metabolism is insulin dependent (Figure 11; Laville and Nazare, 2008; Tappy and Lê, 2010). Several studies were performed to determine which moiety of the sucrose disaccharide may be the primary mediator of insulin resistance. It seems that fructose is the sugar largely responsible for decreased insulin sensitivity, mainly due to its rapid hepatic conversion into glucose, glycogen, lactate, and fat (Thresher *et al*., 2000; Tappy and Lê, 2010).

In short-term controlled feeding studies, dietary fructose significantly increases postprandial triglyceride levels and has little effect on serum glucose concentrations, whereas dietary glucose did not; on the other hand, dietary glucose did increase serum glucose and insulin concentrations in the postprandial state whereas dietary fructose did not (Schaefer *et al*., 2009). Furthermore, the effect of sucrose on glucose and insulin responses is close to the effect of glucose. But its oxidative fate seems to be closer to the fructose one (Laville and Nazare, 2008). Chronically high consumption of fructose in rodents leads to hepatic and extrahepatic insulin resistance, obesity, T2DM and high blood pressure. The evidence is less compelling in humans, but high fructose intake has indeed been shown to cause dyslipidemia and to impair hepatic insulin sensitivity. Hepatic *de novo* lipogenesis and lipotoxicity, oxidative stress and hyperuricemia have all been proposed as mechanisms responsible for these adverse metabolic effects of fructose (Tappy and Lê, 2010).

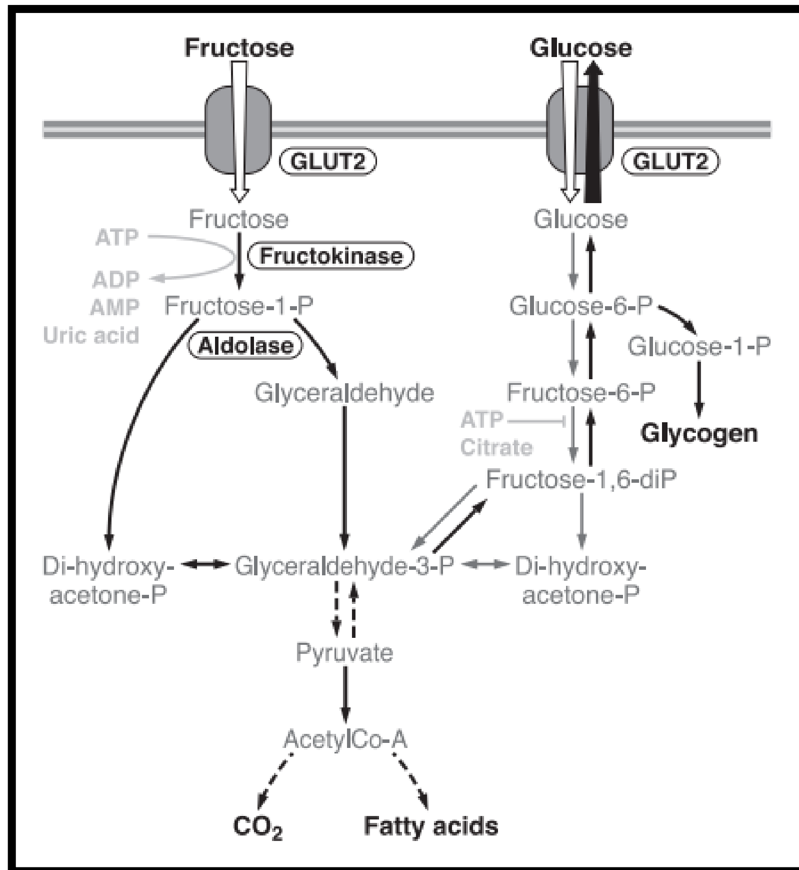


Figure 11: Fructose and glucose metabolism in liver cells. Fructose metabolism (grey arrows) differs from glucose (black arrows) due to 1) a nearly complete hepatic extraction and 2) different enzyme and reactions for its initial metabolic steps. Fructose taken up by the liver can be oxidized to CO_2 and then converted into lactate and glucose; glucose and lactate are subsequently either released into the circulation for extrahepatic metabolism or converted into hepatic glycogen or fat. The massive uptake and phosphorylation of fructose in the liver can lead to a large degradation of ATP to AMP and uric acid (Image taken from Tappy and Lê, 2010).

11.2. Cognitive impairment

The literature is scarce regarding to rodent models of sucrose-induced insulin resistance leading to cognitive impairment (Cao *et al*., 2007; Chepulis *et al*., 2009).

Data from clinical, epidemiological and animal studies have suggested that excessive energy intake adversely affects the brain, particularly during aging. Animal studies have shown that high caloric diets impair the structure and function of the hippocampus (Winocur and Greenwood, 1999; Molteni *et al*., 2002; Wu *et al*., 2003).

Deficits in hippocampal function may arise from peripheral insulin resistance and hyperlipidaemia induced by a high-caloric diet. However, an alternative hypothesis suggested the deleterious effects of this diet on hippocampal plasticity, are independent of peripheral metabolic alterations (Molteni *et al*., 2002; Stranahan *et al*., 2008).

II. Aims

Our main goal was to evaluate the hippocampal function, including learning and memory performance, on an animal model of insulin resistance/pre-diabetes [chronic consumption (9 weeks) of a high-sucrose solution (35 %)]. Two specific tasks were used to assess these cognitive processes:

➔ Morris Water-Maze

One of the most widely used tests to measure hippocampal-dependent spatial-based learning and memory. Various protocols exist for these tests, which are used depending on a plethora of variables (see materials and methods).

➔ Y-Maze

This test is particularly useful to evaluate memory function and the willingness of rodents to explore new environments. The Y-maze function is sensitive to damage in areas such as the hippocampus.

Simultaneously, the following parameters were also analyzed in order to achieve the biochemical characterization of both peripheral and hippocampus phenotype of the employed experimental model:

Table 1: Main goals of the present work.

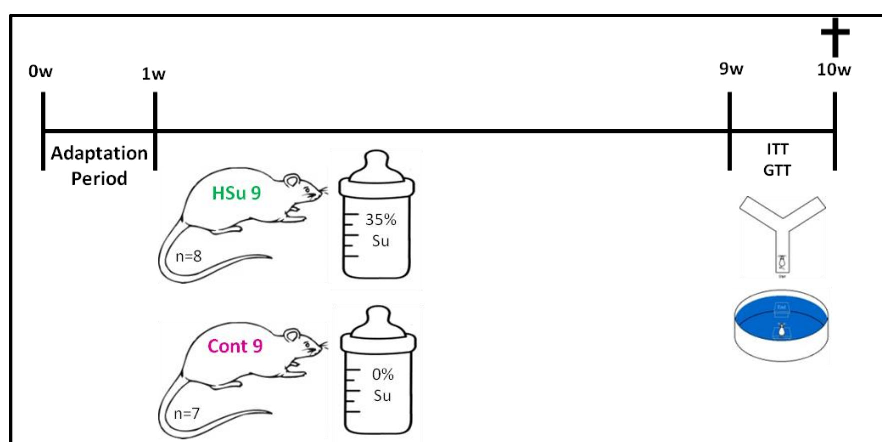
Goal	Specific Markers	Purpose
Peripheral characterization of experimental animals	Glucose	Glycemia
	Insulin	Insulinemia
	Cholesterol Triglycerides	Lipidemia
	GTT ITT HOMA-IR	Insulin sensitivity
	HbA1c	Diabetes diagnosis
Hippocampal Insulin Signaling	IR-β	Insulin receptor subunit
	IRS-1 IRS-1 pSer636/639	Insulin receptor substrate-1 activation
	PI3K	Downstream insulin signaling
Hippocampal Synaptic Plasticity	Synaptophysin	Nerve terminal marker
	PSD-95	Postsynaptic glutamatergic marker
	NR1	NMDAR subunit
	GluR1	AMPAR subunit (LTP and LDP)
Hippocampal neurotoxicty	GFAP GS	Astrogliosis
	TNF-α RAGE	Inflammation/Oxidative stress
	HNE	Lipid peroxidation

III. Material and Methods

1. Animals and high-sucrose diet

Experiments were performed in 4 months old male Wistar rats, weighting 332.9 g ± 9.0 g obtained from Charles River Laboratories Inc. (Barcelona, Spain). The animals were housed two per cage in the Institute of Pharmacology and Experimental Therapeutics (IBILI, Faculty of Medicine, University of Coimbra) under temperature and humidity control (22±1°C, 60 % humidity) and a 12 h light–12 h dark cycle. After an adaptation period of 1 week, the animals initiated the study protocol. All experiments were conducted in accordance with the Portuguese Law nº129/92 (6 July) and under the rules of the European Convention on Animal Care. All efforts were made to minimize animal suffering and to reduce the number of animals used. Throughout the experiments, the health status of all rats was closely monitored for weight loss or other signs of health-related issues.

To assess the effect of a liquid high-sucrose diet, the rats were randomly divided into two groups (Figure 12): 1) Control (Cont) group (n=7) and 2) sucrose-treated (HSu) group (n=8). Both groups had free access (except in the fasting periods) to a standard chow (16.1 % protein; 3.1 % lipids; 3.9 % fiber; 5.1 % minerals) (AO4 Panlab; Charles River, Barcelona, Spain) and to water (Cont) or 35 % sucrose (S0389; Sigma-Aldrich) solution (HSu). As we were interested in hyperglycaemia and insulin resistance-derived brain alterations, we chose to submit the HSu rats to 9 weeks of high-sucrose treatment, since Ribeiro and colleagues found that the rats have shown higher body weight and higher basal glycemia only after 9 weeks of sucrose supplementation.



(GTT, glucose tolerance test; ITT, insulin tolerance test; w, weeks; †, sacrifice)

Figure 12: Experimental design of the present study.

1.1. Body weight

The animals were weighed weekly since their arrival on the institute and throughout the experimental period, on an analytical scale (CB KERN 6 K1, Germany).

2. Cognitive performance tests

The effect of a high sucrose diet for 9 weeks on rats learning and memory performance was assessed using the Morris Water Maze and the Y-Maze tests, during the last week of treatment.

➔ Morris Water-Maze

The water-maze consisted of a large circular pool [120 cm diameter, 50 cm height, filled to a depth of 30 cm with water (room temperature: 23°C ±1)] in which a submerged platform was hidden on a fixed location (Figure 13). The rat could climb onto the platform to emerge from the water and escape from the necessity of swimming. The pool was divided in four quadrants: northeast (NE), southeast (SE), southwest (SW) and northwest (NW). It was placed in a darkened room, illuminated only by sparse red light and with spatial cues for reference, maintained constant throughout the experiment.

The acquisition of the spatial task consisted of placing the rats next to and facing the wall successively in north (N), east (E), south (S), and west (W) positions. The task is carried out across days to determine learning: 4 consecutive trials daily for 5 days. Each rat was given a maximum of 60 s to find the hidden platform (glassy, round, 10 cm diameter, 1 cm below surface, located in the center of the NE quadrant) and was allowed to stay on the platform for 10 s before picked up and to rest outside the maze for 20 s inter trials. Rats that failed to locate the platform were guided to it by the experimenter. The position of the rat in the pool was automatically registered by ANY-maze TM video tracking system (Stoelting Co., Wood Dale IL, USA). The rat must use and remember visual cues to find the platform and latency times were measured for each trial. The capacity of the animal to retrieve and retain information learned can be determined using a probe trial, performed on the 5th day, 24 h after the last training; therefore, we used this test to measure long-term memory. In the probe trial the platform is taken out and the animals are allowed to swim in the pool. Time spent in the region that previously contained the platform (referred to as correct quadrant) and in the opposite

quadrant, crossings over the platform area and time to reach platform location were measured in a single 60 s trial.

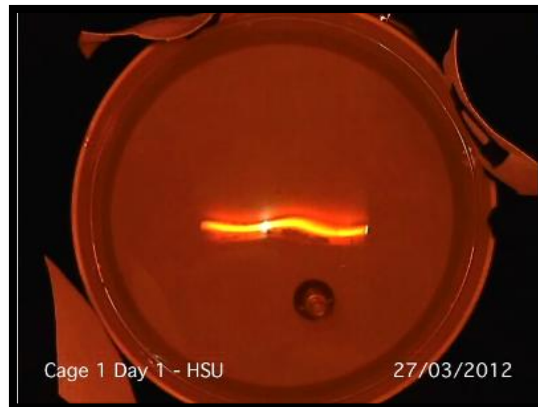


Figure 13: The Water-Maze pool used in the present work.

➔ Y-Maze

The Y-maze was used to assess short-term spatial memory and it is based on the innate preference of animals to explore areas that have not been previously explored.

The Y-maze apparatus consisted of three arms (50 cm long) made of black plastic joined in the middle to form a "Y" shape. The walls of the arms were 8 cm high, allowing the mouse to see distal spatial landmarks on the walls of the room. The inside of the arms were identical, providing no intramaze cues. The maze was cleaned with ethanol between trials to mask any olfactory cues from previous animals. Y-maze testing consisted of two phases. During training trial the rats were placed into the end of one arm (facing the centre) and allowed access to that arm (Start) and to other arm (Other) for 5 min. A removable door made from the same plastic as the maze was used to block access to the third arm (Novel). The rats were removed from the maze and returned to its home cage in the housing room for an intertrial duration of 2 h and then returned to the Y maze by placing them in the start arm, allowed to explore freely all three arms for 5 min (test trial).

The number of entries into and the time spent in each arm were registered from video recordings using ANY-maze TM video tracking system (Stoelting Co., Wood Dale IL, USA). Entry into an arm was defined as placement of all 4 paws into the arm. Animals were excluded from the analyses if they did not leave the start arm for the duration of the test session, or if they spent 75 % of their time in the center area.

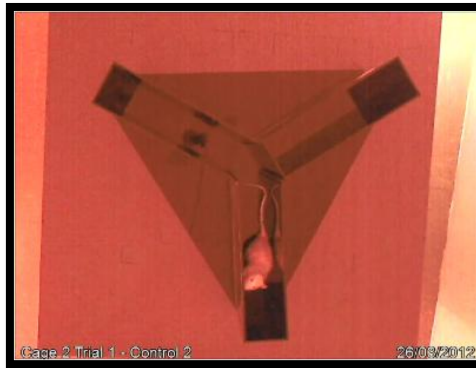


Figure 14: The Y-Maze pool used in the present work.

3. Determination of biochemical peripheral parameters

3.1. Insulin sensitivity

The insulin tolerance test (ITT) and the glucose tolerance test (GTT) were used to assess insulin sensitivity. These tests were performed following the completion of 9 weeks of HSu treatment, after a 6h fasting period.

➔ Insulin Tolerance Test

The ITT is one of the earliest methods developed to assess insulin sensitivity *in vivo* and provides an estimate of overall insulin sensitivity, correlating well with the "gold standard" hyperinsulinaemic–euglycemic clamp. The test was conducted after food deprivation through the administration of an intravenous insulin bolus of 0,75 U/kg body weight, followed by the measure of the decline in plasma glucose concentration over 120 min at 0, 15, 30, 45, 60 and 120 min intervals. Blood samples were collected from the tip of the tail and glucose levels were measured using a glucose analyzer (One Touch Ultra Easy, LifeScan, Johnson and Johnson, Portugal).

➔ Glucose tolerance test

The GTT can be used to estimate insulin secretion and insulin resistance as it determines the clearance of glucose in the blood over a period of time. After food deprivation, a 50 % - glucose solution (2 g/kg body weight) was injected into the animals intraperitoneally. The blood was collected by a small puncture on the tail immediately before and 15, 30, 60 and 120 min after the injection. At each time, glucose was measured by a glucose analyzer (One Touch

Ultra Easy, LifeScan, Johnson and Johnson, Portugal). The area under the serum glucose concentration versus time curve (AUC) has a number of important uses in toxicology, biopharmaceutics and pharmacokinetics. In this specific case, AUC data is a good summary of the body's glucose tolerance, as it represents blood glucose bioavailability. AUC was calculated using the trapezoidal rule.

3.2. Quantification of blood glucose, insulin, triglyceride and cholesterol levels

The day before the final time, after a 6 h fasting period, rats were subjected to intraperitoneal anesthesia (50 mg/kg sodium pentobarbital, Sigma-Aldrich, Portugal) and a blood sample was immediately collected by a venipuncture from the jugular vein into syringes with Heparin-Lithium (Sarstedt, Monovette®) for plasma samples to measure fasting insulin levels or without anticoagulant for serum samples (BD Vacutainer ®; SST™II Advance), to assess fasting glycemia.

At the final time, the rats (26 weeks old) were subjected again to intraperitoneal anesthesia (80 mg/kg sodium pentobarbital) and blood samples were immediately collected by venipuncture from the jugular vein into syringes without anticoagulant for serum samples (glucose, triglycerides and cholesterol measurement; BD Vacutainer ®; SST™II Advance) or with the appropriate anticoagulant (Heparin-Lithium (Sarstedt, Monovette®) for glycated hemoglobin (HbA1c).

The rats were then sacrificed by decapitation and the brains were immediately removed, placed in ice-cold Krebs buffer and carefully dissected. Hippocampal regions were immediately frozen in liquid nitrogen and stored at -80ºC until Western-blot analyses.

Serum glucose, triglycerides and cholesterol levels, plasma insulin concentration as well as blood HbA1c were determined using standard enzymatic and latex immunoagglutination inhibition procedures as indicated in Table 2. The homeostasis model assessment-insulin resistance (HOMA-IR) index was obtained to evaluate insulin resistance.

Table 2:Peripheral biochemical parameters.

Parameters	Samples	Methods
Glucose Triglycerides Cholesterol	Serum	Enzymatic [Hitachi 717 analyzer (Roche Diagnostics)]
Insulin	Plasma	ELISA
Glycated hemoglobin (HbA1c)	Blood	Latex immunoagglutination inhibition [DCA Vantage analyzer (Siemens)]

➔ Glucose

Serum glucose levels were measured using a glucose oxidase commercial kit (Sigma, St. Louis, Mo, USA), based on a colorimetric enzymatic principle. In this assay, glucose oxidase reacts with glucose to form gluconolactone and H_2O_2. H_2O_2 in the presence of peroxidase, originates 4-aminophenazone, an oxygen acceptor, which takes up the oxygen and together with phenol forms a red colored chromogen [4-(benzoquinone-monoamino)-phenazone]. The color intensity is measured by performing a photometric analysis, and it is directly proportional to glucose concentration.

➔ Cholesterol and Triglycerides

Serum total cholesterol and triglycerides were analyzed on a Hitachi 717 analyzer (Roche Diagnostics) using Cholesterol RTU® reagent (bioMérieux®, Lyon, France) and the triglycerides TG PAP 1000 kit (bioMérieux®, Lyon, France), respectively.

These are colorimetric methods in which the staining intensity of the final enzymatic chain compounds is directly proportional to the amount of triglycerides and cholesterol in the sample.

➔ Insulin

Plasma insulin levels were quantified by using a rat insulin ELISA (Enzyme-linked Immunosorbent Assay) kit from Mercodia (solid phase two-site enzyme immunoassay; Uppsala, Sweden).

This ELISA is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. During incubation insulin in the sample reacts with peroxidase-conjugated anti-insulin antibodies and anti-insulin antibodies bound to microtiter well. A simple washing step removes unbound enzyme labeled antibody. The bound conjugate is detected by reaction with 3,3',5,5'-tetramethylbenzidine. The reaction is stopped by adding H_2SO_4 0.5Mto give a colorimetric endpoint that is read spectrophotometrically.

➔ HbA1c

HbA1c is formed in a non-enzymatic glycation pathway by hemoglobin's exposure to plasma glucose, and it is measured primarily to identify the average plasma glucose concentration over prolonged periods of time. This serves as a marker for average blood glucose levels over the previous months prior to the measurement. Thus, HbA1c levels were used as an index of glucose control, through the DCA Vantage latex immunoagglutination inhibition method (Siemens Healthcare Diagnostics, Barcelona, Spain).

For the measurement of total hemoglobin, potassium ferricyanide is used to oxidize hemoglobin present in the sample to methemoglobin. The methemoglobin then complexes with thiocyanate to form thiocyanmethemoglobin, the colored species measured. The extent of color development at 531 nm is proportional to the concentration of total hemoglobin in the sample.

For the measurement of specific HbA1c, an inhibition of latex agglutination assay is used. An agglutinator (synthetic polymer containing multiple copies of the immunoreactive portion of HbA1c) causes agglutination of latex coated with HbA1c specific mouse monoclonal antibody. This agglutination reaction causes increased scattering of light, which is measured as an increase in absorbance at 531 nm. HbA1c in whole blood specimens competes for the limited number of antibody-latex binding sites causing an inhibition of agglutination and a decreased scattering of light. The decreased scattering is measured as a decrease in absorbance at 531 nm. The HbA1c concentration is then quantified using a calibration curve of

absorbance versus HbA1c concentration. The percent HbA1c in the sample is then calculated as follows:

$$\% \, HbA1c = \frac{HbA1c}{Total\ Hemoglobin} * 100$$

Both the concentration of HbA1c specifically and the concentration of total hemoglobin are measured, and the ratio reported as percent HbA1c. All measurements and calculations are performed automatically by the DCA Analyzer, and the screen displays percent HbA1c at the end of the assay.

➔ HOMA-IR

The homeostasis model assessment-insulin resistance (HOMA-IR) index was obtained to evaluate the insulin resistance of rats. The Matthews *et al*. (1985) formula was used, as follow:

$$HOMA - IR = \frac{Glucose\ (mmol/L) * Insulin\ (\mu U/L)}{22{,}5}$$

Plasma insulin and serum glucose values used were obtained after 6 h of food deprivation, as described above.

4. Biochemical characterization of hippocampal phenotype

4.1. Western-Blot Analysis

Total extracts were obtained from left hippocampus as previously described by Simões F. *et al.* (2007)) for measuring protein levels proposed to assess hippocampal insulin signaling, synaptic plasticity, astrogliosis and inflammation and oxidative stress, hippocampi were sonicated in 400 µL of RIPA lysis buffer (150 mM NaCl; 50 mM Tris-HCl pH=8.0; 5 mM EGTA; 1 % Triton X-100; 0.5% DOC; 0.1 % SDS) supplemented with a protease inhibitor cocktail (1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1 µg/mL chymostatin, 1 µg/mL leupeptin, 1 µg/mL antipain, 5 µg/mL pepstatin A, 50 mM sodium fluoride and 1 mM sodium orthovanadate (Sigma-Aldrich) and centrifuged (15000 g, 15 min., 4ºC), leading to a soluble supernatant fraction (corresponding to total extract) and a pellet. The total protein concentration was measured using bicinchonic acid protein assay kit (Thermoscientific®).

Samples were denatured at 95ºC for 5 min in sample buffer 6x (0.5 M Tris-HCl 0.5 M; pH 6.8, 10 % SDS (w/v), 30 % glycerol (v/v), 0.6 M DTT, 0.01 % bromophenol blue (w/v)) and proteins were separated by SDS-polyacrylamide gel electrophoresis (190 V), according to Laemmli procedure (Laemmli U., 1970). 10 % acrylamide gels were used for all proteins analyzed except for TNF-α (≈30 kDa) and IRS-1 (160 kDa) and phosphoylated IRS-1 (Ser636/639) (≈180 kDa) which were separated using a higher (12 %) or lower acrylamide percentage (7.5 %), respectively.

Separated proteins were electroblotted (110 V, 90 min) onto polyvinylidene difluoride (PVDF) membranes (Immobilon PVDF transfer membranes 0.45 µm, Millipore) and blocked by incubation with 1 % BSA in PBS-T (phosphate buffer saline with 0.1 % Tween-20) for 1 h. Blots were then incubated with primary antibodies (Table 3) overnight at 4ºC. The membranes were washed extensively in 0.1 % PBS-T and then incubated for one hour at room temperature with adequate alkaline phosphatase conjugated secondary antibodies (Table 3). After secondary antibody incubation, membranes were washed for one hour in 0.1 % PBS-T. To confirm equal protein loading and sample transfer, blots were reprobed with either rabbit anti-β-actin or mouse anti-GAPDH.

Table 3: Primary and secondary antibodies used for Western-blot analysis.

Antibodies	Molecular Weight (kDa)	Loading (μg)	Dilution	Reference	Company
Rabbit anti-IR-β (C-19)	95	50	1:1000	sc-711	Santa Cruz Biotechnology
Rabbit anti-IRS-1	165	50	1:1000	06-248	Millipore
Rabbit anti-phospho-IRS-1 (Ser636/639)	≈180	50	1:1000	2388	Cell Signaling Technology
Rabbit anti-p85 Pi3K	85	75	1:5000	4292	Cell Signaling Technology
Rabbit anti-HNE	-	60	1:1000	393207	Calbiochem
Rabbit anti-TNF-α	≈28	50	1:600	Ab6671	Abcam
Rabbit anti-RAGE	≈50	60	1:1000	Ab-3611	Abcam
Mouse anti-GFAP	50	20	1:5000	Ab-1540	Millipore
Mouse anti-GS	45	5	1:500	MAB302	Millipore
Rabbit anti-GluR1	110	20	1:1000	AB1504	Millipore
Mouse anti-NR1	139	40	1:1000	05-432	Millipore
Rabbit anti-PSD-95	95	20	1:1000	2507	Cell Signaling Technoloy
Rabbit anti-Synaptophysin	40	5	1:1000	AB9272	Millipore
Rabbit anti-β-actin	42	-	1:5000	A 5441	Sigma life sciences
Mouse anti-GAPDH	38	-	1:5000	Ab9484	Abcam
Goat anti-mouse	-	-	1:5000	A 3562	Sigma life sciences
Goat anti-rabbit	-	-	1:5000	1317	GE Healthcare

After reaction with enhanced chemifluorescence (ECF) (GE Healthcare Life sciences), immunoreactive bands were revealed by scanning blots using a Fluorescent image analyzer Typhoon FLA 900 (GE Healthcare Bio-sciences) imaging system. The relative density of each band was normalized against that of β-actin or GAPDH and quantified in arbitrary units by

software ImageQuant 5.0 (Molecular Dynamics). Data are expressed as percentages of the Cont and are presented as means ± SEM.

5. Data processing and statistical analysis

All values were expressed as the means ± SEM and statistical significance was evaluated by one-way analysis of variance (ANOVA) followed by post test Newman-Keuls multiple comparison test (GTT, ITT and behavioral tests) or by Student's two-tailed t test (GraphPad Prism 5.00.288). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with Cont group (ANOVA and Student's t-test).

IV. Results

1. Body weight monitoring and biochemical characterization of peripheral parameters

➔ Body weight evaluation

Both HSu and Cont groups comprised rats with similar body weight as shown in Figure15. Moreover HSu rats body weight after 9 weeks following a chronic high sucrose consumption (35 %) was not significantly different from Cont (Table 4). It should be noted that the first week corresponds to the adaptation period before the beginning of the experiment and the last week coincides with the behavioral tests which required increased physical effort, explaining the slight decrease in body weight for both groups (Figure 15).

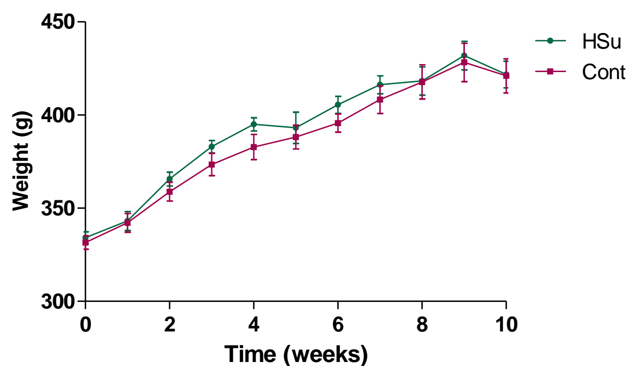


Figure 15: The effect of chronic exposure to sucrose on the weight gain of rats during 9 weeks, after 1 week adaptation period [HSu (n=8), Cont (n=7)]. Data are presented as mean weight ± SEM.

➔ Glucose tolerance and insulin response

Observing all the panels that reflect glucose tolerance and insulin response and that are shown in Figure 2, , one can conclude that the Cont group had higher total insulin sensitivity than the HSu group, which demonstrated impaired glucose tolerance.

In the GTT (Figure 16A), the concentration of blood glucose of both HSu and Cont increased to a maximum at 15 min after glucose injection, and then declined to a steady-state value. However, glucose levels of HSu rats 60 and 120 min after the glucose injection were significantly higher (215.5 ± 48.1 mg/dL; $p < 0.05$ and 168.8 ± 26.1; $p < 0.001$) compared to Cont (159.0 ± 14.27 and 121.4 ± 9.03). Furthermore, the GTT AUC confirms such impaired blood glucose clearance in rats exposed to chronic sucrose consumption (Figure 16B and Table 4), as it represents blood glucose bioavailability. This suggests that insulin secretion was lower in HSu compared to Cont.

In the ITT, the blood glucose levels decreased following insulin injection in both groups (Figure 16C). The blood glucose levels after insulin injection did not differ among groups until 60min after the injection; however, at 120 min HSu rats had significantly higher blood glucose levels (63.9 ±14,8 mg/dL; $p < 0.001$) than Cont (37.2 ± 7.0 mg/dL). This represents an impairment in HSu insulin sensitivity compared to Cont.

Insulin resistance was also assessed by HOMA-IR index (Figure 2D) calculation through serum glucose and plasma insulin levels after food deprivation. Indeed, after 9 weeks of sucrose consumption, HSu HOMA-IR levels were significantly higher than the Cont, as shown in Table 4.

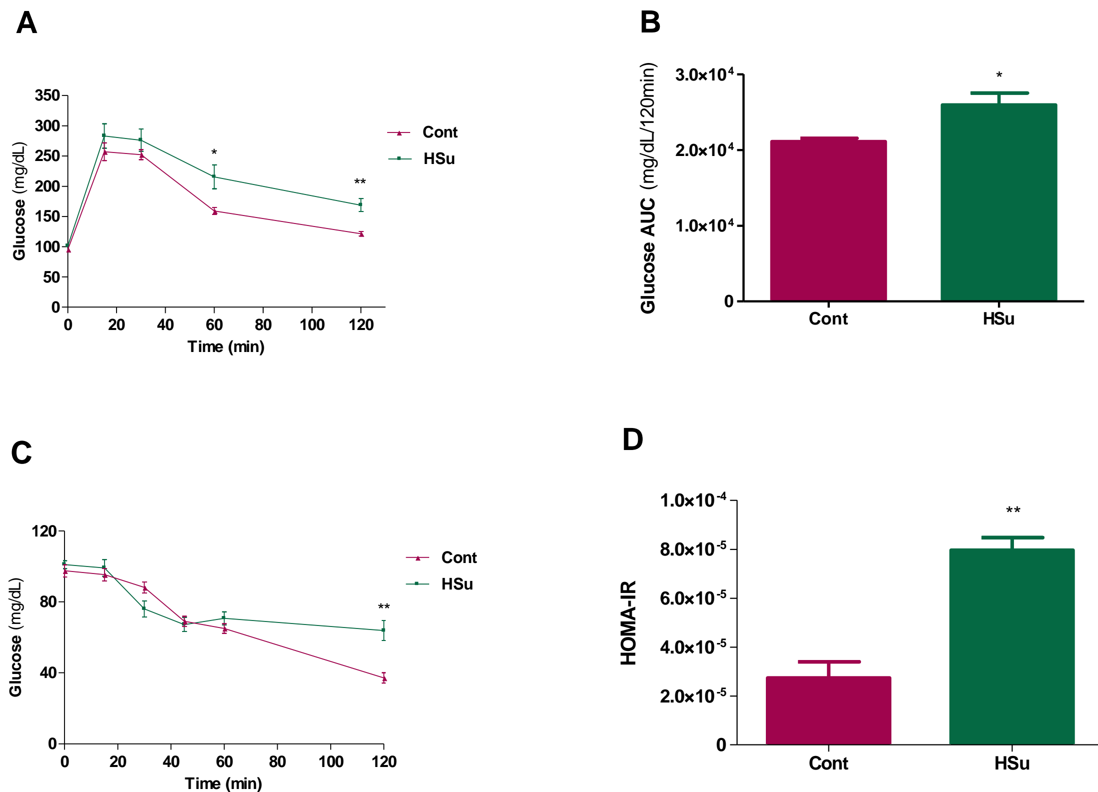


Figure 16: Glucose tolerance and insulin response in high-sucrose (HSu) diet rats and in the control (Cont) group. (A) Glucose tolerance test (GTT) [HSu (n=6), Cont (n=6)]; **(B)** Glucose area under the curve (AUC) for GTT [HSu (n=6), Cont (n=6)]; **(C)** Insulin tolerance test (ITT) [HSu (n=7), Cont (n=6)]; **(D)** Homeostatic model assessment for insulin resistance (HOMA-IR) index [HSu (n=3), Cont (n=5)]. Data are presented as mean ± SEM. *, $p < 0.05$; **, $p < 0.001$ *vs.* Cont.

➔ Peripheral Biochemical Parameters

All peripheral biochemical parameters are summarized in Table 4 and are depicted in figure 17. Briefly, high sucrose consumption during 9 weeks caused elevated postprandial glycemia but no alterations on fasting glycemia compared to Cont group (Figure 17A). HSu rats

also showed elevated plasma triglycerides but no significant difference on plasma total cholesterol between groups (Figure 17D). As it is shown in Figure 17B, insulin levels were influenced by sucrose diet as well, demonstrated by a significant increase in HSu serum insulin. Finally, glycated hemoglobin from HSu rats was slightly higher compared to controls (Figure 17C).

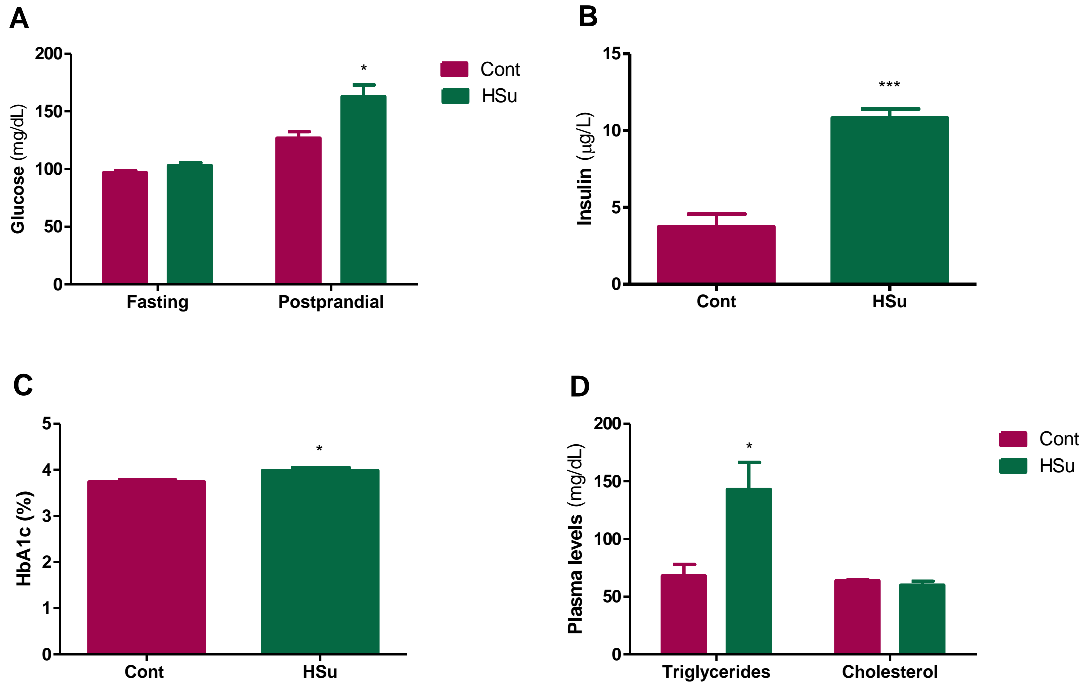


Figure 17: Several peripheral biochemical parameters from high-sucrose (HSu) diet rats and from the control (Cont) group. (A) Glucose levels (mg/dL) of both fasting and postprandial conditions [HSu (n=7), Cont (n=7)]; **(B)** Serum insulin levels (µg/L) [HSu (n=3), Cont (n=5)]; **(C)** Glycated hemoglobin levels (%) [HSu (n=6), Cont (n=5)]; **(D)** Plasma triglycerides and total cholesterol levels (mg/dL) [HSu (n=3), Cont (n=5)]. Data are presented as mean ± SEM. *, $p < 0.05$; ***, $p < 0.0001$ *vs.* Cont.

Table 4: Biochemical peripheral characterization of experimental animals.

Parameters	Cont	n	Hsu	n
Body weight (g)	421,0 ± 24,5	7	421,8 ± 20,3	8
Postprandial glycaemia (mg/dL)	126,8 ± 13,6	7	162,9 ± 26,5*	7
Fasting glycaemia (mg/dL)	96,7 ± 4,5	7	102,9 ± 7,0	7
Fasting Insulin levels (µg/L)	3,7 ± 1,8	5	10,8 ± 1,0***	3
Triglyceride levels (mg/dL)	68,1 ± 26,3	7	143,1 ± 65,7*	7
Cholesterol levels (mg/dL)	63,7 ± 2,5	7	58,2 ± 9,7	7
HbA1c (%)	3,7 ± 0,1	5	4,0 ± 0,2*	6
HOMA-IR	2,7 x10-5± 1,5 x10-5	5	8,0 x10-5 ± 9,0 x10-6**	3
Glucose AUC (mg/dL/120min)	2,1 x104 ± 1,0 x103	6	2,6 x104 ± 3,8 x103*	6

Data represent mean ± SEM. *, p < 0.05; **, p < 0.01; ***, p < 0.001 compared with Cont animals.

2. Cognitive performance assessment

➔ Morris Water Maze

The Morris water maze test was performed to assess rats spatial learning and long-term memory. As shown in Fig. 18A, HSu rats showed no difference in acquisition compared to Cont animals. Indeed, like Cont rats, they were able to locate the hidden platform with progressively reduced search times over the 4 days of training. A one-way ANOVA analysis revealed significant time effects ($F_{3,39} = 26{,}151$; $p < 0.001$) but not significant group effects or interaction. These results suggest an apparently intact acquisition at 9 weeks after the beginning of the experiment.

To assess spatial memory retention, a spatial probe test was performed on the 5th day, by removing the platform from the pool. HSu rats showed significant impairment of spatial memory retention, as they spent less time in the previous correct quadrant (platform) (30.2 ± 2.7 sec.; $p < 0.01$) compared to Cont rats (47.7 ± 3.7sec.) (Figure 18B). Accordingly, the time spent in the opposite quadrant was higher for HSu group (21.9 ± 2.8 sec.; $p < 0.05$) compared to the Cont (12.6 ± 2.2 sec.) (Figure 18C). The latency to reach the previous platform location was greater for HSu rats (15.8 ± 2.3 sec.; $p < 0{,}01$) compared to Cont (5.3 ± 0.6 sec.) (Figure 18D) and the HSu rats made significant fewer target approaches (platform zone) (1.8 ± 0.3; $p < 0.001$) than the Cont ones (4.4 ± 0.5) (Figure 4E).

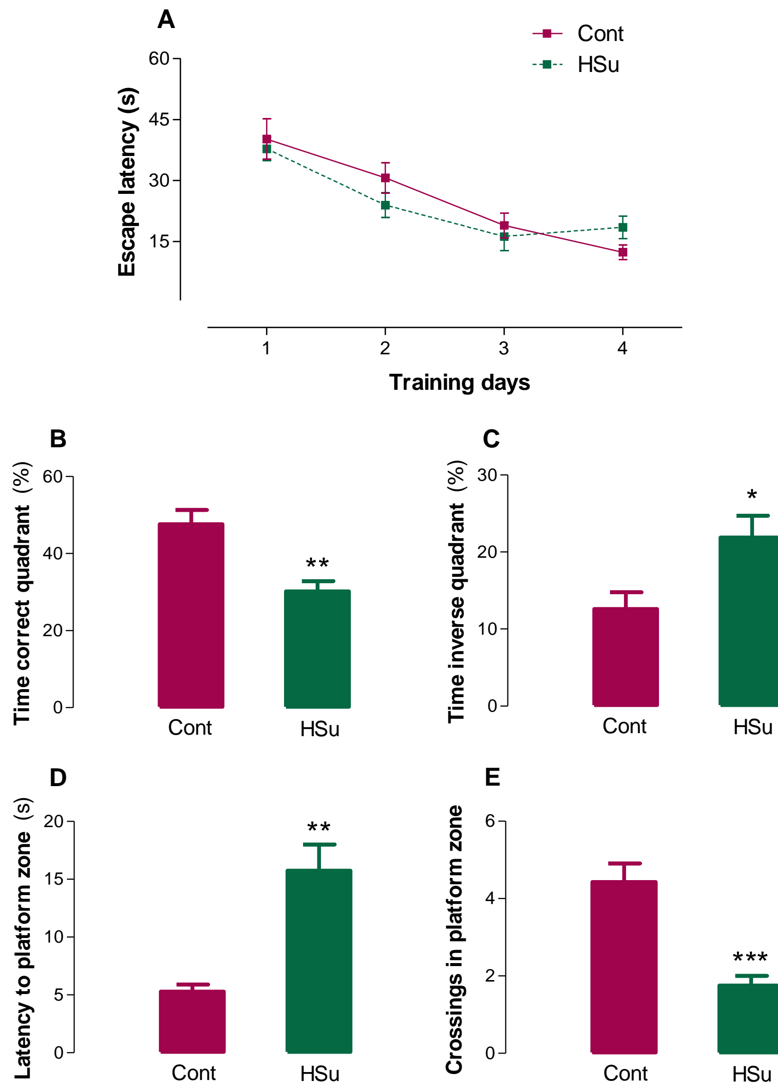


Figure 18: Learning and long-term memory performance of high-sucrose (HSu) diet rats and of control (Cont) group. Morris water-maze test was used to assess learning and memory profiles, through the analysis of multiple parameters. (**A**) Escape latency (s) to the platform over four days of training; **(B)** Time spent (%) in the correct quadrant (probe test); **(C)** Time spent (%) in the opposite quadrant (probe test); **(D)** Latency (s) to platform zone (probe trial); **(E)** Number of crossings in the platform zone (probe test). Data are presented as mean ± SEM [HSu (n=8), Cont (n=7)]. *, $p < 0.05$; **, $p < 0.001$; ***, $p < 0.0001$ *vs.* Cont.

➔ Y-Maze

The Y maze was used to assess spatial short-term memory. To this end, analyses were conducted to determine if the frequency of entries into and duration of time in each arm (Start, Other and Novel) differed among the Cont and HSu groups. Exploration in the training trial was similar for both groups – overall there were no differences in the number of entries and the time spent in both arms of the maze between HSu and Cont groups (Figure 19A, B, C). However, in the Y maze test the sucrose-treated animals showed memory deficits. Total

number of entries did not differ between groups (Figure 19D), but the number of entries in the novel arm was significantly lower for HSu animals (33.8 ± 4.5; $p < 0.05$) compared to Cont ones (46.0 ± 1.6) (Figure 19E). Consistently, the HSu rats made significantly fewer novel arm entries (28.6 ± 4.3, $p < 0.01$) compared to the Cont rats (46.9 ± 1.2) (Figure 19F).

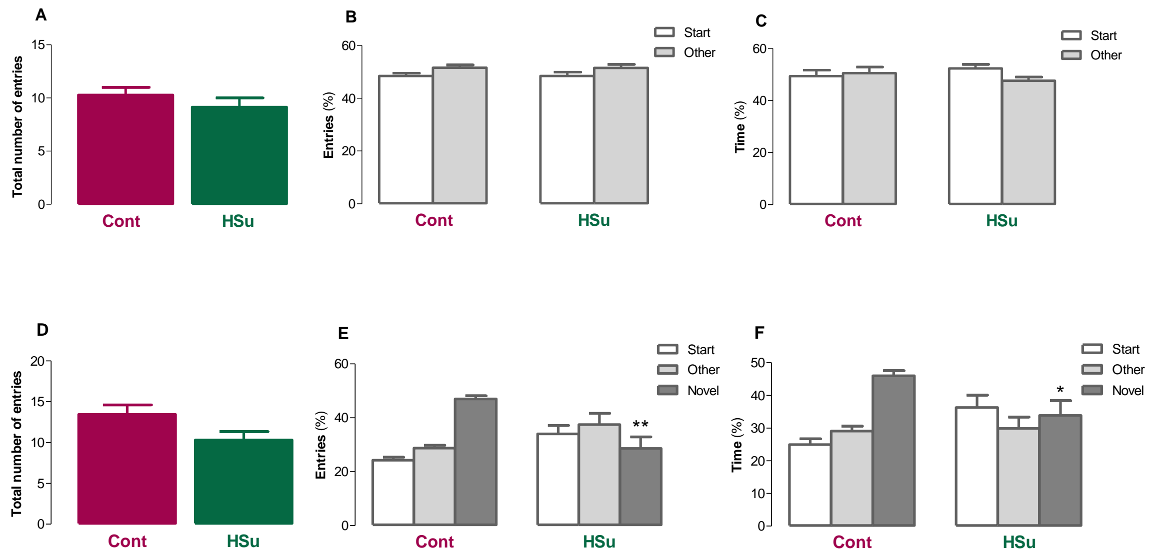


Figure 19: Short-term memory performance of high-sucrose (HSu) diet rats and of control (Cont) group. Y-maze was used to assess learning profiles, through the analysis of multiple parameters. A training trial was performed to test exploratory capacity: **(A)** Total number of entries; **(B)** Entries (%) in both Start and Other arms; **(C)** Time spent (%) in both Start and Other arms. During test trial the same parameters were analyzed: **(D)** Total number of entries; **(E)** Entries (%) in Start, Other and Novel arms; **(F)** Time spent (%) in Start, Other and Novel arms. Data are presented as mean ± SEM [HSu (n=7), Cont (n=7)]. *, $p < 0.05$; **, $p < 0.001$ *vs*. Cont.

3. Hippocampal biochemical analysis

3.1. Insulin signaling

Following peripheral insulin resistance in our animal model it was mandatory to assess insulin signaling in hippocampus from Hsu rats. Moreover insulin has been implicated in memory processes.

➔ IR-β levels

The chronic consumption of sucrose during 9 weeks induced a ≈20 % decrease in hippocampal IR-β levels compared to Cont (Figure 20).

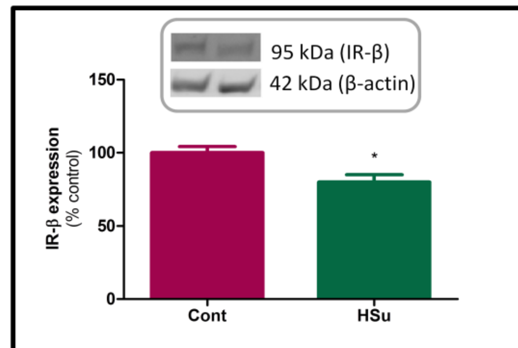


Figure 20: Hippocampal IR-β levels from high-sucrose (HSu) diet rats and control (Cont) group. IR-β levels were assessed by Western-blot analysis and data representing the band of 95 kDa was normalized to β-actin in the same membrane and calculated as percentage of the control and presented as means ± SEM. A decrease in HSu IR-β levels was observed for HSu treated group. [Cont (n=4), HSu (n=4)]. *, $p < 0.05$ vs. Cont.

➔ IRS-1 and serine phosphorylation of IRS-1 Ser(636/639)levels

In spite of changes in IR-β levels, both hippocampal IRS-1 andIRS-1 pSer$^{636/639}$ immunoreactivity (Figures 21A and 21B, respectively) remained unaltered following a sucrose chronic consumption during 9 weeks, compared to Cont.

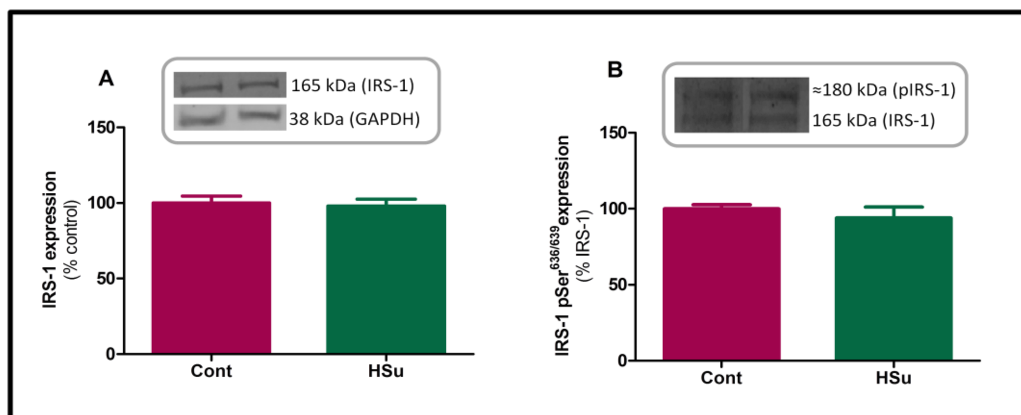


Figure 21: IRS-1 and serine phosphorylation of IRS-1 Ser (636/639) hippocampal levels from high-sucrose (HSu) diet rats and control (Cont) group. IRS-1 and IRS-1 pSer636/639 levels were assessed by Western-blot analysis and are presented as means ± SEM. **(A)** Data representing the band of 165 kDa was normalized to GAPDH in the same membrane and are expressed as percentage of the control, and **(B)** data representing the band of ≈180 kDa was normalized to IRS-1 in the same membrane and are expressed as percentage of the control. No statistical differences were detected in both bands between Cont and HSu treated group. [Cont (n=4), HSu (n=5)].

➔ PI3K levels

PI3K (the main downstream insulin signaling molecule involved in learning and memory) hippocampal levels remained unaltered following a sucrose chronic consumption during 9 weeks, compared to Cont (Figure 22).

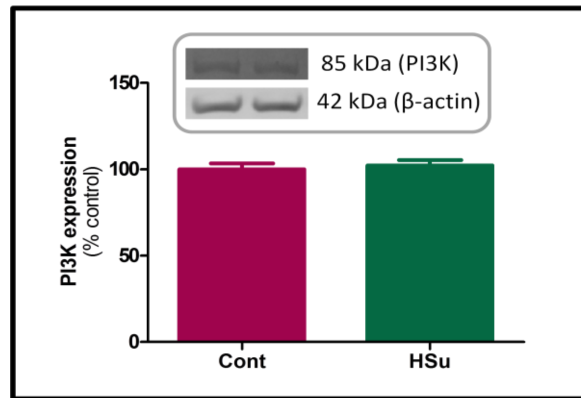


Figure 22: Hippocampal PI3K from high-sucrose (HSu) diet rats and control (Cont) group. PI3K was assessed by Western-blot analysis and data representing the band of 85 kDa was normalized to β-actin in the same membrane. Data was calculated as percentage of the control and presented as means ± SEM. No statistical differences were detected between Cont and HSu treated group PI3K levels. [Cont (n=4), HSu (n=4)].

3.2. Synaptic Plasticity

➔ Glutamatergic Synapses

The memory deficits from Hsu rats seen in the present study warranted the study of GluR1 and NR1 levels on account of their critical role in synaptic plasticity. The chronic consumption of sucrose during 9 weeks induced a ≈31 % and ≈52 % increase, respectively, in GluR1 and NR1 hippocampal levels compared to Cont (Figure 23). In respect to hippocampal PSD-95 (a glutamatergic post-synaptic marker) levels, no statistically significant differences were observed.

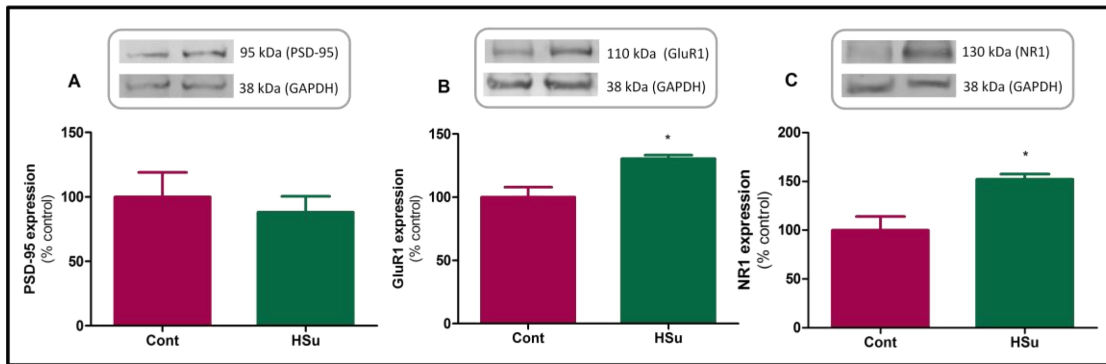


Figure 23: Hippocampal glutamatergic synapses markers from high-sucrose (HSu) diet rats and control (Cont) group. (A) PSD-95 (95kDa), **(B)** GluR1 (110kDa) and **(C)** NR1 (130kDa) levels were assessed by Western-blot analysis and both protein bands were normalized to GAPDH in the same membrane. Data are expressed was calculated as percentage of the control and presented as means ± SEM. No significant changes were observed in PDS-95 hippocampal content between groups but GluR1 expression was higher for HSu group compared to Cont [Cont (n=4), HSu (n=4)].

➔ Nerve terminal marker

Synaptophysin, which is an abundant synaptic vesicle protein, immunoreactivity remained unaltered in hippocampus from HSu animals compared to Cont (Figure 24).

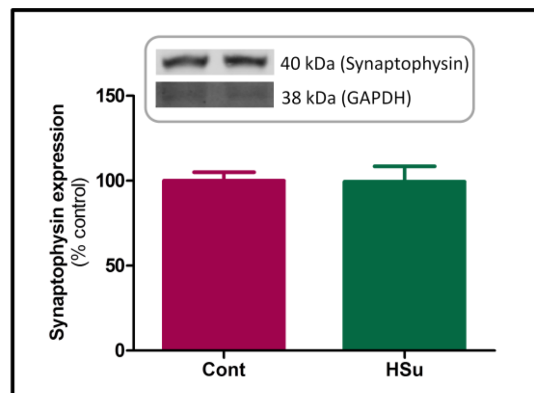


Figure 24: Hippocampal synaptophysin levels from high-sucrose (HSu) diet rats and control (Cont) group. Synaptophysin levels were assessed by Western-blot analysis. Synaptophysin immunoreactivity was normalised to GAPDH in the same membrane and calculated as percentage of control. Data are presented as means ± SEM. No statistical differences were detected in both bands between Cont and HSu treated group. [Cont (n=4), HSu (n=5)].

3.3. Neurotoxicity

➔ Astrogliosis

Herein we set out to evaluate astrogliosis by two reasons: 1) astrocytes are key players in regulating glutamatergic transmission and 2) astrogliosis is a hallmark of neurodegeneration processes. There were no significant alterations in hippocampal GFAP (Figure 25A) and GS (Figure 25B) immunoreactivity after 9 weeks of chronic sucrose exposure compared to Cont.

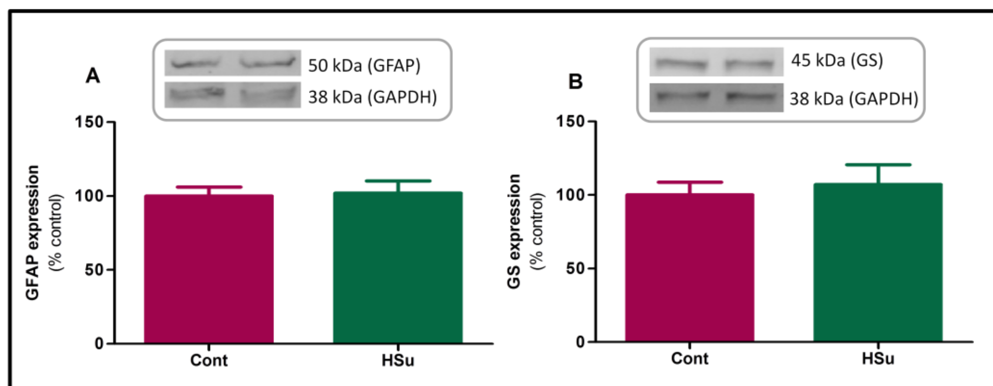


Figure 25: The effect of chronic sucrose consumption during 9 weeks on hippocampal glutamatergic homeostasis. **(A)** GFAP (50kDa) and **(B)** GS (45kDa) expression was assessed by Western-blot analysis and normalized to GAPDH immunoreactivity in same membrane. Data expressed was calculated as percentage of the control and presented as means ± SEM. No statistical differences were detected between Cont and HSu treated group. [Cont (n=4), HSu (n=4)].

➔ Oxidative stress/ Inflammation

Both oxidative stress and inflammation are two key players in diabetic encephalopathy. Therefore it was mandatory to assess hydroxynonenal (HNE, a precursor for advanced lipooxidation endproduct-ALE), RAGE and TNF-� hippocampal levels in the present study. The activation of RAGE (eg. by ALE) leads to the intracellular generation of ROS which, in turn, activate NF-κB. As a consequence of NF-κB activation, the expression of a variety of cytokines is increased, including TNF-α (Wright *et al.*, 2006).

Lipid peroxidation

HNE adducts were detected in a range of different molecular weights. No significant differences were observed for each adduct (Figure 26A, B) between Cont and HSu treated rats.

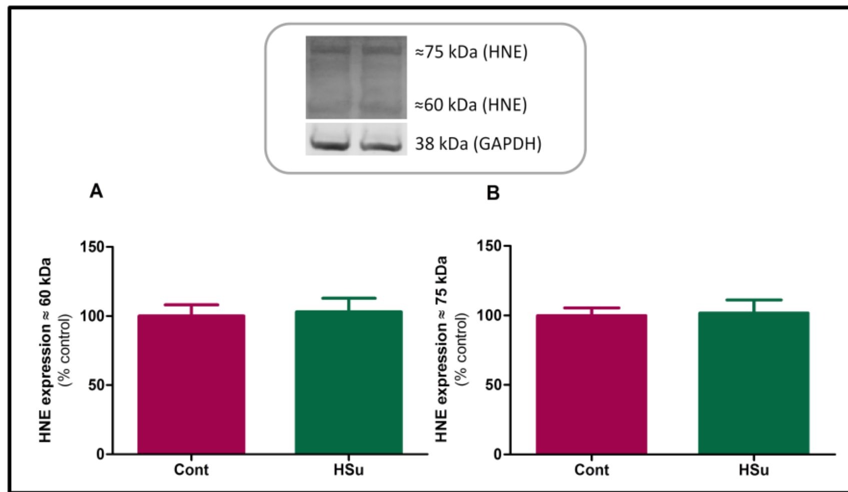


Figure 26: Hippocampal HNE levels from high-sucrose (HSu) diet rats and from control (Cont) group. HNE levels were assessed by Western-blot analysis and data representing bands within **(A)** 75 and **(B)** 60kDa range are expressed as percentage of the control and presented as means ± SEM No statistical differences were detected in both bands between Cont and HSu treated group. [Cont (n=4), HSu (n=5)].

RAGE and TNF-α expression

RAGE (Figure 27A) and TNF-α (Figure 27B) hippocampal levels remained unaltered in HSu animals compared to Cont.

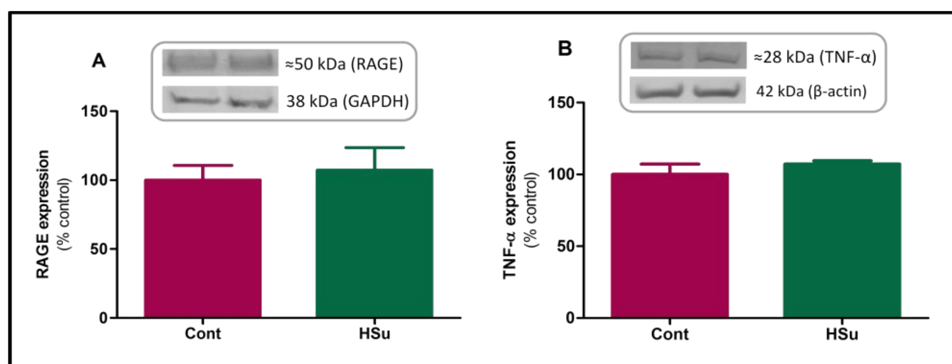


Figure 27: Hippocampal RAGE and TNF-α levels from high-sucrose (HSu) diet rats and from control (Cont) group. RAGE and **TNF-α** levels were assessed by Western-blot analysis and data representing the band above 50 kDa are expressed as percentage of the control and presented as means ± SEM No statistical differences were detected in both bands between Cont and HSu treated group. [Cont (n=4), HSu (n=4)].

V. Discussion

1. High sucrose diet induced insulin-resistance, hyperinsulinemia and glucose intolerance: a prediabetic state?

Several experimental studies demonstrated that diet is an important environmental determinant for life-style-related diseases such as T2DM, (Sumiyoshi *et al*., 2011). In particular, simple sugars are believed to be major components of the Western diet that promote obesity and insulin resistance (Gross *et al*., 2004). Furthermore, numerous rodent studies showed that a high-sucrose diet induces insulin resistance and hypertriglyceridemia (Kanazawa *et al*., 2003; Ribeiro *et al*., 2005; Cao *et al*., 2007;Gadja and Ricci, 2007; Raben *et al*., 2010; Conde *et al*., 2011; Sumiyoshi *et al*., 2011; Carvalho *et al*., 2012).

Along these lines we chose to submit Wistar rats to a high-sucrose diet to model the impact of insulin-resistance on hippocampal function. For that matter we supplemented a standard non purified diet with a sucrose solution replacing water in the diet. The duration of exposure to high-sucrose diet was 9 weeks, since Ribeiro and colleagues (2005) found higher basal glycemia not sooner than 9 weeks of sucrose supplementation. Additionally Conde *et al*. (2011) also affirmed that a shorter duration of sucrose exposure (e.g. 21 days) was not sufficient to induce diminished insulin sensitivity, using a 35 % sucrose solution.

In the present work we show that the addition of sucrose to the drinking water induced insulin resistance, hyperinsulinemia and impaired glucose tolerance, thus confirming that chronic consumption of sugar-sweetened beverages could lead to insulin resistance syndrome independent of dietary fat intake. These results are relevant since they give further support to the compelling evidence indicating that excess consumption of sweet foods, particularly sugar-sweetened beverages, plays an important role in the epidemic of metabolic syndrome and T2DM around the world (Malik *et al*., 2010). Regarding glucose pharmacokinetics, our data demonstrate that the blood glucose clearance in the HSu was impaired already 60 min in the GTT (Figure 16A). Moreover Cont rats had significantly lower blood glucose levels than Hsu, 120 min following insulin injection in ITT (Figure 16C). These data suggest that HSu have peripheral insulin resistance. This was further evidenced by HOMA-IR evaluation and by a compensative hyperinsulinemia which was observed in HSU group compared to Cont, to maintain normoglycemia. These glucose intolerance and hyperinsulinemia could signal potential insulin secretory defects that occur before clinical diagnosis of T2DM (Cefalu, 2006). Furthermore, a considerable proportion of whole-body

insulin-stimulated glucose uptake is dependent upon the hepatic insulin-sensitizing substance (HISS) in a pathway mediated by the hepatic parasympathetic nerves (HPNs) and, according to Ribeiro *et al*. (2005), a high-sucrose diet leads to insulin resistance by rapid impairment of such pathway.

Finally one cannot exclude that a putative defective insulin clearance, which is liver-mediated, could be contributing to the observed hyperinsulinemia (Jimenez-Chillaron *et al*., 2005).

On the other hand, there were no significant differences in total body weight, basal glycemia and total cholesterol levels between the Cont and HSu groups. Thus, these results suggest that feeding a high sucrose diet does not induce obesity in lean rats, which is in agreement with several studies (Kanazawa *et al*., 2003; Ribeiro *et al*., 2005; Cao *et al*., 2007; Sumiyoshi *et al*., 2011). Unless fed for a prolonged period of time, high sucrose diets do not appear to lead to excessive weight gain (Chicco *et al*., 2003). Furthermore, some authors affirm that diet-induced insulin resistance is characterized by fasting normoglycemia (Thresher *et al*., 2000), correlated with our results among others (Ribeiro *et al*., 2005; Sumiyoshi *et al*., 2011).

Significant elevations of HSu postprandial glucose and triglycerides (TG) were also observed herein, which is consistent with many other reports (Kanazawa *et al*., 2003; Cao *et al*., 2007; Sumiyoshi *et al*., 2011; Carvalho *et al*., 2012). The high-sucrose diet could elevate plasma TG by increasing the triglyceride secretion rate and/or decreasing the fractional catabolic rate (Kanazawa *et al*., 2003).

Importantly, sucrose is a disaccharide that is cleaved to a 50:50 molar mixture of glucose and fructose in the intestine. After absorption, glucose and fructose enter the portal circulation and are transported to the liver or pass into the general circulation. As fructose is more lipogenic than glucose, because it bypasses a major rate-controlling step in glycolysis, it is converted to fatty acids in the liver at a greater rate (see section 11.1). This might contribute to the increased TG levels seen in Hsu rats. Some authors suggested that increased TG levels would foster its deposition in adipose tissue, liver and muscle, eventually resulting in impaired insulin signaling and dyslipidaemia, increasingly associated with proatherogenic conditions (have to indicate at least one reference here). Other factors related with the fructose moiety of sucrose can contribute to reduced insulin sensitivity such as decreased mitochondrial fatty acid oxidation and the accumulation of hepatic diacylglycerol (DAG), which in turn inhibited insulin signaling via activation of PKC-ζ (Dekker *et al*., 2010). Fructose-induce hyperuricemia is another mechanism implicated to play a role in the metabolic syndrome by inhibiting nitric oxide bioavailability which is required by insulin to stimulate glucose uptake (Nakagawa *et al*., 2006).

Consequently, large amount of evidence suggests that fructose is the primary nutrient mediator of sucrose-induced hypertriglyceridemia, insulin resistance and glucose intolerance (Thresher *et al*., 2000; Gadja *et al*., 2007). Furthermore, fructose feeding has now been shown to alter gene expression patterns, mainly those involved in de novo lipogenesis (eg. carbohydrate regulatory element-binding protein (ChREBP) activity and nuclear SREBP-1), satiety factors in the brain, increase inflammation, reactive oxygen species and portal endotoxin concentrations via Toll-like receptors, and induce leptin resistance (Dekker *et al*., 2010). Nevertheless, sucrose-rich diets instead of fructose alone stems from the fact that they closely mimic the human condition, because in most cases, the dietary exposure to fructose comes through the coingestion of glucose via sucrose (glucose and fructose) or industrial blends of fructose and glucose (high-fructose corn syrup, HFCS) in ratios very similar to sucrose (Dekker *et al*., 2010).

Overall, only fructose (and sucrose containing fructose) has a lipogenic effect in liver and could potentially modify fatty acids' balance in very low density lipoproteins (VLDL) and induce harmful secondary effects like hypertriglyceridemia or insulin resistance (Laville and Nazare, 2008) and has little effect on serum glucose concentrations; whereas dietary glucose increases serum glucose and insulin concentrations in the postprandial state (Shaeffer *et al*., 2009). Thus, the higher postprandial glucose response on the HSu group can be explained by the large amount of available glucose from both sucrose and starch on this diet.

Finally, these data suggest that the consumption of a 35 % sucrose solution for an extended period of time (9 weeks) produces a less beneficial glycemic and insulinemic response and a risk of developing insulin resistance compared with standard-fed animals. This is suggestive of a pre-diabetic state, characterized by fasting normoglycemia accompanied by hyperinsulinemia and impaired insulin-stimulated glucose uptake. In fact, insulin resistance precedes and contributes to the development of T2DM and, even in the absence of hyperglycemia, it is part of the clustering of cardiovascular risk factors termed metabolic syndrome (see Figure 3).

Furthermore, although the main aim was to assess the effect of adding sucrose to the diet, one cannot discard that the total energy intake which was increased by the sucrose consumption, can contribute to the differences seen between the sucrose group and control. In fact, it was also affirmed that other dietary factors, such as caloric excess, may be more important determinants of insulin action than carbohydrate type (Black *et al*., 2006). Finally, animals were given *ad libitum* access to food and drink, so they may have consumed different amounts of energy, which may also have influenced the response to the diet, being a confounding factor.

2. High Sucrose intake induced memory deficits that were accompanied by down-regulation of IR-β and up-regulation of GluR1 and NR1 glutamate receptors

Several studies have linked insulin resistance and T2DM to deficits in hippocampal declarative memory (Strachan *et al*., 1997; Convit, 2005; Winocur *et al*., 2005; Gold *et al*., 2007). Coherently, our results convincingly showed that the metabolic condition resulting from adding sucrose to the diet had a significant influence on the rats' memory performance. HSu group showed normal acquisition of the standard, fixed-location of the platform in the hidden-platform water-maze task as well as similar exploratory activity in the Y-maze test compared to Cont; however, HSu rats were dramatically impaired on hippocampus-dependent spatial memory tasks. In other words, both groups had learned to an equal extent about the location of the platform, but Cont rats showed a strong preference for the quadrant of the pool in which the platform had been located (long-term memory assessment); and, in the Y-maze test (short-term memory assessment), a significantly greater proportion of Cont rats recognized the novel arm as the unvisited arm of the maze compared to HSu. These results strongly suggest that the prediabetic state coincide with cognitive deficits. According to several authors, these memory deficits are probably due to a disruption amongst insulin activity and glucose metabolism (Ristow, 2004; van der Heide *et al*., 2006). Moreover, Gold *et al*. (2007) affirm that hippocampal damage associated memory impairments are most probably the early brain complications of T2DM.

There are few studies addressing specifically the detrimental effects of sucrose in rodents' memory performance (Cao *et al*., 2007; Chepulis *et al*., 2009), nevertheless consistent findings have also been reported for other types of diet-induced metabolic alterations. For instance, Stranahan *et al*. (2008) have shown that mice on hypercaloric resembling Western diets over 8 months had impaired memory, reduced dendritic spine density, and impaired LTP. These adverse effects on brain function were associated with reduced levels of BDNF in the hippocampus and suggest that "Western" diets impair synaptic function and cognition. Furthermore, Wu and colleagues (2003) found that the previous exposure of the rats to a high-fat/refined sugar (HFS) diet, during only 4 weeks, exacerbated the spatial learning and memory impairment induced by traumatic brain injury. Additionally Molteni *et al*. (2002) also found such HFS-induced memory deficits over 8 weeks of consumption (similar exposure length with our study). Moreover, others have also reporter similar findings for high-fat diets over 12 and

20 weeks (Winocur and Greenwood, 1999; McNay *et al*., 2010) and for excessive fructose consumption (18 weeks) (Ross *et al*., 2009).

As previously discussed for peripheral insulin-resistance, we have to distinguish which moiety of the sucrose disaccharide may be the most detrimental for sucrose-induced memory impairments observed in our experimental animals. For example, as fructose crosses the BBB (Cha and Lane, 2009), fructose-specific neuronal effects were evaluated., In facts, fructose-fed rodents showed hippocampal insulin resistance associated with impaired memory retention and decreased LTD formation in hippocampal neurons (Mielke *et al*., 2005; Ross *et al*., 2009). On the contrary, focal application of glucose seems to enhance cognition (Dash *et al*., 2007) by directly or indirectly increasing the release of acetylcholine (Ragozzino *et al*., 1996). Furthermore, fructose-induced hypertriglyceridemia as seen in the HSu group may also contribute to the memory dysfunction observed herein. In support of this idea, direct injection of triglycerides into the brain has detrimental consequences for learning and memory (Farr *et al*., 2008) and affect leptin, ghrelin, and insulin transport through the BBB (Banks *et al*., 2004; Banks *et al*., 2008; Urayama *et al*., 2008). It is noteworthy that along with insulin, leptin and ghrelin enhance hippocampal synaptic plasticity and improves performance of rodents in learning and memory tasks (Harvey, 2007; McNay, 2007). Besides obesity, insulin resistance is associated with fluctuations in such energy regulating signaling peptides (Stylianou *et al*., 2007; Selenscig *et al*., 2010). As DM is associated with leptin resistance (Myers *et al*., 2008), it is possible that impaired leptin signaling contributes to the diet-induced deficits in hippocampal plasticity in the present study, as well as impaired ghrelin transported through the BBB. The involvement of leptin and ghrelin on memory deficits in our model warrants further analysis.

Regarding insulin, chronic peripheral hyperinsulinemia down-regulates BBB insulin receptors and reduces insulin transport into the brain (Wallum *et al*., 1987; Banks, 2004). Based on these facts, we cannot directly correlate HSU peripheral hyperinsulinemic condition to brain hippocampal levels. Therefore, the fact that we did not measure the levels of hippocampal insulin content represents the first limitation of the current study.

However, we cannot exclude the hypothesis that insulin signaling is also affected in the hippocampus thus contributing to cognitive deficits exhibited buy HSu rats. Furthermore, *in vivo* studies have shown that spatial memory is directly related with the expression of hippocampal IRs (Zhao *et al*., 1999; Zhao *et al*., 2004). Furthermore there is recent evidence towards the involvement of the insulin signaling through PI3K in spatial memory (McNay *et al*., 2010). Therefore we set out for the study of the IR-β→IRS-1→PI3K signaling pathway, by measuring their total basal hippocampal levels. Indeed, we found a reduction in IR-β expression for HSu rats compared to Cont. However we report herein an absence of

alterations in the further signaling molecules: IRS-1 and PI3K. Furthermore, as IRS-1 total basal levels were normal we decided to ascertain if there were any differences in insulin-dependent IRS-1 signaling transduction. For that, we measured the IRS-1 $pS^{636/639}$ total levels, a candidate biomarker of brain insulin resistance (Talbot *et al*., 2012), and again, no differences were found between groups. So, besides the downregulation of the insulin receptor, the further signaling pathway seems to be unaffected on basal conditions. One possible explanation is that could be difficult to see the differences in the signaling pathway transduction at basal conditions; maybe should we have stimulated the rats with exogenous insulin prior to sacrifice [or stimulated the hippocampus by insulin intracerebroventricular (ICV) injection], the insulin-induced phosphorylation would have been amplified and thus the differences detectable, as IRS-1 activation includes its recruitment and further phosphorylation by the insulin receptor upon insulin binding.

In line with our results, Youngren *et al*. (2011) found that HFS feeding (8 weeks of exposure) induced insulin resistance in muscle concomitant with diminished IR levels. However they also saw IRS-1 diminished levels, which we did not. It is noteworthy that chronic hyperinsulinemia (e.g. during T2DM and obesity) downregulates IR expression in the BBB and this represents central insulin resistance, as it was reviewed by Craft and Watson (2004). Likewise, T1DM animal models show significantly reduced hippocampus IR protein levels, probably due to a persistent deficiency of insulin leading to a disuse receptor down-regulation (Dou *et al*., 2005).But Winocur and colleagues (2005) found no differences in the hippocampal IR-β expression on hyperinsulinemic ZDF rats, with 6 months of age. Alternatively, an increased IR turnover and/or more frequent receptor internalization may have contributed to a lower level of IR protein (Dou *et al*., 2005). On the other hand, we cannot exclude the fact that, as shown in Figure 6, upon extracellular insulin binding, the insulin receptor can recruit and activate other cellular substracts, including those of the MAPK signaling pathway, also involved in the regulation of learning and memory processes (Cardoso *et al*., 2009; Zhang *et al*., 2010). The status of this signaling pathway was not evaluated in the present study.

Hyperglycemia could also play a role in the impairment of insulin signaling, including underexpression of IR-β. Nevertheless, sucrose-sweetened water did not increase fasting glycemia and thus, glucose neurotoxicity should not be held responsible for the neuronal insulin dysfunction (Tomlinson and Gardiner, 2008). One of the major key players that translate hyperglycemia into glucotoxicity is RAGE signaling. RAGE upregulation fuels oxidative stress and inflammation pathways, leading to sustained cellular dysfunction (see section 6.3.1). Consistent with normoglycemia, HSu RAGE expression levels were comparable to Cont. Furthermore we did not found any signs of hippocampal oxidative stress in such early pre-

diabetic stage as seen by normal HNE levels in HSU hippocampi when compared with Cont. rats. Additionally, no signs of inflammation were found in HSu hippocampi, as seen by normal TNF-α levels compared with Cont. animals. Commonly, systemic inflammation exacerbates CNS inflammation and correlates with cognitive decline (Leonard and Myint, 2006). Interestingly we failed to see signs of astrogliosis as shown by similar GFAP and GS levels between hippocampi from HSU and Cont, unlike other studies which found increased hippocampal astrogliosis for both T1DM and T2DM animal models (Coleman *et al*., 2004; Duarte *et al*., 2012), excluding this hypothesis as a possible influencing factor in HSu memory deficits as others have found LTP alterations in GFAP knockout mice, probably involved in such learning and memory processes (McCall *et al*., 1996). The possible involvement of synaptotoxicity and astrogliosis (see section glial cells) in the mechanism of diabetes-induced memory impairment is supported by the main finding of the Duarte *et al*., (2012) work, which demonstrate that long-term caffeine consumption prevented diabetes-associated memory impairments, astrogliosis and loss of nerve terminal markers in the hippocampus. Along with this we did not found either any alteration in band intensity for synaptophysin between groups, opposed to other studies which reported a reduction of this nerve terminal marker in a diet-induced insulin resistant animal model (Stranahan *et al*., 2008).

Therefore, one can suggest that HSu memory impairments are independent of inflammation and oxidative stress mechanisms. Other mechanism should be sought, namely glutamatergic transmission that underlies neuronal synaptic plasticity. Interestingly, Carvalho *et al*. (2012) have recently reported that wild-type mice fed 20 % sucrose-sweetened water for 7 months resulted in metabolic alterations associated to diabetes, which contributed to the development of AD-like pathologic features, namely a significant increase in amyloid β protein levels. Moreover, high-fat or high-sucrose diet-induced insulin resistance promoted amyloidosis and was also associated with poor learning and memory performance in AD mouse models (Ho *et al*., 2004; Cao *et al*., 2007). This is consistent with large population studies that confirm such an association between T2DM molecular and biochemical features and AD (Leibson *et al*., 1997; Ott *et al*., 1999), a neurological disorder characterized by profound memory loss and progressively cognitive and behavioral decline. Data from clinical, epidemiological and animal studies have suggested that excessive energy intake adversely affects the brain, particularly during aging. Studies suggest that individuals with a high energy intake are at increased risk of Alzheimer's disease (Luschsinger *et al*., 2002). Although we did not measure Aβ levels we believe that 9 weeks of sucrose-sweetened water consumption has not been sufficiently long-enough to induce amyloidosis as in Carvalho *et al*. (2012). Furthermore, in the CNS, Aβ is another known RAGE ligand, involved in RAGE upregulation and

pro-oxidant/inflammatory events (Peppa *et al*., 2003; Ding and Keller, 2005; Ramasay *et al*., 2005; Sparvero *et al*., 2009), which we did not found to be increased in HSu rats.

Animal studies have shown that high-calorie diets impair the structure and function of the hippocampus, a brain region critical for learning and memory (Winocur and Greenwood, 1999; Molteni *et al*., 2002; Wu *et al*., 2004; Farr *et al*., 2008). The adverse effects of high calorie diets on learning and memory have been associated with impaired hippocampal synaptic plasticity and neurogenesis (Farr *et al*., 2008; Lindqvist et al., 2006).

In the present work we found no alterations in PSD-95, one of the fundamental glutamatergic scaffolding proteins accompanied by an upregulation of GluR1 and NR1 subunits in hippocampal total extracts of HSu rats compared to Cont. Along these lines, we have to clarify that we measured total protein levels in total hippocampal extracts by Western-Blot analysis and thus, we could ascertain GluR1 and NR1 expression in the whole hippocampus, but not its localization or activity. Synaptosomal extracts as well as imunnohistochemistry protocols could provide a better understanding of the role of this AMPAR and NMDAR subunits upregulation, seen in HSu rats, on functioning and localization.

Interestingly, Turringiano and colleagues showed that inhibition of synaptic transmission upregulates AMPAR transcription (Turrigiano et al., 1998), presumably as a means of compensation. The interaction between insulin synaptic plasticity effects and DM has been primarily demonstrated in the context of T1DM rodent model, where a significant deficit in LTP was observed (Biessels *et al*., 1996; Stranahan *et al*., 2008). Indeed, DM seems to induce cognitive impairment and defects of LTP in the hippocampus, an important mechanism of learning and memory in mammals, known to require regulation of the glutamate receptor properties (Kamal *et al*., 2000; Huang *et al*., 2010). Diabetic alterations on both excitatory and inhibitory neurotransmission (Kamal *et al*., 2000) are potentially under the direct regulation of insulin (McNay and Recknagel, 2011). Coherently, our results demonstrate that HSu rats had abnormal glutamate receptors subunits levels resulting from peripheral hyperinsulinemia among other metabolic alterations. These alterations may be responsible for LTP/LDP impairment and consequently memory deficits. Moreover, abundant IR are found in both cell bodies and synapses and besides very little is known about the functional significance of synaptic IR in the neurons, several studies have drawn links between IR signaling and plasma membrane expression of ion channels as well as neurotransmitter receptors at the CNS synapses (Chiu and Cline, 2010). Thus, we might speculate that HSu IR-β downregulation may be involved in an increase in GluR1 and NR1 subunits expression. Other possible responsible mechanism for glutamate receptors upregulation and memory deficits is glutamate neurotoxicity found by other authors (Atlante *et al*., 2001; Valastro *et al*., 2002). In fact, the

breakdown of glucose yields a number of compounds (such as acetyl CoA, glutamate and ATP) that are necessary for normal neuronal structure and function. Acetyl CoA is a constituent of acetylcholine synthesis, that together with glutamate constitute neurotransmitters involved in the acquisition and maintenance of behavior parameters (e.g. learning and memory capacities) (Shulingkamp *et al*., 2000). This is consistent with postprandial hyperglycemia but not with hippocampal neurotoxicity as we did not found any signs of astrogliosis, oxidative stress or inflammation.

VI. Conclusion

In the present study, we confirm the deleterious effect of adding sucrose to a normal rodent diet, resulting in a pre-diabetic state, mainly characterized by fasting normoglycemia, postprandial hyperinsulinemia, insulin resistance, hypertriglyceridemia and impaired glucose tolerance compared with the cont rats. These metabolic changes were associated with memory impairments which were underlied by reduced IR-β levels as well as GluR1 and NR1 increased expression in hippocampi from Hsu animals. Interestingly, we failed to see evidences of hippocampal neurotoxicity. Therefore, one might conclude that deficient central insulin signaling together with altered glutamatergic transmission might interact to trigger memory deficits in the HSu rats.

These data underscore the potential role of dietary sugar in the early central diabetic complications and suggest that controlling the consumption of sugar-sweetened beverages may be an effective way to curtail the risk of developing T2DM. Furthermore, identifying the mechanisms by which insulin and glutamatergic signaling contribute to "diabetic encephalopathy" is of paramount clinical relevance.

VII. References

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