DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

# Effects of vitamin D deficient and high-fat diets in the diabetic brain: focus on insulin signaling

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica da Professora Doutora Paula Moreira e do Professor Doutor António Moreno (Universidade de Coimbra).

Guilherme Alvarinhas de Assis Loureiro

2015

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# Effects of vitamin D deficient and high-fat diets in the diabetic brain: focus on insulin signaling

Trabalho realizado no grupo "Cell Metabolism and Quality Control" do consórcio CNC.IBILI, sob a orientação laboratorial da Doutora Cristina Carvalho

Guilherme Alvarinhas de Assis Loureiro

2015

Aos meus pais,

À Manuela,

## Agradecimentos

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**Um grande obrigado a todos vocês!**

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# I – List of Abbreviations

- $1,25(OH)_2D$ – 1,25 dihydroxyvitamin D
- 25(OH)D – 25 hydroxyvitamin D
- 7-DHC – 7-dehydrocholesterol
- ABC – avidin-Biotin-Peroxidase complex
- AchE – acetylcholinesterase
- AD – Alzheimer's disease
- AGEs – advanced glycation end products
- AKT – Protein Kinase B
- AMPK – AMP-activated protein kinase
- ANOVA – analysis of variance
- ApoE – alipoprotein E
- ATP – adenosine triphosphate
- Aβ – amyloid beta peptide
- AβPP – amyloid beta protein precursor
- BAD – Bcl-2-associated death promoter protein
- BBB – blood brain barrier
- BCA – bicinchoninic acid
- BDNF – brain-derived neurotrophic factor
- BSA – bovine serum albumin
- $Ca^{2+}$ – calcium
- CaMKIIδ – calcium/calmodulin-dependent protein kinase IIδ
- cAMP – adenosine 3',5'-cyclic monophosphate
- CDK5 – cyclin-dependent kinase 5

- CNS – central nervous system
- CREB – cAMP response element-binding protein
- $Cu^{1+}$ – cuprous cation
- $Cu^{2+}$ – cuprous
- DBP – vitamin D binding protein
- DNA – deoxyribonucleic acid
- DTT – dithiothreitol
- ECF – enhanced chemifluorescense
- EGTA – ethylene glycol tetraacetic acid
- ELISA – enzyme-linked immunosorbent assay
- ERK – extracellular-signal regulated kinase
- FELASA - Federation of Laboratory Animal Science Association
- FVB – Friend virus-b type mouse
- GABA – gamma-aminobutyric acid
- GDNF – glial cell line-derived neurotrophic factor
- GK – Goto-Kakizaki
- Glut 1 – Glucose transporter 1
- Glut 3 – Glucose transporter 3
- Glut 4 – Glucose transporter 4
- GSK3β – Glycogen synthase kinase 3 beta
- $H_2O_2$ – hydrogen peroxide
- $HbA_{1c}$ – glycated hemoglobin
- HDL – high-density lipoprotein
- HF-C – high-fat coconut fat
- HF-F – high-fat fish oil

- HF-L – high-fat lard
- HF-O – high-fat olive oil
- HFD – high-fat diet
- HRP – horseradish peroxidase
- IDE – insulin degrading enzyme
- IDF – international diabetes federation
- IGF-1 – insulin-like growth factor 1
- IGF-1R – insulin-like growth factor 1 receptor
- iNOS – inducible nitric oxide synthase
- IR – insulin receptor
- IRS – insulin receptor substrate
- IRS1 – insulin receptor substrate 1
- IRS2 – insulin receptor substrate 2
- JNK – c-Jun N-terminal kinases
- LTD – long-term depression
- LTP – long-term potentiation
- MAPK – mitogen-activated protein kinase
- MAPK/ERK 1/2 – mitogen-activated protein kinase / extracellular-signal regulated kinase 1/2
- MARRS – membrane associated rapid response steroid-binding
- MOPS – 3-(N-morpholino) propanesulfonic acid
- mRNA – messenger ribonucleic acid
- mTOR – mammalian target of rapamycin
- $NA_2EDTA$ – ethylenediaminetetraacetic acid
- $Na_3VO_4$ – sodium orthovanadate

- $Na_4P_2O_7$ – sodium pyrophosphate
- NaCl – sodium chloride
- NF-kB – factor nuclear-kappa B
- NGF – nerve growth factor
- NMDA – N-methyl-D-aspartate
- NO – nitric oxide
- NT-3 – neurotrophin-3
- OD – optical density
- OXPHOS – oxidative phosphorylation
- PD – Parkinson's disease
- PI3K – phosphatidylinositol 3-kinase
- PKA – protein kinase A
- PKC – protein kinase C
- PLC – phospholipase C
- PMSF – phenylmethanesulfonylfluoride
- PSD95 – postsynaptic density-95 protein
- PVDF – polyvinylidenedifluoride
- RAF – Rapidly accelerated fibrosarcoma
- RAF/MAPK - Rapidly accelerated fibrosarcoma / mitogen-activated protein kinase
- RAGE – receptor for advanced glycation end products
- ROS – reactive oxygen species
- SAFE – Scientific Animal Food & Engineering
- SDS – sodium dodecyl sulfate
- SEM – standard error of the mean

- Shc – Src-homology-2-containing protein
- SNAP25 – synaptosomal-associated protein 25
- STZ – streptozotocin
- T1D – type 1 diabetes
- T2D – type 2 diabetes
- TBS-T – tween in tris-buffered saline
- TMB – tetramethylbenzidine
- UV – ultraviolet
- VDR – vitamin D receptor
- VDREs – vitamin D response elements
- vitD – vitamin D

# II – Abstract

Type 2 diabetes (T2D) is a chronic metabolic disorder that reached epidemic proportions, affecting almost 390 million people around the globe. Most cases of T2D result from unhealthy eating habits. In fact, consumption of high-fat diets and high intake of saturated fat are associated with an increased risk of obesity, metabolic syndrome and T2D. Additionally, hypovitaminosis D has also been described as a major health problem. Interestingly, a common characteristic found in T2D patients is the low levels of serum vitamin D. Vitamin D is a fat-soluble hormone that shares common signaling pathways with insulin, modulating glucose homeostasis and organs function such as the brain, among other things. Given the growing evidence that T2D is a risk factor for the development of neurodegenerative disorders, particularly Alzheimer's disease (AD), we hypothesize that high-fat and/or low vitamin D diets potentiate brain insulin signaling alterations in T2D brain cortex and hippocampus predisposing to neurodegenerative events. To test our hypothesis, we evaluated the insulin signaling pathway and its effects in synaptic integrity and AD-related hallmarks in brain cortex and hippocampus of 6-month-old Goto-Kakizaki (GK) rats, a commonly used non-obese T2D animal model, subjected to distinct dietary regimens as follows: 1) GK rats fed a standard diet (GK); 2) GK rats fed a low vitamin D diet (GK low vitD); 3) GK rats fed a high-fat diet (GK HFD) and 4) GK rats fed a high-fat, low vitamin D diet (GK HFD low vitD). Animals were subjected to these diets during 4 months. 6-month-old Wistar rats were used as control animals. Besides the biochemical characterization of the animals, brain cortical and hippocampal

homogenates were used to evaluate the protein levels of glucose transporters, insulin/insulin-growth factor 1 (IGF-1) levels and respective receptors (IR and IGF-1R), vitamin D receptor (VDR), several molecules modulated by insulin/IR activation, markers of synaptic integrity and AD-related hallmarks. GK rats showed increased fasting glycemia and glycated hemoglobin ($HbA_{1C}$) and a decrease in body weight, typical features of this animal model. HFD increased body weight and $HbA_{1C}$, total cholesterol, non-high-density lipoprotein cholesterol and triglycerides levels. Interestingly, low vitD diet attenuated HFD-induced alterations particularly in body weight and total cholesterol, non-HDL and triglycerides levels. HFD and low vitamin D diet altered brain glucose transporters, particularly the insulin sensitive glucose transporter 4 (Glut 4), whose levels in the hippocampus were decreased by low vitD and/or HDF while in brain cortex, low vitD increased the levels of Glut 4, an effect that was abrogated by the combination with HFD. In brain cortex the combination HFD and low vitD decreased the levels of VDR and cholesterol and increased IR. Also in brain cortex, low vitD diet and HFD per se increased insulin levels while in the hippocampus only HFD increased the levels this hormone. HFD also increased phosphorylated insulin receptor substrate 2 (ρIRS2) levels in brain cortex. Additionally, in both brain cortex and hippocampus, HFD increased the levels of phosphorylated extracellular signal-regulated kinases (ρERK) and phosphorylated tau, these effects being abrogated by low vitD. Phosphorylated protein kinase B (ρAKT) was decreased by HFD in brain cortex while in the hippocampus the levels of this protein were decreased by the combination HFD and low vitD. The levels of the phosphorylated form of 5’ adenosine monophosphate-activated protein

kinase (ρAMPK) and active protein kinase A (PKA) were decreased by low vitD diet and/or HFD in both brain cortex and hippocampus. In the hippocampus, HFD increased the levels of phosphorylated mammalian target of rapamycin (ρmTOR), an effect that was abolished by the combination HFD and low vitD. Interestingly, the levels of phosphorylated cAMP response element-binding protein (ρCREB) were significantly increased in brain cortex by the combination HFD and low vitD while in the hippocampus the opposite effect was observed. The combination of HFD and low vitD also decreased the levels of inactive glycogen synthase kinase 3 beta (GSK3β) and p35 in the hippocampus. No statistically significant alterations were observed in all the other parameters analyzed. Our findings demonstrate that high-fat and/or low vitD diets affect cerebral insulin signaling pathway. Nevertheless, some compensation mechanisms seem to occur to avoid metabolic disturbances-induced brain damage.

**Keywords:** Brain cortex and hippocampus, high-fat diet, insulin signaling, low vitamin D diet, neurodegeneration, type 2 diabetes

# III – Resumo

A diabetes tipo 2 (T2D) é uma doença metabólica crónica que atingiu proporções epidémicas, afetando cerca de 390 milhões de pessoas em todo o mundo. A maior parte dos casos de T2D resultam de hábitos alimentares pouco saudáveis. De facto, a ingestão excessiva de alimentos ricos em gorduras, nomeadamente gorduras saturadas, está associada a um aumento do risco de obesidade, síndrome metabólico e T2D. A hipovitaminose D também está associada a várias doenças. Curiosamente, a T2D está normalmente associada a níveis baixos de vitamina D no plasma de indivíduos acometidos pela doença. A vitamina D é uma hormona lipossolúvel que partilha vias de sinalização com a insulina, modulando a homeostasia da glucose e o funcionamento de vários órgãos, como o cérebro, entre outras coisas. Uma vez que a T2D é um importante fator de risco para o desenvolvimento de doenças neurodegenerativas, nomeadamente a doença de Alzheimer (AD), colocamos a hipótese de que uma dieta gorda e/ou uma dieta deficiente em vitamina D afeta(m) as vias de sinalização da insulina no cérebro diabético predispondo para processos neurodegenerativos. Para testar a nossa hipótese, avaliámos as alterações nas vias de sinalização da insulina e os seus efeitos na integridade sináptica e marcadores relacionados com AD no córtex cerebral e no hipocampo de ratos Goto-Kakizaki (GK), um modelo animal não obeso de T2D comummente utilizado, com 6 meses de idade, sujeitos a dietas distintas: 1) ratos GK sujeitos a uma dieta regular (GK); 2) ratos GK sujeitos a uma dieta deficiente em vitamina D (GK low vitD); 3) ratos GK sujeitos a uma dieta gorda (GK HFD) e 4) ratos GK sujeitos

a uma deita gorda, deficiente em vitamina D (GK HFD low vitD). Os animais estiveram sujeitos a estas dietas durante 4 meses. Foram usados ratos Wistar da mesma idade como animais controlo. Além da caracterização bioquímica dos animais, foram utilizados homogeneizados de córtex cerebral e hipocampo dos ratos para avaliar os níveis proteicos dos transportadores de glucose, insulina/fator de crescimento semelhante à insulina 1 (IGF-1) e dos respetivos recetores (IR e IGF-1R), recetor da vitamina D (VDR), várias moléculas moduladas pela ativação de insulina/IR, marcadores de integridade sináptica e marcadores relacionados com AD. Os ratos GK apresentaram um aumento da glicemia em jejum, na hemoglobina glicada ($HbA_{1C}$) e uma diminuição do peso corporal, características deste modelo animal. A dieta gorda aumentou o peso corporal e os níveis plasmáticos de $HbA_{1C}$, colesterol total, colesterol não-HDL (lipoproteínas de alta densidade) e triglicerídeos. Curiosamente, a dieta deficiente em vitamina D atenuou as alterações induzidas pela dieta gorda relativamente ao peso corporal e aos níveis de colesterol total, colesterol não-HDL e triglicerídeos. A dieta gorda e a dieta deficiente em vitamina D alteraram os níveis dos transportadores de glucose, particularmente o transportador de glucose 4 (Glut 4), o qual é sensível à insulina, cujos níveis no hipocampo foram diminuídos por ambas as dietas, independentemente ou em conjunto, enquanto no córtex cerebral, a dieta deficiente em vitamina D aumentou os níveis de Glut 4, um efeito que foi anulado pela combinação com a dieta gorda. No córtex cerebral a combinação da dieta gorda com deficiência em vitamina D diminuiu os níveis de VDR e de colesterol e aumentou os níveis de IR. No córtex cerebral, a deficiência em vitamina D ou a dieta gorda aumentaram os níveis de insulina

enquanto no hipocampo apenas a dieta gorda aumentou os níveis desta hormona. A dieta gorda aumentou ainda os níveis do substrato do recetor de insulina 2 fosforilado (ρIRS2) no córtex cerebral. Tanto no córtex cerebral como no hipocampo, a dieta gorda aumentou os níveis das proteínas cinase regulada por sinais extracelulares fosforilada (ρERK) e tau fosforilada, sendo estes efeitos anulados pela deficiência em vitamina D. A proteína cinase B (AKT) fosforilada foi diminuída pela dieta gorda no córtex cerebral enquanto no hipocampo os níveis desta proteína estavam diminuídos pela combinação da dieta gorda com deficiência em vitamina D. Os níveis proteicos da proteína cinase ativada por 5' adenosina monofosfato fosforilada (ρAMPK) e da proteína cinase A (PKA) ativa foram diminuídos pela dieta deficiente em vitamina D e/ou pela dieta gorda no córtex cerebral e hipocampo. No hipocampo, a dieta gorda aumentou os níveis do alvo da rapamicina nos mamíferos fosforilado (ρmTOR), um efeito que foi abolido pela combinação da dieta gorda com deficiência em vitamina D. Curiosamente, os níveis do elemento de resposta à adenosina monofosfato cíclico fosforilado (ρCREB) foram significativamente aumentados no córtex cerebral pela combinação de ambas as dietas, enquanto no hipocampo se observou o efeito oposto. A combinação de uma deita gorda e deficiência em vitamina D também diminuiu os níveis de cinase 3 beta da sintetase do glicogénio (GSK3β) e de p35 no hipocampo. Não foram observadas alterações estatisticamente significativas nos outros parâmetros analisados. Estes resultados mostram que uma dieta gorda e/ou deficiente em vitamina D afeta(m) as vias de sinalização da insulina no cérebro. No entanto, foram também observados alguns mecanismos de compensação que possivelmente visam limitar a

lesão cerebral provocada pelas alterações metabólicas associada T2D e/ou dietas desequilibradas/hipercalóricas.

**Palavras-chave:** Córtex cerebral e hipocampo, diabetes tipo 2, dieta com baixo teor de vitamina D, dieta gorda, neurodegenerescência, sinalização da insulina

# *Chapter 1 – Introduction*

## 1.1 – Diabetes

Diabetes is a complex chronic metabolic disease with disturbing epidemic proportions (Sebastiao et al., 2014), spreading rapidly worldwide. With an estimated incidence of 387 million people worldwide, according to the International Diabetes Federation (IDF), it is predicted an increase of 205 millions new cases until 2035 **(Figures 1.1 and 1.2)**. Besides unhealthy lifestyle habits that include low physical activity and high calories intake that frequently lead to obesity, the increasing incidence of diabetes can also result from population growth and increased life expectancy (van Eersel et al., 2013).

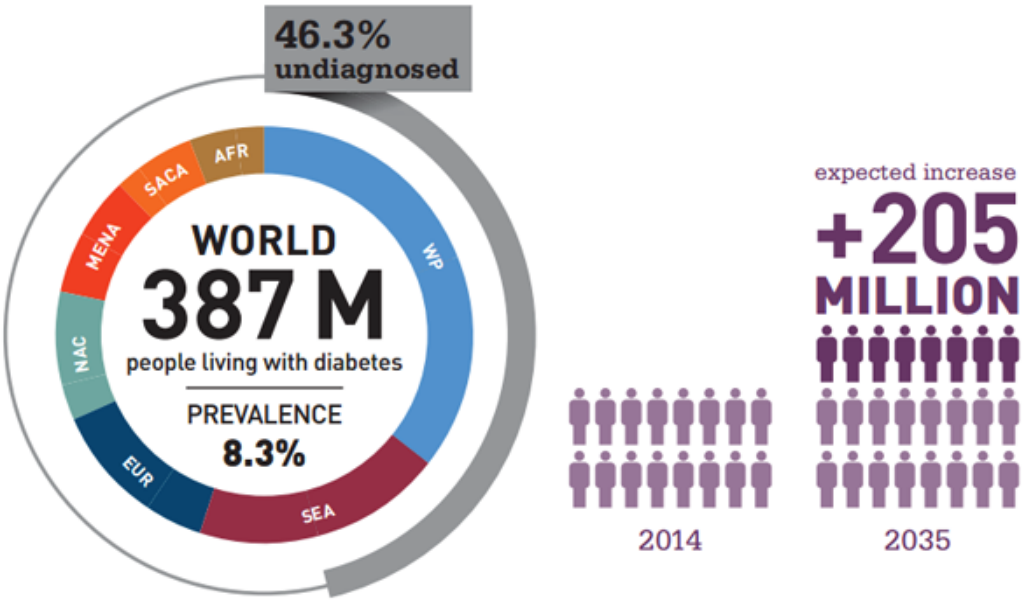


**Figure 1.1 –** ***Diabetes mellitus incidence worldwide.*** Prevalence of diabetes in 2014 and predicted incidence in 2035 (from www.idf.org).

According to the National Diabetes Observatory (2014), 13% of the Portuguese population between 20 and 79 years old has diabetes, with almost 50% (5,7% of this age group) of cases being undiagnosed.

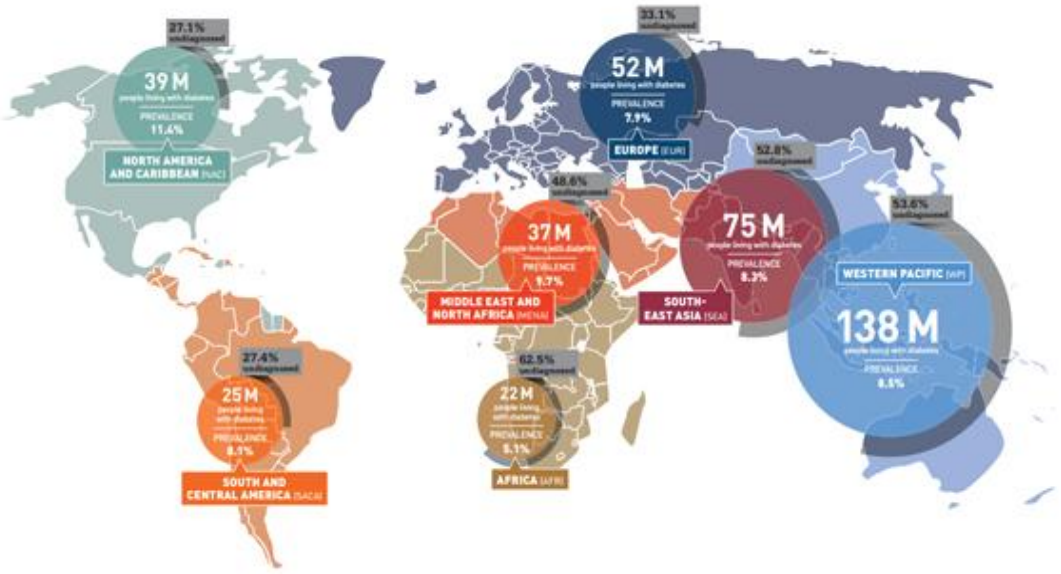


**Figure 1.2 – *Geographic distribution of diabetes.*** World distribution of diabetes cases (from www.idf.org)

There are two main types of diabetes: type 1 diabetes (T1D) and type 2 diabetes (T2D). Both types are characterized by chronic hyperglycemia (Correia et al., 2012) and dysfunctional energy metabolism, due to insulin deficiency and/or loss of insulin action (Holt et al., 2010, Umegaki, 2012, American Diabetes, 2014). Chronic hyperglycemia leads to long-term complications because it negatively affects several organs such as the brain, kidney, eyes and heart (Correia et al., 2012, American Diabetes, 2014).

The incidence of diabetes, particularly T2D, increased dramatically in the last years mainly due to unhealthy lifestyles such as poor diets and lack of physical exercise (Moreira, 2013). Indeed, a connection between genetics,

environmental factors and predisposition and diabetes as been established. For example, it has been demonstrated that defects in human leukocyte antigen class II genes, that regulate the immune response, may play a role in T1D while the hepatocyte nuclear factor 1α, which influences insulin transcriptional activity and secretion (Holt et al., 2010) may be involved in T2D.

In the next chapters we will give an overview about the two main types of diabetes giving focus to T2D and its role as one of the main risk factors for neurodegenerative diseases.

### 1.1.1 – Type 1 diabetes: insulin deficiency

In most people with T1D, the high levels of blood glucose are caused by the autoimmune destruction of pancreatic β-cells leading to the loss of insulin production (Atkinson and Eisenbarth, 2001, Zimmet et al., 2001, Holt et al., 2010). Epidemiologically, T1D represents 5 to 10% of all diabetes cases (Daneman, 2006) with higher incidence in younger people. However, recent studies revealed that T1D could also occur in older people. In fact, 5 to 30% of diabetes cases initially diagnosed as T2D were actually T1D (Atkinson and Eisenbarth, 2001).

The incidence of T1D seems to be related not only with genetic but also with environmental factors such as diet, psychological stress or viruses, among others (Holt et al., 2010, Skyler, 2012).

The management of T1D is based on constant monitoring of blood glucose levels and insulin injections to suppress insulin deficiency, a therapy

that is associated with hypoglycemic episodes and glycemic fluctuations, which may affect the structure and function of several organs (Daneman, 2006).

### 1.1.2 – Type 2 Diabetes: defects in insulin action and secretion

T2D is an age-related disease and the most common form of diabetes, representing up to 90-95% of all diabetes cases (Umegaki, 2012, Moreira, 2013, Nunes et al., 2014). Insulin resistance, commonly accompanied by progressive impairment in insulin secretion, due to a gradual dysfunction of pancreatic β-cells, is one of the main characteristics of T2D (Butler et al., 2003, Umegaki, 2012, Nunes et al., 2014). As a compensation mechanism, there is an overproduction of insulin leading to hyperinsulinemia and pancreatic β-cells exhaustion (Mehran et al., 2012). As a consequence of an insulin resistant state, which implies a less effective response to insulin, body glucose metabolism is largely affected on target tissues such as the liver, adipose tissue, skeletal muscle and also the brain (Umegaki, 2012, Nunes et al., 2014).

Apart from aging, sedentary lifestyle and obesity are also major risk factors for T2D development (Stumvoll et al., 2005). In fact, obesity leads to an 80-85% increase in the risk of developing T2D through processes like inflammation and increased insulin resistance (Stumvoll et al., 2005, Holt et al., 2010). In obesity, increased free fatty acids may also interfere with pancreatic β-cells function due to chronic exposure of these cells to fatty

acids, which may impair insulin secretion and decrease insulin biosynthesis (Holt et al., 2010). Moreover, a feedback loop occurs with hyperglycemia-derived reactive oxygen species (ROS) leading to pancreatic β-cells dysfunction and death, through nuclear factor kappaB (NF-κB) activation and islet amyloid polypeptide (also known as amylin) accumulation (Butler et al., 2003, Stumvoll et al., 2005).

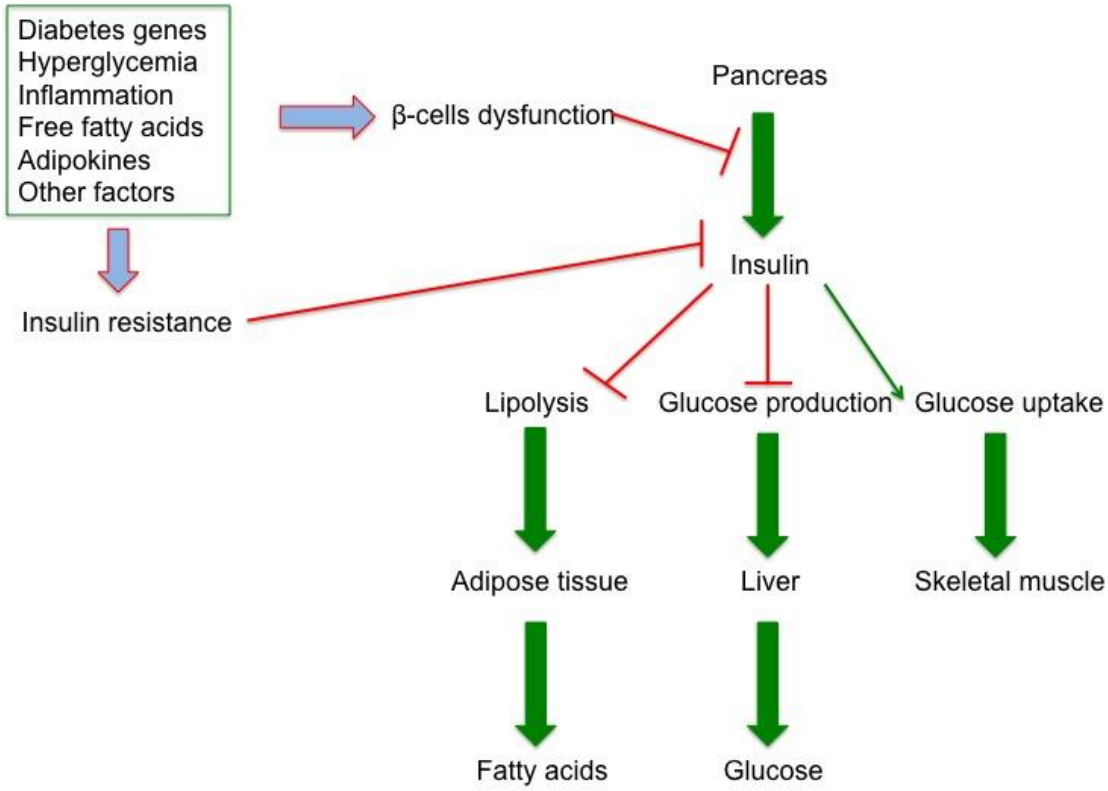


**Figure 1.3 –** ***Effects of insulin resistance and hyperglycemia.*** In normal conditions, insulin inhibits lipolysis, decreasing fatty acids release, glucose production in the liver and promotes glucose uptake by skeletal muscle and adipose tissue. Several factors associated to T2D (e.g. genetic risk factors, adipokines, inflammation) affect both insulin secretion and action. Insulin resistance leads to an increase in the blood levels of free fatty acids and glucose, among other things, aggravating insulin resistance (adapted from Stumvoll et al., 2005).

## 1.1.3 – The complications of diabetes

Diabetes is associated with several macrovascular (stroke, cerebrovascular disease, heart disease and peripheral vascular disease) and microvascular (retinopathy, nephropathy and neuropathy) complications (Daneman, 2006, Holt et al., 2010) **(Figure 1.4).** In general, poor glycemic control, hypertension and hyperlipidemia are the factors underlying the development of these complications (Daneman, 2006, Holt et al., 2010).

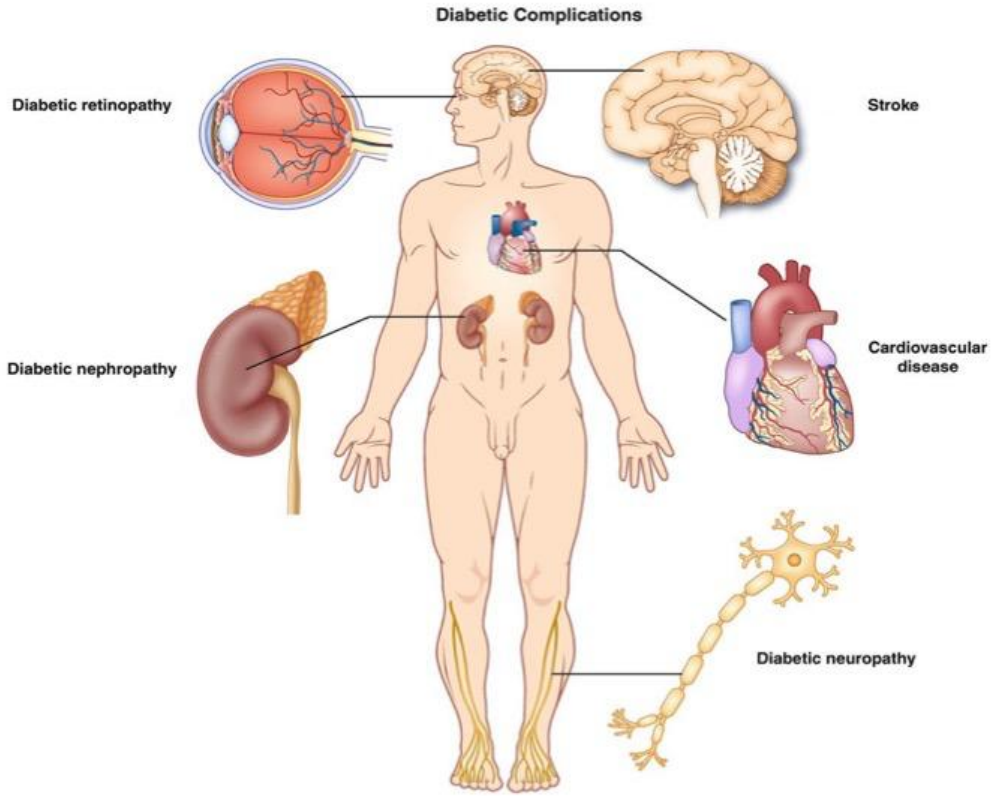


**Figure 1.4 – *Diabetes-associated complications.*** Diabetes has multiple deleterious effects throughout the body. Macrovascular alterations are associated with stroke, cardiovascular disease and neuropathy while microvascular alterations are associated with diabetic retinopathy and nephropathy, among other consequences of diabetes (adapted from Skyler, 2012).

Of relevance to our work, are diabetic encephalopathy, cognitive decline and dementia (Sima, 2010). Brain defects promoted by diabetes include alterations in structure and function such as brain atrophy, impaired cerebral signal conduction, neurotransmission and synaptic plasticity and deficits in learning and memory abilities (Sima, 2010). Diabetes-associated alterations in the central nervous system (CNS) have been widely scrutinized.

There is a hypothesis that states a close association between diabetes, specially T2D and neurodegenerative diseases such as Alzheimer's disease (AD). In fact, it is described that T2D patients have a 50% to 150% higher risk of developing dementia. Additionally, T2D and AD seem to share several features as, for example, impaired glucose metabolism, vascular and mitochondrial dysfunction, oxidative stress and amyloidogenesis (Arab et al., 2011, Correia et al., 2012). In 2010, there were 35,6 million people living with dementia with a predicted increase to 115,4 million in 2050, with AD prevailing and accounting for 60% to 80% of all cases (Li et al., 2015).

As a highly metabolic active tissue, that uses glucose as the main energy substrate, brain is expected to be greatly affected by diabetes (Correia et al., 2011, Cardoso et al., 2013).

Indeed, hyperglycemia induces several metabolic alterations leading to oxidative phosphorylation (OXPHOS) impairment (Correia et al., 2012, Cardoso et al., 2013, Blazquez et al., 2014), increased oxidative stress (Cardoso et al., 2013) and damage (Duarte et al., 2000, Correia et al., 2012) and advanced glycation end products (AGEs) formation that coupled with free radicals exacerbate oxidative damage and an unbalance between pro-oxidants and antioxidants (Umegaki, 2012). However, some compensatory

mechanisms may occur, particularly in the early stages of diabetes, in order to overcome diabetes-associated alterations (Santos et al., 2014).

Patients and animal models of diabetes, particularly T2D, exhibit cognitive impairments characterized by a decline in memory and learning abilities that increase the risk of developing dementia (e.g. AD) (Cardoso et al., 2009, Sima, 2010, Candeias et al., 2012, Carvalho et al., 2013a, 2015, Sridhar et al., 2015). These cognitive defects are intimately associated to neuronal degeneration and loss (Hussain et al., 2014).

Insulin resistance seems to be a major contributor for diabetes-related brain alterations. Indeed, insulin of both peripheral and central origin, and insulin receptors (IR) have been observed in several brain regions and insulin signaling play a myriad of functions in the CNS (Cardoso et al., 2009, Duarte et al., 2012). This aspect will be further discussed in the next subsection.

Moreover, oxidative stress has also been described as a characteristic of diabetic brain and AD (Correia et al., 2012, Mondragon-Rodriguez et al., 2013). Previous studies from our laboratory demonstrated that T2D and AD rodent models present similar cognitive anomalies, mitochondrial alterations, increased levels of oxidative stress and damage, and vascular/endothelial dysfunction (Carvalho et al., 2012, 2013a). Interestingly, AD and T2D models also presented a defect in nitric oxide (NO)-dependent vasodilation of the aorta that was related with increased inflammation and oxidative stress, including a significant increase in the levels of the receptor for advanced glycation end products (RAGE) (Sena et al., 2015). Also, in AD brain, the presence of AGEs is a common observation that is closely associated to

impaired energy metabolism and increase oxidative stress (Dukic-Stefanovic et al., 2001).

### 1.1.4 – Brain insulin and its "neuro" importance

Insulin is a polypeptide mainly synthesized in pancreatic β-cells, however, a small portion is also synthetized in the brain (Schechter et al., 1996). Besides its peripheral and crucial function in maintaining glucose homeostasis, it is widely known that functional insulin signaling pathways regulate a broad spectrum of cellular mechanisms that are essential for normal brain functioning (Cardoso et al., 2009, Correia et al., 2011, Candeias et al., 2012, 2012, Sebastiao et al., 2014). Due to its neuromodulatory and neuroprotective effects, insulin also protects against cognitive impairment associated with diabetes, a situation that is more common in the elderly (Biessels and Kappelle, 2005, van Eersel et al., 2013, Blazquez et al., 2014). Insulin pleiotropic actions are modulated by binding to its receptors. Insulin binding to IR promotes an autophosphorylation of the intracellular domain of the receptor, activating the intrinsic tyrosine kinase activity. This event triggers the phosphorylation of several endogenous substrates, including insulin receptor substrates (IRS). Then, two main pathways can be activated: the phosphatidylinositol 3-kinase (PI3K) pathway and the mitogen-activated protein kinases (MAPK/ERK1/2) pathway.

The MAPK/ERK1/2 pathway is responsible for the activation of several transcription factors, modulating protein expression, and consequently, regulating the processes underlying learning and memory (Cardoso et al.,

2009). In fact, insulin signaling seems to modulate long-term potentiation (LTP) and long-term depression (LTD) as well as synaptic plasticity. Besides, insulin also regulates neurotransmitter release, the activity of excitatory and inhibitory receptors such as glutamate and γ-aminobutyric acid (GABA) and N-methyl-D-aspartate (NMDA) receptors. Moreover, insulin also activates protein kinase B (AKT), via PI3K, which regulates pro-survival mechanisms. Indeed, AKT regulates the phosphorylation of glycogen synthase kinase 3β (GSK3β) and the mammalian target of rapamycin (mTOR) (Cardoso et al., 2009). When activated, GSK3β inhibits the activation of several cell survival transcription factors, promoting neuronal death. Therefore, GSK3β inactivation by AKT is crucial for neuronal survival. The PI3K pathway also promotes glucose uptake by facilitating the translocation of glucose transporter 4 (Glut 4) to the plasma membrane.

From the above, it is easy to infer an involvement of insulin signaling dysfunction in the development of dementia, particularly in T2D patients. Prolonged peripheral hyperinsulinemia seems to affect the blood-brain barrier (BBB) and IR structure and/or function, inducing an insulin resistance state in the brain (Kim et al., 2011a, Correia et al., 2012). Central insulin resistance play an important role in cognitive impairment, a phenomenon widely documented in diabetic patients and experimental models (Biessels and Kappelle, 2005, Candeias et al., 2012, Blazquez et al., 2014, Hussain et al., 2014, Nunes et al., 2014, Li et al., 2015, Sridhar et al., 2015). In fact, alterations in brain insulin signaling is associated with an increased activity of GSK3β, which potentiates tau protein hyperphosphorylation and amyloid beta protein formation, the two neuropathological hallmarks of AD (Cardoso et al.,

2009) **(Figure 1.5)**. Of extreme importance is also the fact that tau is a microtubule-associated protein, needed for the correct axonal transport (Cardoso et al., 2009). So, the increased levels of phosphorylated tau will compromise transport along the axons (Correia et al., 2011), which may lead to degeneration and death of neurons. Altogether, this evidence supports the idea of a close link between T2D and insulin resistance and neurodegenerative diseases.

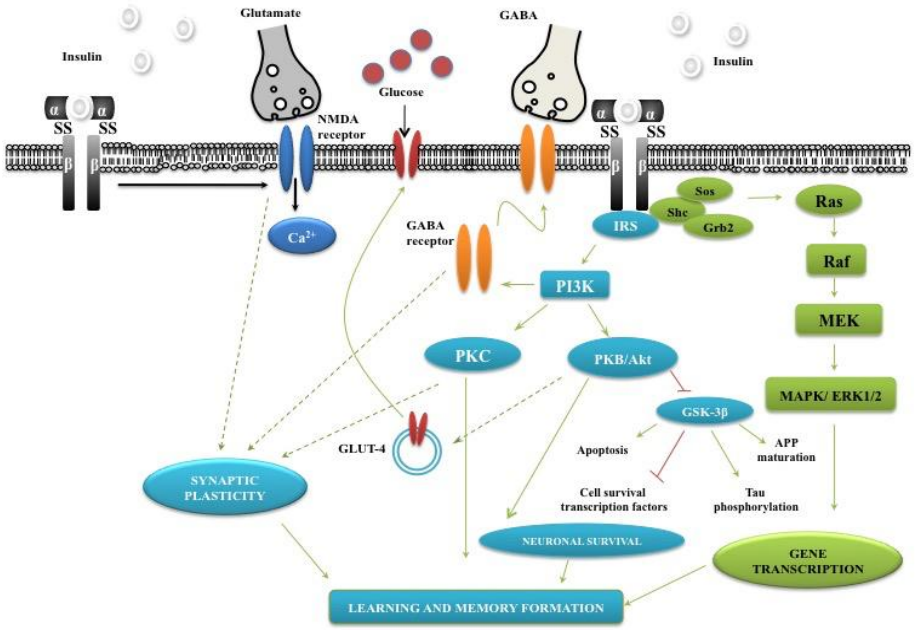


**Figure 1.5 –** ***Insulin signaling pathways.*** Schematic illustration of the potential molecular mechanisms of insulin signaling in the brain. Insulin binds to the insulin receptor activating the intrinsic tyrosine kinase, which phosphorylates endogenous substrates, such as the insulin receptor substrates (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. PI3K mediates the activation of the protein kinase B (AKT) favoring neuronal survival. Activated AKT can interfere with the apoptotic machinery, inactivating the pro-apoptotic proteins, BAD, and caspase-9. Furthermore, PI3K/AKT activation inhibits glycogen synthase kinase 3β (GSK3β), 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 (Glut 4) 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. In summary, insulin has an important role in the regulation of neuronal cell survival/death, synaptic plasticity, learning and memory (adapted from Cardoso et al., 2009).

## 1.2 – Vitamin D: one hormone with multiple functions

The fat-soluble vitamin D can be found in two forms: ergocalciferol or vitamin $D_2$, present essentially in plants and some fish, and cholecalciferol or vitamin $D_3$, produced in the skin upon sunlight exposure (Kulie et al., 2009, Thacher and Clarke, 2011, Vuolo et al., 2012, DeLuca et al., 2013, Eyles et al., 2013). Vitamin D metabolization begins with the interaction of ultraviolet (UV) B radiation (290-315 nM) with 7-dehydrocholesterol present in epidermis. After a spontaneous isomerization, cholecalciferol is formed and enters the circulation, being transported by vitamin D binding protein (DBP) (Kulie et al., 2009). To achieve its active form, vitamin D suffers two hydroxylation steps. The first one, occurs in the liver by 25-hydroxylase action, which produces a more stable form of vitamin D, the 25-hydroxyvitamin D (25(OH)D), commonly used to assess vitamin D status in humans (Eyles et al., 2013). Then, a second hydroxylation occurs in different organs, including the brain, producing the active form of vitamin D, 1,25-dihydroxyvitamin D [$1,25(OH)_2D$] through the action of $1\alpha$-hydroxylase (Mathieu and Badenhoop, 2005, Kiraly et al., 2006, Eyles et al., 2013).

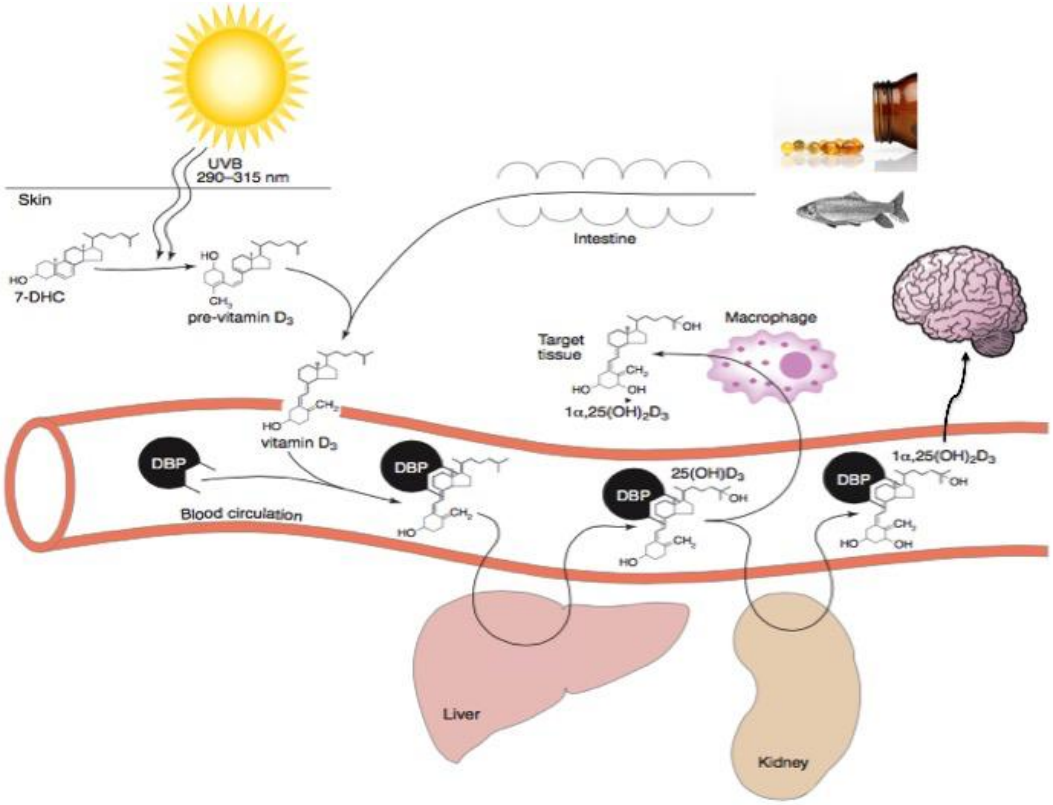


**Figure 1.6 – *Vitamin D metabolism.*** Vitamin D is taken up from food (vitamin D2 and D3) or synthesized in the skin [sun light with the photon energy wavelength of 290–315 nm causes photolysis of 7-dehydrocholesterol (7-DHC; provitamin D3) to pre-vitamin D3, the immediate precursor in the biosynthetic pathway to vitamin D3]. In the circulation, all vitamin D metabolites are bound to vitamin D-binding protein (DBP). To become active, vitamin D3 is hydroxylated first in the liver (via 25-hydroxylase) and successively in the kidney (and other organs via 1α-hydroxylase) to its final form, $1\alpha,25(OH)_2D_3$. Note that macrophages and other cells are also capable of hydroxylate vitamin D to $1\alpha,25(OH)_2D_3$, thereby facilitating locally regulated tissue concentrations (adapted from Mathieu and Badenhoop, 2005).

In order to exert its functions vitamin D needs to bind its specific receptor (VDR), a member of nuclear hormone receptors. When activated, VDR acquires a heterodimeric form and interacts with vitamin D response elements (VDREs), specific deoxyribonucleic acid (DNA) sequences normally in the promoter region, regulating transcription processes (Deeb et al., 2007,

Bikle, 2009, Vuolo et al., 2012). Furthermore, besides its genomic signaling (through interaction with nuclear VDR and consequent interaction with VDREs, resulting in gene transcription or repression) vitamin D can also exert its action through non-genomic signaling [through interaction with caveolae or $1,25D_3$ MARRS (membrane-associated rapid response steroid) binding] resulting on a signaling cascade with activation of second messengers systems, as for example PI3K and MAPK pathways. Another example are, Rapidly accelerated fibrosarcoma/MAPK second messenger that can be activated and promote a crosstalk with the nucleus, leading to the previous mentioned genomic effects **(Figure 1.7)** and extracellular signal-regulated kinase (ERK) activation that may also improve transcriptional activity of VDR (Vuolo et al., 2012).

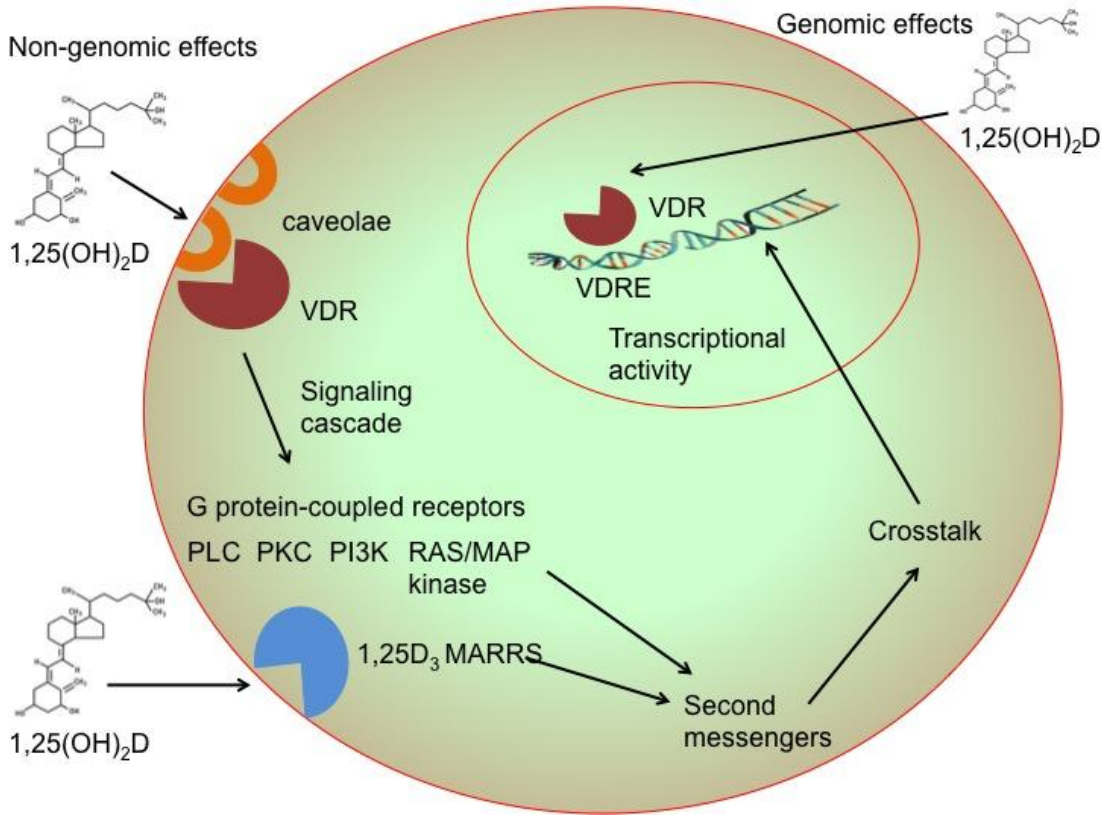


**Figure 1.7 – *Genomic and non-genomic effects of vitamin D.*** 1,25$(OH)_2$D (active vitamin D) interacts with the vitamin D receptor (VDR) localized in the nucleus to generate genomic effects or in caveolae of the plasma membrane to generate non-genomic effects (rapid responses, RR). Moreover, 1,25$(OH)_2$D can directly interact with a novel receptor for 1,25$(OH)_2$D called 1,25$D_3$ MARRS (membrane-associated, rapid response steroid-binding). Binding of 1,25$(OH)_2$D at plasma membrane may activate one or more second messenger systems, including phospholipase C (PLC), protein kinase C (PKC), G protein-coupled receptors, or PI3K. Possible outcomes include opening of the voltage-gated calcium or chloride channels or generation of second messengers. Some of these second messengers, particularly RAF/MAPK, may modulate a crosstalk with the nucleus for gene expression regulation (adapted from Vuolo et al., 2012).

Vitamin D plays an important role throughout the organism with its deficiency being a potential risk factor for obesity (Kiraly et al., 2006) and the development of T2D (Menon et al., 2013), as well as for the development of

cognitive impairment and dementia, such as Parkinson's disease (PD) and AD (Evatt et al., 2008, Johansson et al., 2013, Littlejohns et al., 2014). Indeed, cross-sectional studies showed that patients with one of these diseases frequently present hypovitaminosis D (Annweiler et al., 2012, Johansson et al., 2013, Annweiler et al., 2014). This relation probably arises from vitamin D important functions in mediating insulin synthesis and secretion, insulin sensitivity (Norman et al., 1980, Mathieu et al., 2005a, Chagas et al., 2012) and modulating inflammatory process, characteristic of both diabetes and AD as well as its neuroprotective and neurotrophic actions (Kiraly et al., 2006, Harms et al., 2011, DeLuca et al., 2013, Eyles et al., 2013).

Vitamin D is widely known for its role in calcium homeostasis and bone metabolism, enhancing calcium absorption in the intestines and increasing calcium mobilization to the bone, activating osteoblasts and osteoclasts, thus improving bone density (Thacher and Clarke, 2011). On the other hand, increasing evidence reveals that vitamin D can influence vital functions in several organs, such as the brain and pancreas (Mathieu and Badenhoop, 2005, Kulie et al., 2009, Eyles et al., 2013). Previous studies showed that vitamin D reduces cardiovascular disease and hypertension incidence due to an interaction with renin-angiotensin system, a decrease in coagulation and modulates vascular smooth muscle cells proliferation (Thacher and Clarke, 2011, Danik and Manson, 2012). Additionally, it seems to have great influence in the immune system, playing a role in reducing the risk of cancer development (Vuolo et al., 2012) and inhibits T-cells proliferation and cytokines transcription (Danik and Manson, 2012). The anti-proliferative and

differentiation effects of vitamin D are a target of attention given its potential in cancer prevention or treatment (Bikle, 2009). It was also shown that vitamin D, in keratinocytes, is capable of promoting UV-induced DNA damage repair (Bikle, 2009).

Eyles and coworkers (2005) showed that both VDR as well as 1$\alpha$-hydroxylase are present in several regions of the brain, suggesting that vitamin D may have an important role at CNS (Kiraly et al., 2006, DeLuca et al., 2013). In agreement, it was already described that vitamin D has neurotrophic properties in both developing and adult brain (Eyles et al., 2005) and could be involved in neurotransmission, neuroplasticity and neuroprotection. Recently, it was postulated that probably those effects may last longer than before assumed through epigenetic regulation and metabolic imprinting (DeLuca et al., 2013). Moreover, also differentiation, maturation and growth seem to be modulated by 1,25$(OH)_2$D action. Indeed, 1,25$(OH)_2$D upregulates the production of growth factors like glial cell line-derived neurotrophic factor (GDNF), nerve growth factor (NGF) and mediates the synthesis of a long range of neurotransmitters (Eyles et al., 2013). For example, GDNF can increase the differentiation of dopaminergic neurons in animal models of PD treated with 1,25$(OH)_2$D. Indeed, vitamin D seems to attenuate neurotoxicity induced by 6-hydroxydopamine, possibly through its capacity of enhancing GDNF expression (Wang et al., 2001). Furthermore, vitamin D can also be a neuroprotective agent upregulating the expression of several neurotrophin factors such as neurotrophin-3 (NT-3), responsible for the protection of nerve transmission. Neurotrophins are important in spatial navigation and when they are downregulated, spatial navigation, learning and

processing events are affected (Buell and Dawson-Hughes, 2008). Moreover, neuroplasticity regulation by vitamin D occurs through transcription regulation of multiple genes linked to cytoskeleton maintenance, mitochondrial function, synaptic plasticity and cell growth and proliferation (DeLuca et al., 2013). Also, vitamin D exerts neuroprotective and detoxificant functions concerning to oxidative and nitrosative stress, inhibiting the synthesis of inducible nitric oxide synthase (iNOS) (Garcion et al., 1997), increasing glutathione levels (Kiraly et al., 2006, Buell and Dawson-Hughes, 2008), a powerful antioxidant, and modulating L-type sensitive calcium channels (Buell and Dawson-Hughes, 2008). iNOS is capable of generate NO that is associated with inflammatory diseases (Garcion et al., 1997) and vitamin D deficiency enhances nitrosative stress (Keeney et al., 2013). As a major calcium regulator, vitamin D prevents the excitotoxicity promoted by excess calcium levels associated with aging and age-related diseases (Buell and Dawson-Hughes, 2008, DeLuca et al., 2013). Also, increase in acetylcholine levels in the brain due to an increase in choline acetyltransferase activity was showed after a treatment with vitamin D (Sonnenberg et al., 1986). Given the major role of vitamin D in neuronal development it is legitimate to conclude that vitamin D deficiency during young age, or even gestational stage, could have repercussions in the elderly, specifically related with cognitive impairment and neurodegenerative disorders development. In fact, epidemiological studies reveal that AD patients present low plasmatic levels of vitamin D (Przybelski and Binkley, 2007, Evatt et al., 2008, Littlejohns et al., 2014), while low concentrations of vitamin D appear to impair brain function (Holick, 2015) and people with hypovitaminosis D are more prone to present cognitive

dysfunctions (Annweiler et al., 2014). Moreover, several studies reveal that vitamin D optimal concentration improves neuronal and cognitive function in rats (Annweiler et al., 2012, Briones and Darwish, 2012, Latimer et al., 2014) through anti-inflammatory actions, regulation of neurotrophic agents, choline acetyltransferase (Annweiler et al., 2014) and calcium/calmodulin-dependent protein kinase IIδ (CaMKIIδ) activity (Latimer et al., 2014). For instance, this kinase activates adenosine 3',5'-cyclic monophosphate (cAMP) response element binding protein (CREB), enhancing synaptic strength and memory formation and improving neurogenesis and dendritic outgrowth by potentiating brain-derived neurotrophic factor (BDNF) (Latimer et al., 2014) **(Figure 1.8)**. Also, vitamin D may play a role in decreasing Aβ levels in the brain since it stimulates macrophages and phagocytosis involved in the clearance of this peptide (Durk et al., 2014).

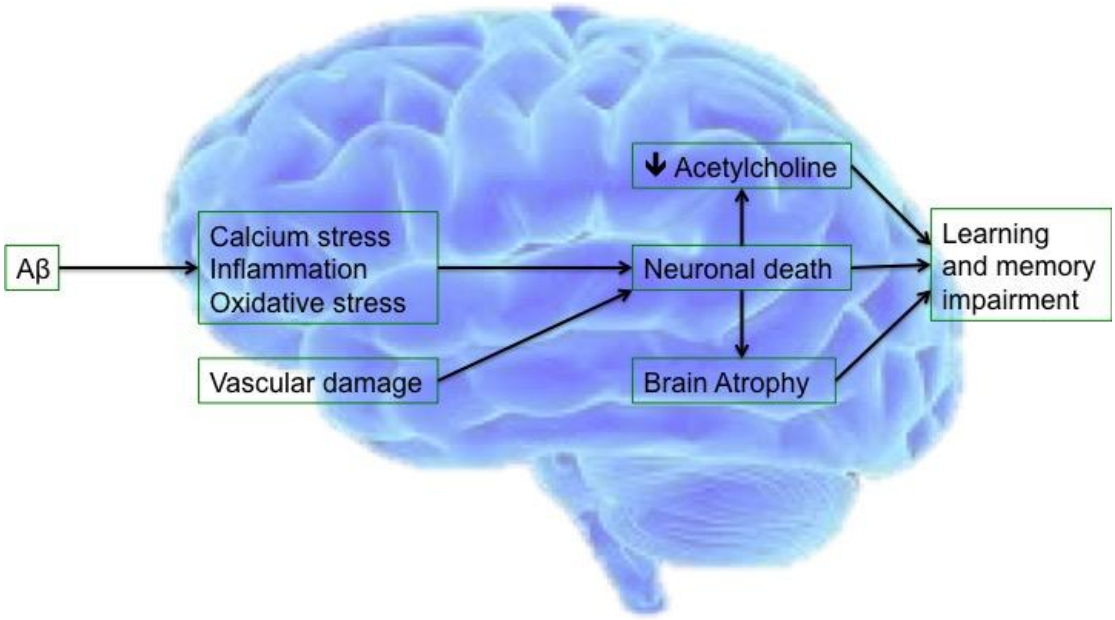


**Figure 1.8 –** ***Potential neuroprotective effects of vitamin D.*** Vitamin D has many neuroprotective effects contributing to the maintenance of neuronal function and preventing the development of neurodegenerative disorders. This large range of actions may be implicated in amyloid beta peptide (Aβ) clearance, through phagocytosis, and regulation of calcium levels by the prevention of excitotoxic events. Vitamin D has also antioxidant actions, by the increase of glutathione levels and anti-inflammatory potential. Finally, the regulation of acetylcholine levels, a neurotransmitter important in learning and memory formation and the regulation of neurotrophic factors are also mediated by vitamin D (adapted from Annweiler et al., 2014).

## 1.2.1 – Vitamin D deficiency and the incidence of T2D: are they related?

Low levels of plasma vitamin D were already described in some longitudinal observational studies as a predictive factor for T2D development. However, the existence of epidemiologic studies is still scarce and it remains

a controversial subject (Forouhi et al., 2008, Knekt et al., 2008, Pittas and Dawson-Hughes, 2010, Mitri et al., 2011, Peeyush Kumar et al., 2011, Kabadi et al., 2012, Kostoglou-Athanassiou et al., 2013, Hirani et al., 2014, Nigil Haroon et al., 2015). Indeed, vitamin D has been described as a modulator of blood glucose levels, insulin secretion mediated by glucose and as a regulator of calcium levels, important to promote insulin sensitivity in target tissues (Muscogiuri et al., 2012, Sung et al., 2012, Alkharfy et al., 2013, Bachali et al., 2013, Pilz et al., 2013, Badawi et al., 2014, Bellan et al., 2014). So, vitamin D deficits are linked to diabetes, specifically to T2D (Norman et al., 1980, Bourlon et al., 1999, Mathieu et al., 2005a). Moreover, insulin synthesis was shown to improve in the presence of higher levels of $1,25(OH)_2D$ (Mathieu et al., 2005a) demonstrating a connection between insulin and vitamin D actions. When administered to obese people, vitamin D seems to improve insulin sensitivity, mainly due to its anti-inflammatory properties and may also improve insulin secretion and insulin receptor expression (Peeyush Kumar et al., 2011, Alkharfy et al., 2013). Moreover, it also seems to play an important role in reducing lipid profile in obese diabetic patients (Eftekhari et al., 2014).

Although it is widely shown that vitamin D has positive actions throughout the body, supplementation with vitamin D in diabetes patients remains a controversial question with studies revealing the inexistence of benefits in parameters such as inflammatory markers, glycemic control and insulin sensitivity (Jorde and Figenschau, 2009, Kampmann et al., 2014, Krul-Poel et al., 2014).

As discussed above, a connection between T2D and dementia do exist. Despite the observation that T2D and dementia, including AD, are

characterized by low levels of vitamin D, as far as we know, no studies exist concerning the impact of vitamin D deficiency in T2D brain. Due to the crosstalk of insulin and vitamin D pathways, we hypothesize that vitamin D deficiency may exacerbate brain alterations promoted by the insulin resistant state that characterizes T2D.

# *Chapter 2 – Objectives*

## 2.1 – Objectives

As abovementioned, high-fat intake and vitamin D deficiency increase the risk of insulin resistance, T2D and neurodegenerative diseases. Appropriate insulin signaling is required for normal brain functioning and disturbances in its pathways have been implicated in several neurodegenerative processes.

In this line, this study aimed to uncover the effects of high-fat and/or low vitamin D diets in the brain of T2D rats, putting the focus on brain insulin signaling. Because AD-related hallmarks and synaptic integrity depend on insulin signaling, these possible alterations were also studied in T2D brains.

For this purpose we evaluated the insulin signaling pathway and its effects in synaptic integrity and AD-related hallmarks in brain cortex and hippocampus of T2D Goto-Kakizaki (GK) rats subjected to distinct diets, during 4 months, as follows: 1) GK rats fed a standard diet (GK); 2) GK rats fed a low vitamin D diet (GK low vitD); 3) GK rats fed a high-fat diet (GK HFD) and 4) GK rats fed a high-fat, low vitamin D diet (GK HFD low vitD).

# *Chapter 3 – Material and Methods*

## 3.1 – Materials

All the chemicals were of the highest grade of purity commercially available. The specific enzyme-linked immunosorbent assay (ELISA) kits, antibodies, reagents and other specific materials used in this work are described in the following subsections.

## 3.2 – Animals and experimental design

Male wistar and GK rats (2-month-old) were maintained in our animal facilities (Faculty of Medicine, University of Coimbra) under controlled light (12 hours day/night cycle) and humidity with ad libidum access to food and water.

GK rats are a non-obese type 2 diabetes animal model, characterized by an early life development of the disease, presenting mild hyperglycemia, insulin resistance and secondary hyperinsulinemia (Santos et al., 2014). GK rats were randomly divided into four groups and fed: 1) regular (standard) diet (GK), 2) vitamin D deficient diet (GK Low vitD), 3) high-fat diet (GK HFD) and 4) high-fat diet deficient in vitamin D (GK HFD Low vitD), for four months **(Figure 3.1).** Wistar rats were fed with a regular diet and used as control group.

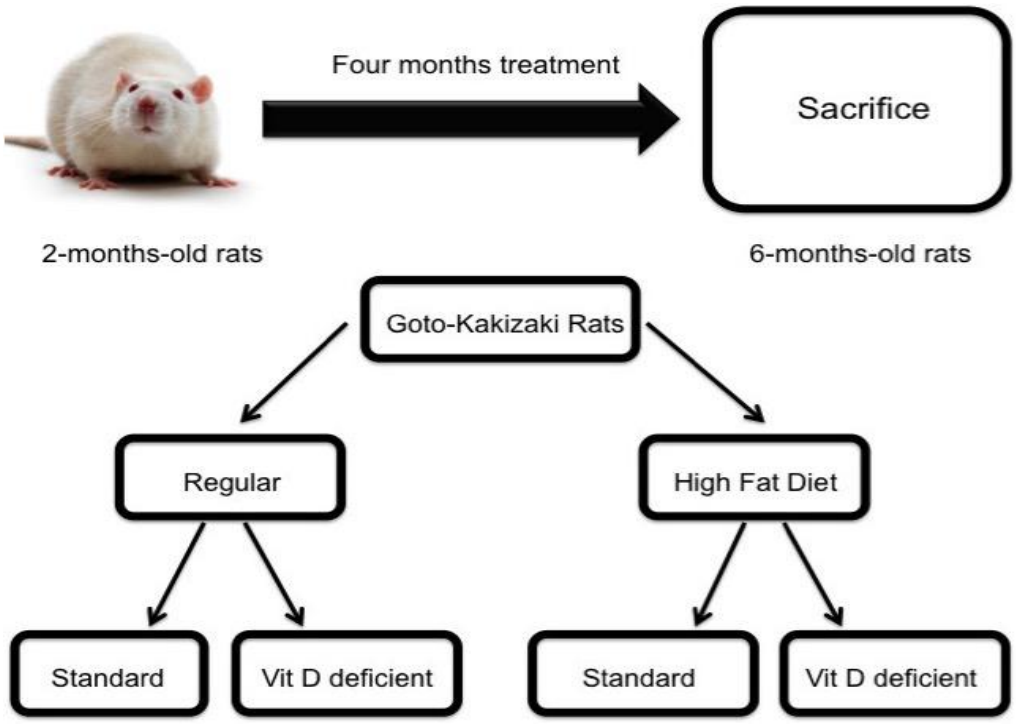


**Figure 3.1 -** ***Experimental design.*** GK rats were randomly divided into four groups and fed 1) regular (standard) diet; 2) vitamin D (vitD) deficient diet; 3) high-fat diet; 4) high-fat diet deficient in vitamin D. The animals were sacrificed four months after the treatment.

Diets were purchased from Scientific Animal Food & Engineering (SAFE) and the diets composition is described in **tables 3.1 and 3.2**.

According to procedures approved by the Federation of Laboratory Animal Science Association (FELASA) and Directive 2010/63/EU, four months after the treatment, animals were anesthetized with ketamine/chlorpromazine [ketamine chloride (75 mg/kg, im, Parke-Davis, Ann Arbor, MI, USA) and chlorpromazine chloride (2,65 mg/kg, im, Lab. Vitória, Portugal)] and sacrificed by decapitation. Animals' manipulation was performed by qualified persons (accredited by FELASA and Direção Geral de Alimentação e Veterinária, DGAV).

**Table 3.1 –** ***Standard diet composition***

| Animal diet constitution (AO3 mouse maintenance feed) | |
|---|---|
| Cereals | 69,2 % |
| Vegetable proteins | 20,2 % |
| Animal proteins | 6 % |
| Vitamin and mineral mixture | 4.6 % |
| **Average analysis** | |
| Nitrogen-free extracts | 51.7 % |
| Proteins | 21.40 % |
| Moisture | 12.2 % |
| Minerals | 5.7 % |
| Lipids | 5.1 % |
| Fibres | 3.9 % |

**Table 3.2 –** ***Vitamin D deficient high-fat diet composition***

| High-fat vitamin D deficient diet | |
|---|---|
| AO3 Standard mouse maintenance feed | 68.12 % |
| Caseine (85% protein) | 7.5 % |
| Dextrose | 3 % |
| Saccharose (=sucrose) | 1.62 % |
| Dextrin corn | 1.63 % |
| Cocao butter | 7.5 % |
| Cholestrol | 1.25 % |
| Cellulose | 1.25 % |
| Premix mineral safe | 7 % |
| Premix vitamin safe | 1 % |
| Chlorure choline | 0.13 % |
| Standard Vitamin D level | 3000 UI/Kg |
| Deficient Vitamin D level | <200 UI/Kg |

## 3.3 – Animals' biochemical characterization

Immediately before animal sacrifice, and following body weight measurement, blood glucose levels were determined by a glucose oxidase reaction, using a commercial glucometer (Glucometer – Elite Bayer, Portugal) and compatible reactive tests (Ascencia Elite Bayer, Portugal) and blood glycated hemoglobin ($HbA_{1C}$) levels were determined using a commercial A1CNow® System (Bayer HealthCare, 81611395).

Serum fasting plasma lipids [total and high-density lipoprotein (HDL) cholesterol and triglycerides] were evaluated by using commercial kits in an automatic system (Olympus-Diagnóstica Portugal, Produtos de Diagnóstico SA, Portugal) in blood collected by heart puncture in deeply anesthetized rats. Serum was obtained by centrifugation of blood at 13 000 rpm for 10 minutes at 4ºC (Sigma 2-16 PK centrifuge).

## 3.4 – Brain samples homogenization

The brain cortices and hippocampus were separately homogenized in a lysis buffer (Cell Signaling #9803) containing 20 mM Trizma hydrochloride (Tris-HCl; pH 7.5), 150 mM sodium chloride (NaCl), 1 mM disodium salt of ethylenediaminetetraacetic acid ($Na_2EDTA$), 1 mM ethylene glycol tetraacetic acid (EGTA), 1% Triton, 2.5 mM sodium pyrophosphate ($Na_4P_2O_7$), 1 mM beta-glycerophosphate, 1 mM sodium orthovanadate ($Na_3VO_4$), 1 µg/ml leupeptin supplemented with a protease and phosphatase inhibitors cocktail from Roche, 0.1 M phenylmethanesulfonylfluoride (PMSF) and 0.2 M

dithiothreitol (DTT). To favor disruption, the homogenates were frozen in liquid nitrogen and thawed 3 times followed by centrifugation at 14 000 rpm for 10 minutes at 4ºC (Eppendorf Centrifuge 5415C). The supernatants were collected and stored at -80ºC.

## 3.5 – Protein concentration determination

Protein concentration was determined through bicinchoninic acid (BCA) protein assay (Pierce Thermo Fisher Scientific, Rockford, IL). This quantification method is based on a colorimetric detection of protein concentration, using biuret reaction principle, in which protein is capable of reducing cuprous ($Cu^{2+}$) to cuprous cation ($Cu^{1+}$) in an alkaline medium and a highly sensitive detection of the ($Cu^{1+}$), using a reagent containing BCA. Bovine serum albumin (BSA) is used to perform a standard curve, preparing a series of dilutions of known protein concentrations.

## 3.6 – Western Blot Analysis

The samples (50μg of protein) were resolved by electrophoresis in 8%, 10% or 15% sodium dodecyl sulfate (SDS)–polyacrylamide gels, according the molecular weight of proteins of interest, and then transferred into polyvinylidene difluoride (PVDF) membranes. Non-specific bindings were blocked by gently agitating the membranes in 5% BSA and 0.1% Tween in Tris-buffered saline (TBS-T) for 1 hour at room temperature. Subsequently, membranes were incubated with the respective primary antibodies **(table 3.3)**

diluted in 1% BSA, overnight at 4ºC, with gentle agitation. After, membranes were washed (3x 5 minutes) with 0,05% TBS-T, and then incubated with the specific secondary antibody for 2 hours **(table 3.4),** at room temperature, with gentle agitation. After incubation, membranes were washed (3x 5 minutes) with TBS-T and incubated with enhanced chemifluorescence (ECF) for 5 minutes. The specific bands of immunoreactive proteins were visualized in a VersaDoc Imaging System (Bio-Rad) and the density of protein bands were calculated using the Quantity One Software (Bio-Rad).

## Table 3.3 - *Primary antibodies*

| Primary Antibody | Molecular Weight (kDa) | Dilution | Reference | Secondary Antibody Species |
|---|---|---|---|---|
| IDE (K-20) | 118 | 1:500 | Santa Cruz Biotechnology (sc-27266) | Goat |
| Insulin Receptor β (4B8) | 95 | 1:1000 | Cell signaling (#3025) | Rabbit |
| Anti-VDR | 46 | 1:750 | Sigma (SAB2501098) | Goat |
| IGF-IRβ (H-60) | 92 | 1:1000 | Santa Cruz Biotechnology (sc-9038) | Rabbit |
| Anti-phospho-IRS1 (Ser307 mouse/ Ser312 human | 160 | 1:750 | Millipore (05-1087) | Rabbit |
| IRS1 | 180 | 1:1000 | Cell signaling (#2382S) | Rabbit |
| Anti-IRS2 (phospho S731) | 200 | 1:1000 | Abcam (ab3690) | Rabbit |
| IRS-2 (M-19) | 165-185 | 1:750 | Santa Cruz Biotechnology (sc-1555) | Goat |
| Phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (197G2) | 44/42 | 1:1000 | Cell signaling (#4377) | Rabbit |
| p44/42 MAPK (ERK1/2) | 44/42 | 1:1000 | Cell signaling (#9102) | Rabbit |
| PI3 Kinase p110α (C73F8) | 110 | 1:1000 | Cell signaling (#4249) | Rabbit |
| Phospho-Akt (Ser473) (587F11) | 60 | 1:750 | Cell signaling (#4051) | Mouse |
| Anti-Akt | 59 | 1:1000 | BD Transduction Laboratories TM (610861) | Mouse |
| Anti-Glucose Transporter GLUT-1, CT | 46 | 1:750 | Millipore (CBL242) | Rabbit |
| Anti-Glucose Transporter GLUT3 | 45-47 | 1:750 | Abcam (ab41525) | Rabbit |
| Glut4 (1F8) | 50 | 1:1000 | Cell signaling (#2213S) | Mouse |
| Phospho-SAPK/JNK (Thr183/Tyr185) (G9) | 46/54 | 1:750 | Cell signaling (#9255) | Mouse |
| SAPK/JNK | 46/57 | 1:1000 | Cell signaling (#9252) | Rabbit |
| Phospho-AMPKα (Thr172) (40H9) | 62 | 1:1000 | Cell signaling (#2535) | Rabbit |
| AMPKα | 62 | 1:1000 | Cell signaling (#2532) | Rabbit |
| Phospho-mTOR (ser2448) | 289 | 1:1000 | Cell signaling (#2971) | Rabbit |
| mTOR (L27D4) | 289 | 1:1000 | Cell signaling (#4517) | Mouse |
| Phospho-GSK-3β (Ser9) | 46 | 1:1000 | Cell signaling (#9336) | Rabbit |
| p-GSK-3β (Tyr 216) | 46 | 1:1000 | Santa Cruz Biotechnology (sc-135653) | Rabbit |
| GSK-3β (11B9) | 46 | 1:1000 | Santa Cruz Biotechnology (sc-81462) | Rabbit |
| 06-519 \| Anti-phospho-CREB (Ser133) | 45 | 1:1000 | Millipore (06-519) | Rabbit |
| CREB (48H2) | 43 | 1:1000 | Cell signaling (#9197) | Rabbit |
| Cdk5 (C-8) | 35 | 1:1000 | Santa Cruz Biotechnology (sc-173) | Rabbit |
| p35/25 (C64B10) | 35/25 | 1:1000 | Cell signaling (#2680) | Rabbit |
| p-Tau (Ser 396) | 46-80 | 1:1000 | Santa Cruz Biotechnology (sc-101815) | Rabbit |
| p-Tau (Thr 181) | 46-68 | 1:750 | Santa Cruz Biotechnology (sc-101816) | Rabbit |
| Tau (BT2) | 46-80 | 1:1000 | Thermo Scientific (MN1010) | Mouse |
| Monoclonal Anti-Synaptophysin | 38 | 1:1000 | Sigma (S5768) | Mouse |
| Monoclonal Anti-SNAP-25 | 25 | 1:1000 | Sigma (S5187) | Mouse |
| PSD95 (D27E11) XP® | 95 | 1:1000 | Cell signaling (#3450) | Rabbit |
| Monoclonal Anti-β-Actin | 42 | 1:5000 | Sigma (A5441) | Mouse |

**Table 3.4** - ***Secondary antibodies***

| Antibody | Dilution | Reference |
|---|---|---|
| Mouse | 1:10000 (1:20000 for actin) | GE Healthcare ECF Western Blotting Reagent Pack mouse. (RPN5781) |
| Rabbit | 1:10000 | GE Healthcare ECF Western Blotting Reagent Pack mouse. (RPN5783) |
| Anti-Goat | 1:10000 | Santa Cruz Biotechnology (sc-2771) |

## 3.7 – Evaluation of adenosine 3',5'-cyclic monophosphate (cAMP) levels

cAMP levels were determined using a commercial colorimetric kit (cAMP Direct Immunoassay Kit, BioVision Incorporated, #K371-100), according to the manufacturer's instructions. The kit utilizes recombinant Protein G coated 96-well plate to efficiently anchor cAMP polyclonal antibody on to the plate. cAMP - horseradish peroxidase (HRP) conjugate directly competes with cAMP from sample binding to the cAMP antibody on the plate. After incubation and washing, the amount of cAMP-HRP bound to the plate was determined by reading the optical density (OD) at 450 nm. The absorbance is inversely proportional to the concentration of cAMP in samples. The results are expressed as pmol/mg of protein.

## 3.8 – Evaluation of protein kinase A (PKA) levels

PKA levels were assessed using a commercial kit (PKA kinase activity kit, Enzo Life Science Inc., ADI-EKS-390A), following the manufacturer's instructions. In the assay, the substrate, which is readily phosphorylated by PKA, is pre-coated on the wells of the provided PKA substrate microtiter plate. The samples were added to the appropriate wells followed by the addition of ATP to initiate the reaction. The kinase reaction was terminated and a phosphospecific substrate antibody was added to the wells, which binds specifically to the phosphorylated peptide substrate. The phosphospecific antibody was subsequently bound by a peroxidase conjugated secondary antibody. The assay was developed with tetramethylbenzidine (TMB) substrate and a color developed in proportion to PKA phosphotransferase activity. The color development was stopped with acid stop solution and the intensity of the color was measured in a microplate reader at 450 nm (OD). The results are expressed as ng/mg of protein.

## 3.9 – Evaluation of insulin levels

Insulin levels were measured using a commercial colorimetric kit [Insulin (mouse/rat) EIA kit, Bertin pharma, A05105], following the manufacturer's instructions. In brief, insulin levels were determined based in a competition mechanism between unlabelled insulin present in samples and acetylcholinesterase (AChE), linked to rat insulin (tracer), for specific binding sites in Guinea-Pig anti-rat insulin antiserum sites. Samples were added to

anti-insulin antibody coated plate followed by the addition of an enzymatic substrate for AchE (Ellman's reagent) to initiate the reaction. Color intensity was measured at 450 nm (OD). The color intensity is inversely proportional to the amount of insulin in the samples. The results are expressed as ng/mg of protein.

## 3.10 – Evaluation of insulin-like growth factor 1 (IGF-1) levels

IGF-1 levels were determined using a commercial available kit (Rat insulin-like growth factor I ELISA Kit, Biosensis® - BEK-2150-2P), following the manufacturer's instructions. The IGF-I Kit is a sandwich ELISA. The capture antibody is a monoclonal rat IGF-I antibody pre-coated onto the 96-well strip plates provided in the kit. Rat test samples and standards of known IGF-I concentration were added to these wells and allowed to complex with the bound IGF-I antibody. A biotinylated rat IGF-I polyclonal antibody was then added. This detection antibody bound to the antigen thus completing the sandwich. After washing, an enzyme Avidin-Biotin-Peroxidase complex (ABC) was added for binding to the second antibody. The peroxidase substrate TMB was added to induce a colored reaction product. The intensity of this colored product, read at 450 nm (OD), is directly proportional to the concentration of IGF-I present in the samples. The results are expressed as pg/mg of protein.

## 3.11 – Evaluation of amyloid beta $(A\beta)_{1-42}$ levels

$A\beta_{1-42}$ levels were assessed using a commercial available kit (Amyloid beta 42 ELISA kit, mouse, Invitrogen, life technologies, #KMB3441), according to the manufacturer's instructions. This kit is a solid phase sandwich ELISA. A monoclonal antibody to the $NH_2$-terminus of mouse Aβ has been coated onto the wells of the microtiter strips provided. Samples, including standards of known mouse Aβ42 content and control specimens, were pipetted into these wells. The Aβ antigen binds to the immobilized (capture) antibody. After washing, a rabbit monoclonal antibody specific for the COOH-terminus of the $A\beta_{1-42}$ sequence was added to the wells. Bound rabbit antibody was detected by the use of a HRP-labeled anti-rabbit antibody. After removal of excess anti-rabbit antibody, a substrate solution was added, which was acted upon by the bound enzyme to produce color. The intensity of this colored product (450 nm) is directly proportional to the concentration of $A\beta_{1-42}$. The results are expressed as pg/mg of protein.

## 3.12 – Evaluation of cholesterol levels

Brain cholesterol levels were measured using a commercial available kit (Cholesterol RTU$^{TM}$, bioMérieux SA) following the manufacturer's instructions. The principle of the reaction is based on the production of hydrogen peroxide ($H_2O_2$) in cholesterol oxidation by cholesterol oxidase. In a first step, cholesterol esters are hydrolyzed to free cholesterol and fatty acids by cholesterol esterase. Then, free cholesterol is oxidized by cholesterol oxidase,

producing cholest-4-en-3-one and hydrogen peroxide. Finally, quinoneimine dye is formed when $H_2O_2$ oxidizes phenol and 4-aminoantipyrine. Briefly, 1 ml of the kit's reagent [3-(N-morpholino)propanesulfonic acid (MOPS) buffer (pH 7), phenol, sodium cholate, magnesium chloride, a surfactant agent, 4-aminoantipyrine, peroxidase, cholesterol oxidase and cholesterol esterase) was added to 10 μl of sample. Color intensity was measured (500 nm) after 10 minutes of incubation at room temperature. Color intensity is directly proportional to the cholesterol concentration present in the samples. The results are expressed as μg/mg of protein.

## 3.13 – Statistical analysis

Results are presented as mean ± SEM of the number of the indicated number of animals. Statistical significance was determined using the two-way analysis of variance ANOVA test for multiple comparisons, followed by the post-hoc Tukey test. A p-value <0.05 was considered statistically significant.

# *Chapter 4 – Results*

## 4.1 – Characterization of the experimental animals

We measured several T2D-associated parameters in order to validate our experimental models. We observed an increase in fasting glycemia and $HbA_{1C}$ and a significant decrease in body weight in all groups of GK rats, characteristic of this animal model, when compared with Wistar control group **(Table 4.1)**. Nevertheless, HFD promoted a more pronounced increase in $HbA_{1C}$ levels and body weight. Also, HFD induced a significant increase in total cholesterol levels, mainly associated with the increase in non-high density lipoproteins (non-HDL) cholesterol, as well as in triglycerides levels **(Table 4.1)**. Interestingly, the deficiency in vitD attenuated HFD-induced alterations particularly body weight and total cholesterol, non-HDL and triglycerides levels **(Table 4.1)**. No significant alterations were observed in brain weight **(Table 4.1)**.

**Table 4.1 – *Animals Characterization***

| Groups / Parameters | Wistar | GK | GK Low vitD | GK HFD | GK HFD Low vitD |
|---|---|---|---|---|---|
| **Glycemia** | 66.13±1.46 | 105.30±5.05**** | 87.75±6.27* | 82.38±3.06$ | 88.50±4.59* |
| **$HbA_{1c}$** | 4.70±0.03 | 7.31±0.49** | 7.07±0.57** | 8.23±0.45**** | 8.14±0.40*** |
| **Body weight** | 463.40±21.41 | 379.00±5.43*** | 377.30±6.40*** | 394.00±7.00** | 372.10±7.20**** |
| **Brain weight** | 1.97±0.02 | 2.02±0.05 | 1.83±0.14 | 2.06±0.017 | 2.06±0.03 |
| **Total Cholesterol** | 86.25±4.44 | 72.63±3.58 | 85.00±3.91 | 176.60±15.76****$$$$#### | 147.90±13.33**$$$## |
| **HDL Cholesterol** | 50.50±1.98 | 47.38±1.71 | 54.00±2.46 | 53.00±3.05 | 60.63±4.55$ |
| **Non-HDL Cholesterol** | 35.75±2.75 | 25.25±1.89 | 31.00±1.71 | 123.60±13.43****$$$$#### | 87.25±9.40***$$$$###& |
| **Triglycerides** | 85.50±13.74 | 70.75±4.39 | 75.75±5.01 | 191.00±2.63**$$$### | 134.60±3.89 |

Data shown are the means ± SEM of 7-8 animals from each condition studied. Statistical significance: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 when compared with Wistar rats; $^{\$}$p<0.05; $^{\$\$\$}$p<0.001; $^{\$\$\$\$}$p<0.0001 when compared with GK rats; $^{\#\#}$p<0.01; $^{\#\#\#}$p<0.001; $^{\#\#\#\#}$p<0.0001 when compared with GK Low vitD; $^{\&}$p<0.05 when compared with GK HFD.

## 4.2 – High-fat and low vitD diets alter glucose transporters levels

In the brain we can found the insulin sensitive Glut 4, the partially insulin sensitive Glut 1 and the insulin insensitive Glut 3. Concerning Glut 1, no significant alterations were observed in the brain cortex of all experimental groups while low vitD alone increased significantly the levels of Glut 1 in the hippocampus **(Figure 4.1 A and B).**

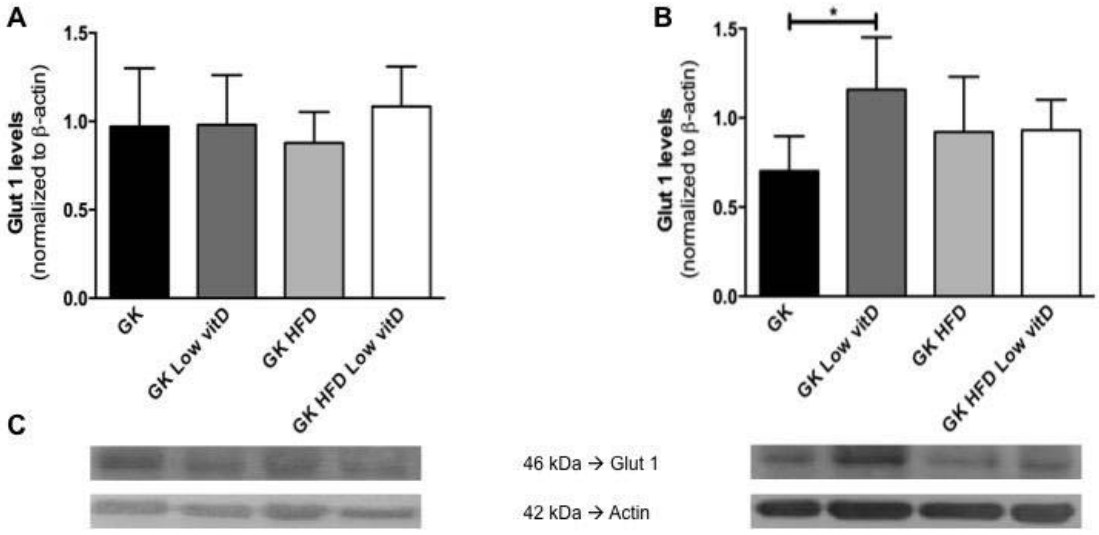


**Figure 4.1 –** ***Effect of high-fat and/or low vitD diets in brain cortex and hippocampus Glut 1 levels.*** Brain cortex **(A)** and hippocampus **(B)** Glut 1 levels and western blot representative images **(C).** Data shown are the means ± SEM of 6 animals from each condition studied. Statistical significance: *p<0.05.

Furthermore, in brain cortex Glut 3 levels were significantly increased in HFD low vitD rats when compared with GK low vitD rats **(Figure 4.2 A).** Curiously, in the hippocampus Glut 3 levels were significantly decreased by HDF alone or in combination with low vitD **(Figure 4.2 B)**.

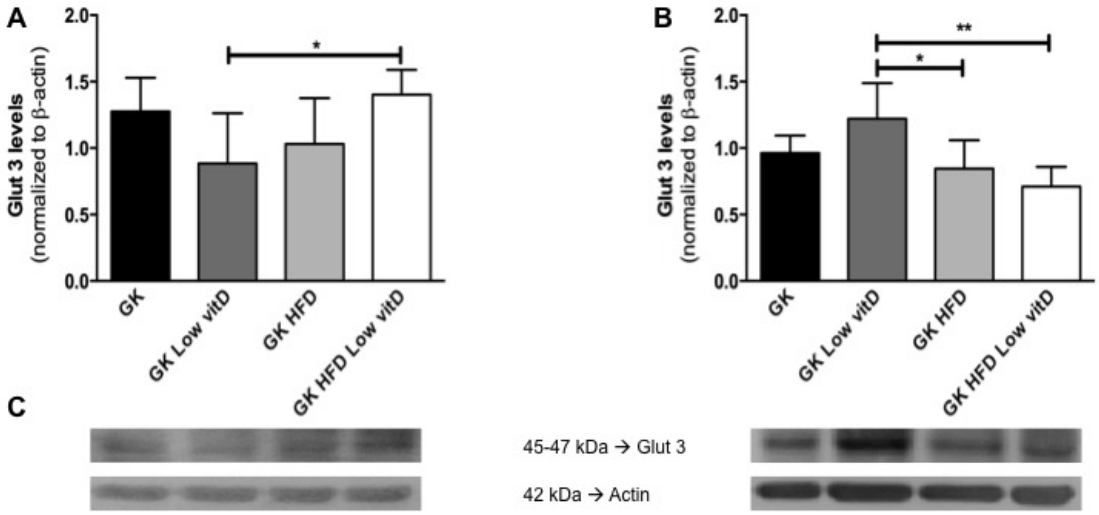


**Figure 4.2 – *Effect of high-fat and/or low vitD diets in brain cortex and hippocampus Glut 3 levels.*** Brain cortex **(A)** and hippocampus **(B)** Glut 3 levels and western blot representative images **(C).** Data shown are the means ± SEM of 6 animals from each condition studied. Statistical significance: *p<0.05; **p<0.01.

Concerning the insulin-sensitive Glut 4, in brain cortex a significant decrease was observed in HFD and HFD low vitD groups when compared with GK low vitD group **(Figure 4.3 A).** In the hippocampus, all dietary regimens decreased the levels of this transporter **(Figure 4.3 B).**

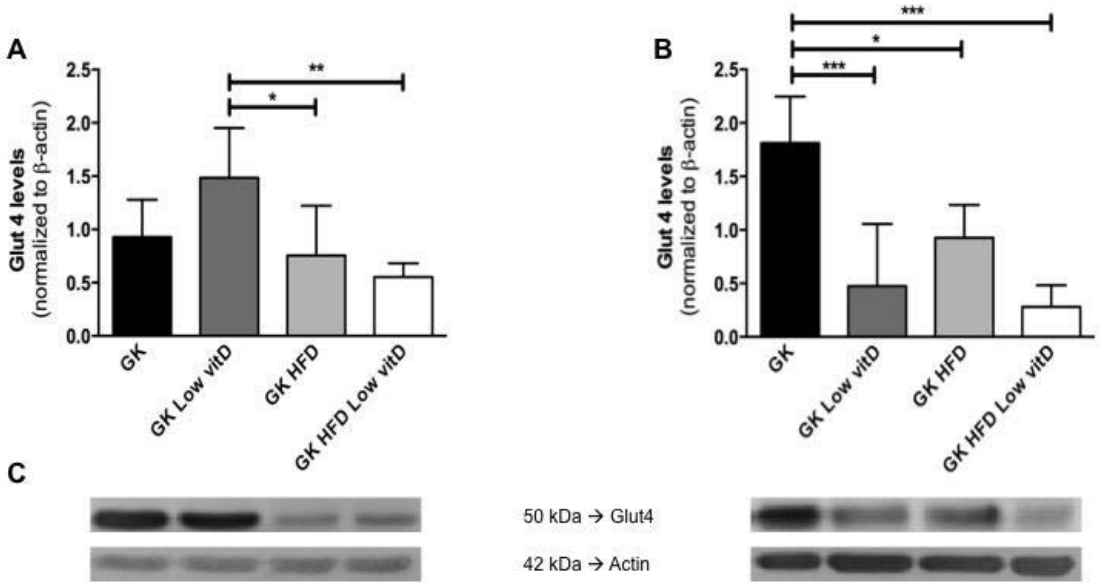


**Figure 4.3 –** ***Effect of high-fat and/or low vitD diets in brain cortex and hippocampus Glut 4 levels.*** Brain cortex **(A)** and hippocampus **(B)** Glut 4 levels and western blot representative images **(C).** Data shown are the means ± SEM of 6 animals from each condition studied. Statistical significance: *p<0.05; **p<0.01; ***p<0.001.

## 4.3 – High-fat and low vitD diets decrease vitamin D receptor expression

In order to exert its effects, vitamin D binds to VDR, activating a signaling cascade that includes PI3K (p110) activation. A slight increase of VDR levels was observed in brain cortex of HFD group, although it was not statistically significant when compared with GK rats group **(Figure 4.4 A)**. However, in the hippocampus, no significant alterations were observed in the same experimental group **(Figure 4.4 B)**. Interestingly, in both brain cortex and hippocampus a decrease in VDR levels was observed in animals

exposed to low vitD and HFD, when compared with the HFD group, although only statistically significant in brain cortex **(Figure 4.4 A and B)**.

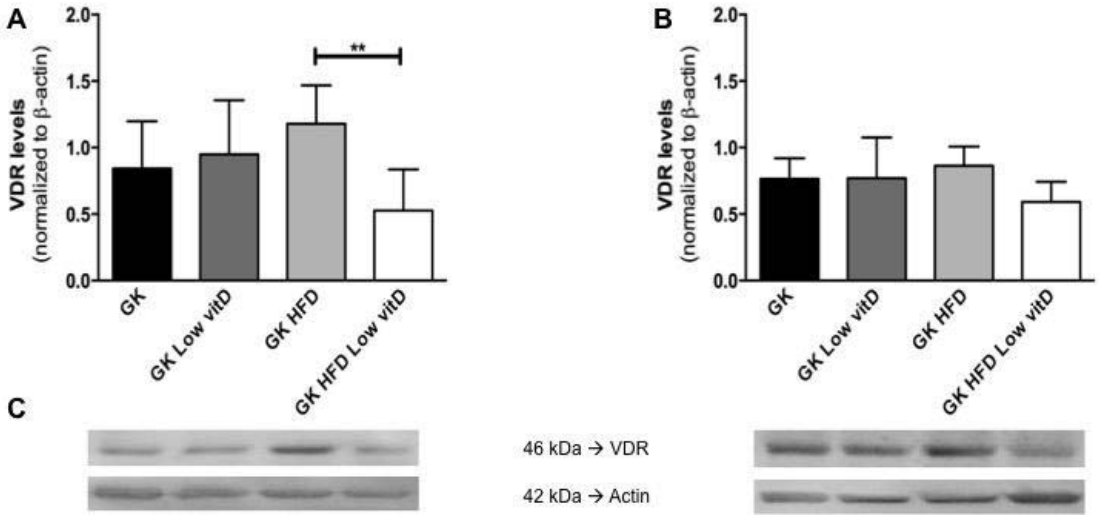


**Figure 4.4 – *Effect of high-fat and/or low vitD diets in VDR levels.*** Brain cortex **(A)** and hippocampus **(B)** VDR levels and western blot representative images **(C).** Data shown are the means ± SEM of 7 animals from each condition studied. Statistical significance: **p<0.01.

## 4.4 – The combination of low vitD and high-fat diet increases insulin receptor levels in brain cortex

The binding of a ligand to its receptor is a crucial step to signaling pathways activation. Our results showed that in brain cortex, although low vitD or HFD per se did not affect IR levels, the combination of low vitD and HFD induced a significant increase in IR levels **(Figure 4.5 A)**. However, no significant alterations in the expression of IR in hippocampus were observed (**Figure 4.5 B)**.

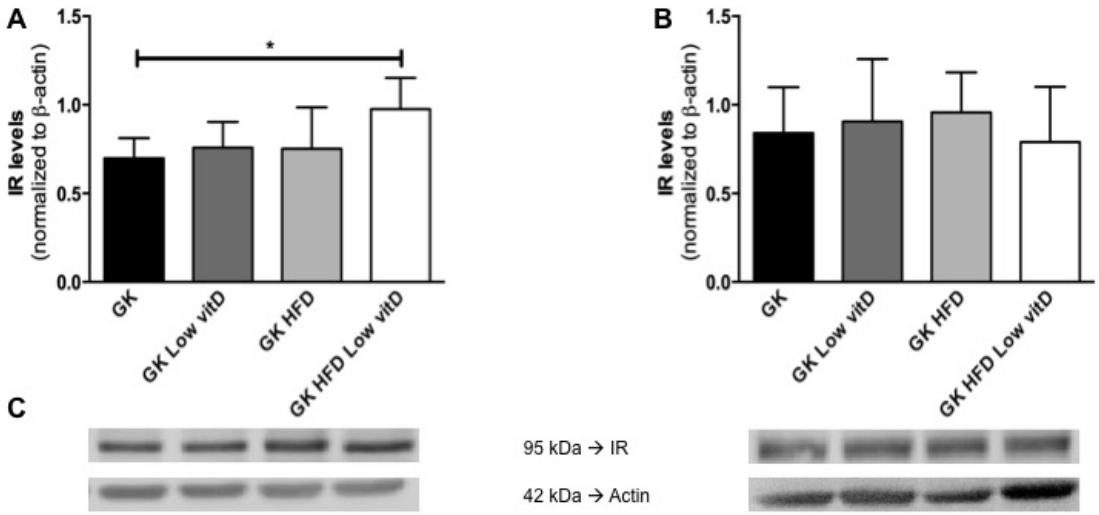


**Figure 4.5 –** ***Effect of high-fat and/or low vitD diets in IR levels.*** Brain cortex **(A)** and hippocampus **(B)** IR levels and western blot representative images **(C).** Data shown are the means ± SEM of 7 animals from each condition studied. Statistical significance: *p<0.05.

In agreement with IGF-1 levels, also IGF-1R levels were not significantly affected in any of the experimental groups **(Figure 4.6 A and B)**.

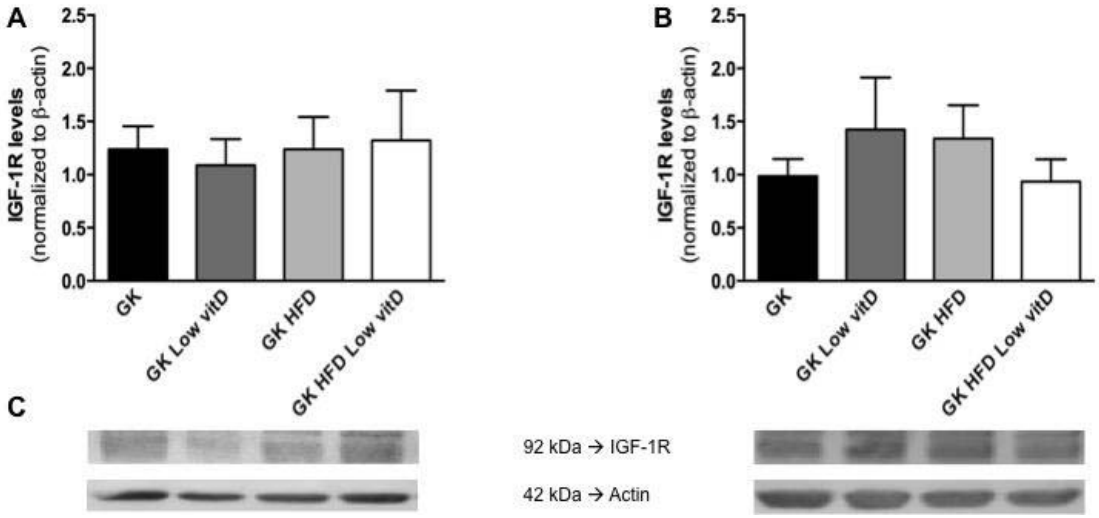


**Figure 4.6 –** ***Effect of high-fat and/or low vitD diets in IGF-1R levels.*** Brain cortex **(A)** and hippocampus **(B)** IGF-1R levels and western blot representative images **(C).** Data shown are the means ± SEM of 7 animals from each condition studied.

## 4.5 – High-fat and low vitD diets promote brain insulin levels increase but not in IGF-1 levels

Insulin and IGF-1 are two hormones closely associated with T2D due to their major role in glucose utilization and metabolism. In the brain, they modulate neuronal excitability, nerve cell metabolism and cell survival. An increase in insulin levels in brain cortex was observed in GK rats exposed to HFD and low vitD diets when compared with GK rats exposed to a standard diet **(Figure 4.7 A)**. In the hippocampus, low vitD diet, by itself, did not increase insulin levels. However, HFD or HFD combined with low vitD, led to an increment in insulin levels **(Figure 4.7 B).**

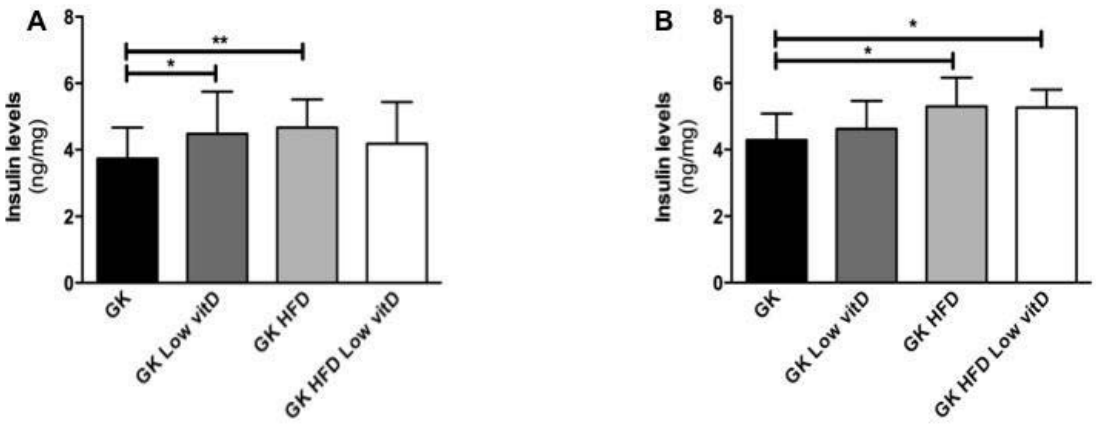


**Figure 4.7 – *Effect of high-fat and/or low vitD diets in insulin levels.*** Brain cortex **(A)** and hippocampus **(B)** insulin levels**.** Data shown are the means ± SEM of 7 animals from each condition studied. Statistical significance: *p<0.05; **p<0.01.

Furthermore, no significant alterations in IGF-1 levels were observed neither in brain cortex nor in hippocampus **(Figure 4.8 A and B)**.

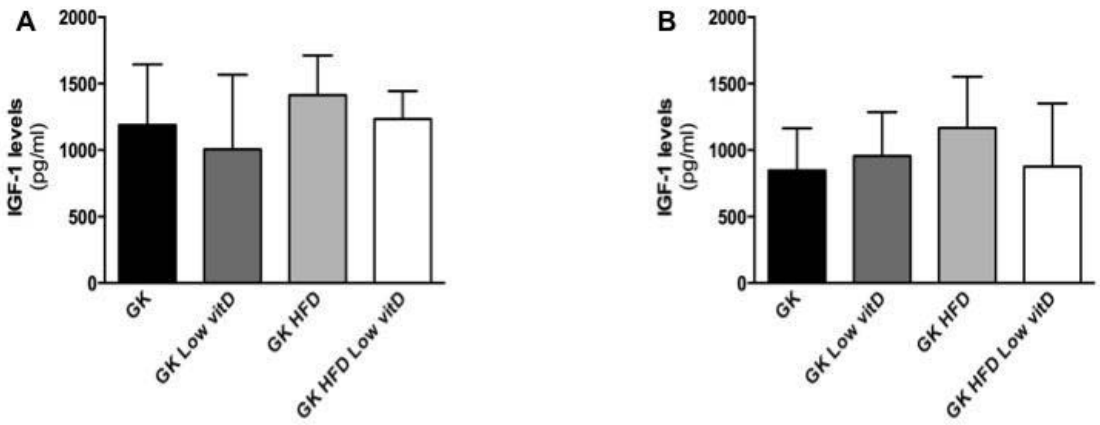


**Figure 4.8 –** ***Effect of high-fat and/or low vitD diets in IGF-1 levels.*** Brain cortex **(A)** and hippocampus **(B)** IGF-1 levels. Data shown are the means ± SEM of 6 animals from each condition studied.

## 4.6 – High-fat and low vitD diets do not affect IDE protein levels

One of the principal functions of insulin degrading enzyme (IDE) is, as its name suggests, the degradation of insulin, regulating the activation of its downstream pathways. Here, we observed that IDE levels were not altered, neither in the brain cortex nor in the hippocampus **(Figure 4.9 A and B).**

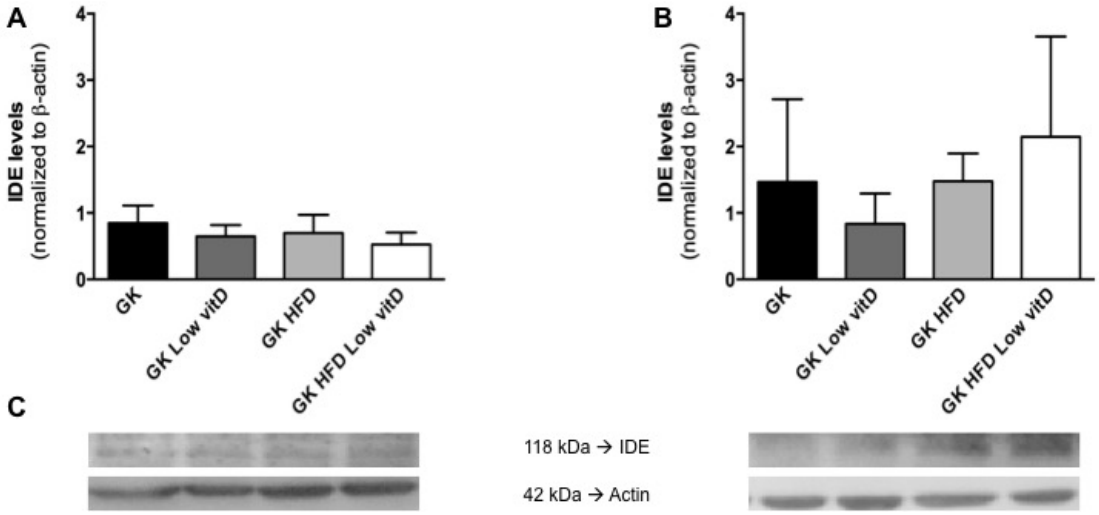


**Figure 4.9 –** ***Effect of high-fat and/or low vitD diets in IDE levels.*** Brain cortex **(A)** and hippocampus **(B)** IDE levels and western blot representative images **(C).** Data shown are the means ± SEM of 3-5 animals from each condition studied.

## 4.7 – High-fat and low vitD diets modulates insulin receptor substrate 2 phosphorylation

In the presence of insulin, IR phosphorylates IRS proteins that activate the PI3K/AKT pathway. In this line, we evaluated ρIRS1 levels and no significant alterations were observed in our experimental groups. However, GK rats exposed to low vitD presented a slight increase in its phosphorylation levels in brain cortex **(Figure 4.10 A)** while in hippocampus no significant alterations were observed **(Figure 4.10 B).**

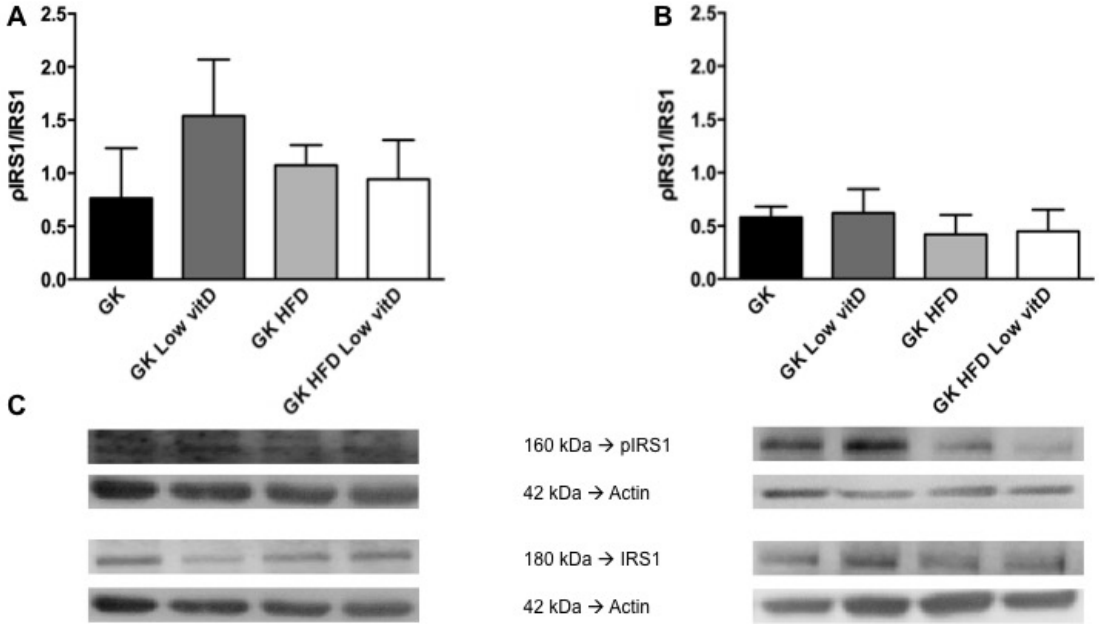


**Figure 4.10 –** ***Effect of high-fat and/or low vitD diets in brain cortex and hippocampus IRS1 phosphorylation.*** Brain cortex **(A)** and hippocampus **(B)** ρIRS1/IRS1 levels and western blot representative images **(C).** Data shown are the means ± SEM of 3-5 animals from each condition studied.

On the other hand, phosphorylation of IRS2 was significantly increased in brain cortex of GK HFD rats when compared with GK group**.** In respect to low vitD diet, in brain cortex, by itself it did not induce any alterations but together with HFD it lead to a decrease in pIRS2 levels when compared with GK HFD **(Figure 4.11 A)**. However, this alteration was not conserved in hippocampus. By opposite, when HFD and low vitD diet were combined, it seemed to slightly increase IRS2 phosphorylation **(Figure 4.11 B)**.

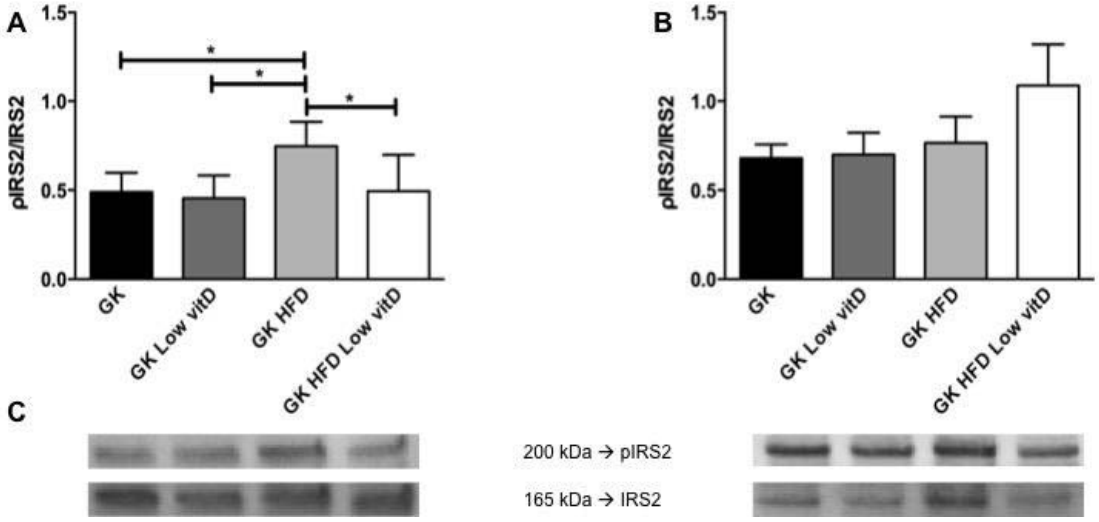


**Figure 4.11 –** ***Effect of high-fat and/or low vitD diets in brain cortex and hippocampus IRS2 phosphorylation.*** Brain cortex **(A)** and hippocampus **(B)** ρIRS2/IRS2 levels and western blot representative images **(C).** Data shown are the means ± SEM of 3-7 animals from each condition studied. Statistical significance: *p<0.05.

Moreover, c-Jun N-terminal kinase protein (JNK) is a stress-activated kinase that inhibits IRS1 phosphorylation decreasing the activation of insulin pathways. In accordance with ρIRS1/IRS1 levels, no significant alterations were observed ρJNK/JNK levels in brain cortex **(Figure 4.12 A)**, although a tendency to decrease ρJNK/JNK levels were observed in low vitD and HDF brain cortex. In the hippocampus, low vitD alone or in combination with HFD induced a slight decrease in the phosphorylation levels of JNK **(Figure 4.12 B)**.

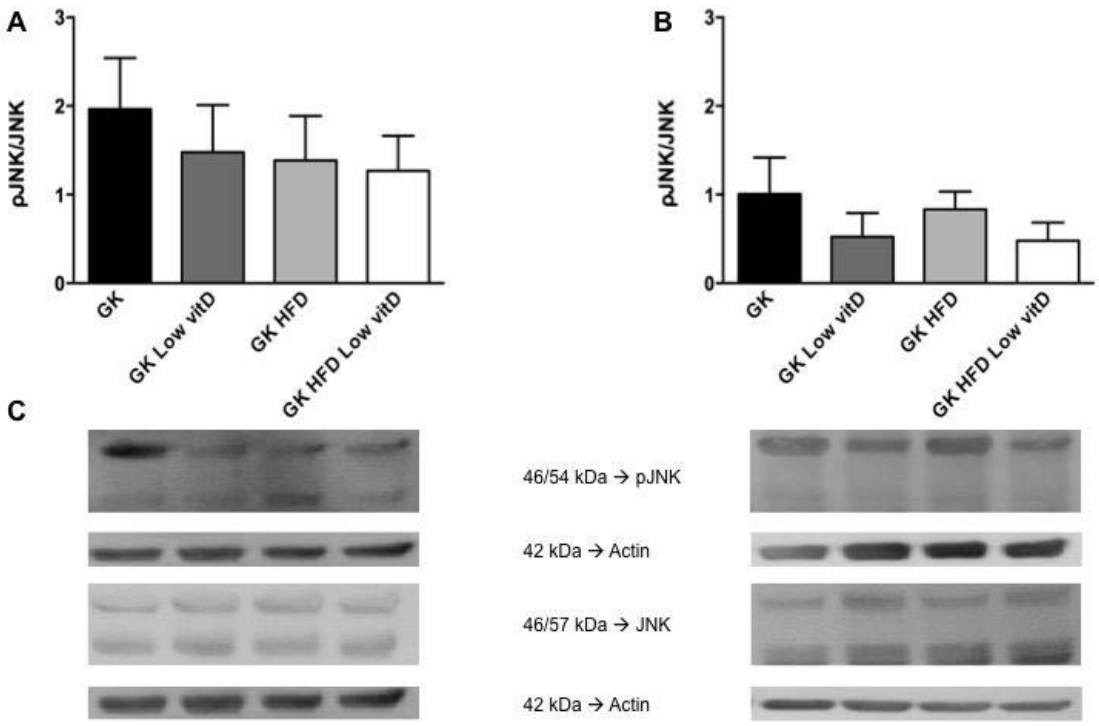


**Figure 4.12 –** ***Effect of high-fat and/or low vitD diets in brain cortex and hippocampus JNK phosphorylation.*** Brain cortex **(A)** and hippocampus **(B)** ρJNK/JNK levels and western blot representative images **(C).** Data shown are the means ± SEM of 3-7 animals from each condition studied.

## 4.8 – High-fat diet increases ERK activation while low vitD diet decreases its activation

ERK phosphorylation gives us an idea about MAPK pathway activation, since it is responsible for several cellular processes activated in response to stimuli that modulate gene expression, and cell growth, proliferation, differentiation, survival and apoptosis. Interestingly, we observed a significant decrease in ERK activation, in both brain cortex and hippocampus of rats exposed to low vitD alone or combined with HFD **(Figure 4.13 A and B)**.

On the other hand, HFD seems to induce the opposite effect, promoting an increase in ERK phosphorylation in both brain cortex and in hippocampus **(Figure 4.13 A and B)**.

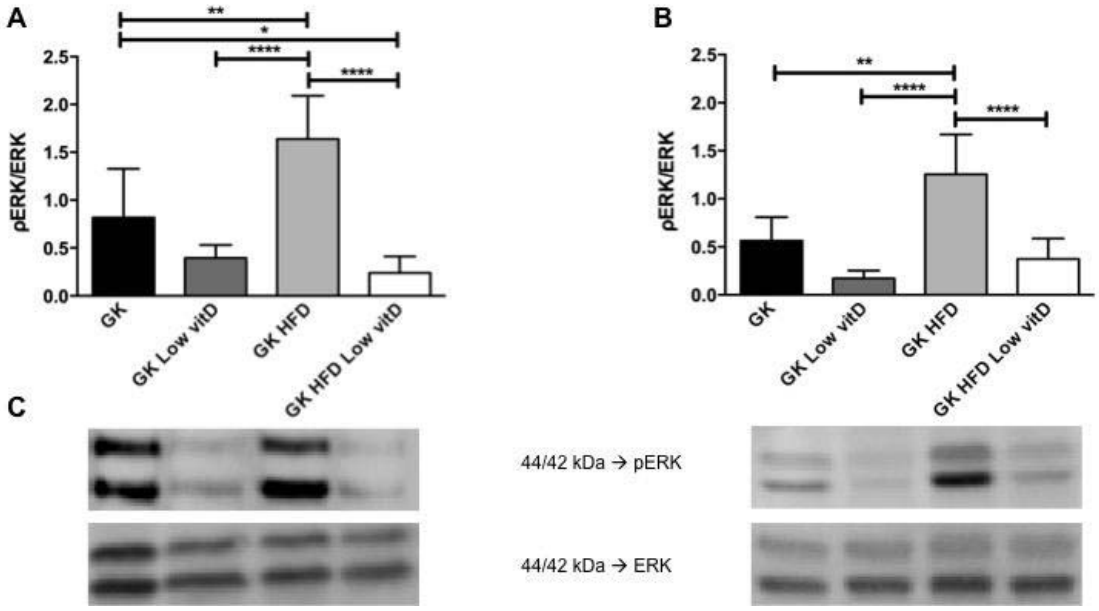


**Figure 4.13 –** ***Effect of high-fat and/or low vitD diets in brain cortex and hippocampus ERK phosphorylation.*** Brain cortex **(A)** and hippocampus **(B)** ρERK/ERK levels and western blot representative images **(C).** Data shown are the means ± SEM of 7 animals from each condition studied. Statistical significance: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

## 4.9 – High-fat and low vitD diets interfere with PI3K pathway

PI3K/AKT is one of the main pathways activated by insulin. Consequently, we analyzed the levels of PI3K, AKT, AMPK and mTOR. No significant alterations in PI3K (p110) levels were observed in both brain cortex and hippocampus **(Figure 4.14 A and B).**

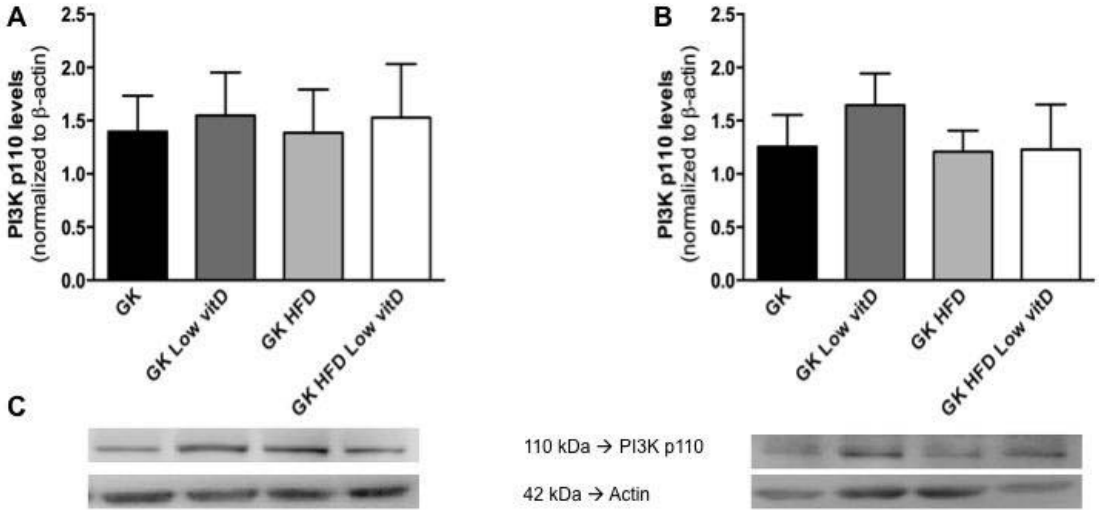


**Figure 4.14 –** ***Effect of high-fat and/or low vitD diets in brain cortex and hippocampus PI3K (p110) levels.*** Brain cortex **(A)** and hippocampus **(B)** PI3K (p110) levels and western blot representative images **(C).** Data shown are the means ± SEM of 7 animals from each condition studied.

However, a decrease in ρAKT/AKT was observed in brain cortex of GK rats treated with HFD **(Figure 4.15 A)** while low vitD diet alone or combined with HFD decreased hippocampal ρAKT/AKT levels but only reached statistical significance when HFD was combined with low vitD **(Figure 4.15 B).**

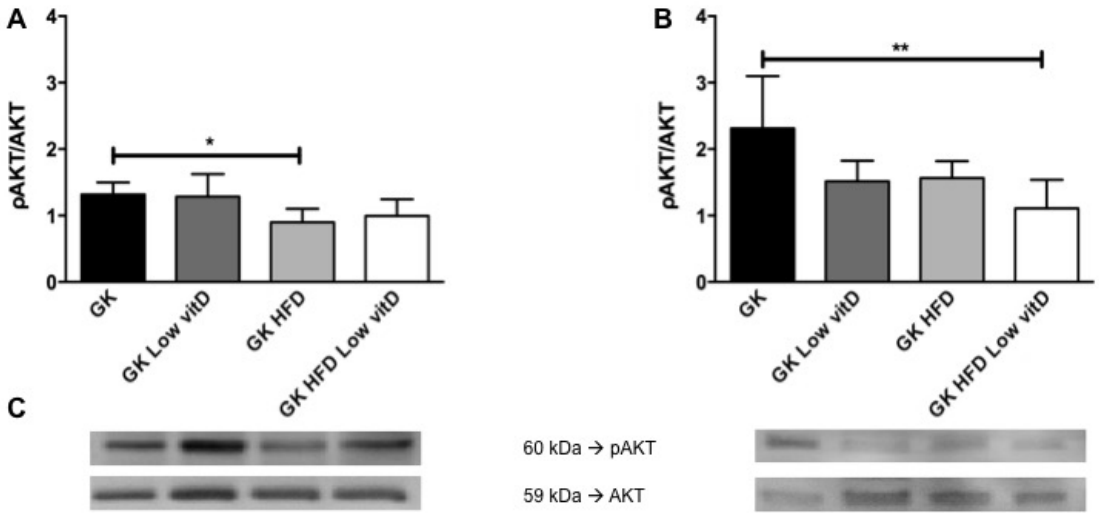


**Figure 4.15 –** ***Effect of high-fat and/or low vitD diets in brain cortex and hippocampus AKT phosphorylation.*** Brain cortex **(A)** and hippocampus **(B)** ρAKT/AKT levels and western blot representative images **(C).** Data shown are the means ± SEM of 6-7 animals from each condition studied. Statistical significance: *p<0.05; **p<0.01.

Concerning to AMPK activation, a significant decrease was observed in in the brain cortex and hippocampus of all groups of animals, this effect being more pronounced when low vitD was combined with HFD **(Figure 4.16 A and B)**.

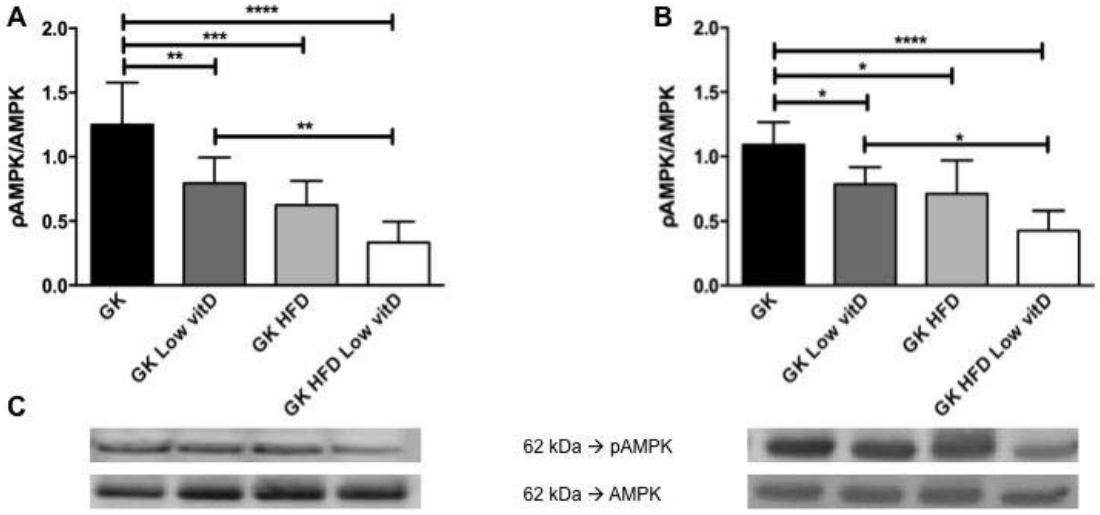


**Figure 4.16 – *Effect of high-fat and/or low vitD diets in brain cortex and hippocampus AMPK phosphorylation.*** Brain cortex **(A)** and hippocampus **(B)** ρAMPK/AMPK levels and western blot representative images **(C).** Data shown are the means ± SEM of 7 animals from each condition studied. Statistical significance: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

Furthermore, it is known that AMPK is responsible for the inhibition of mTOR activity that in turn regulates IRS1 phosphorylation, and insulin signaling pathway. Regarding the activation of mTOR, a slight increase in brain cortex of the HFD group was observed **(Figure 4.17 A).** However, this increase was only statistically significant in the hippocampus, when compared with low vitD treated group **(Figure 4.17 B)**. In the hippocampus, low vitD attenuates the increase in ρmTOR/mTOR levels promoted by HFD **(Figure 4.17 B)**.

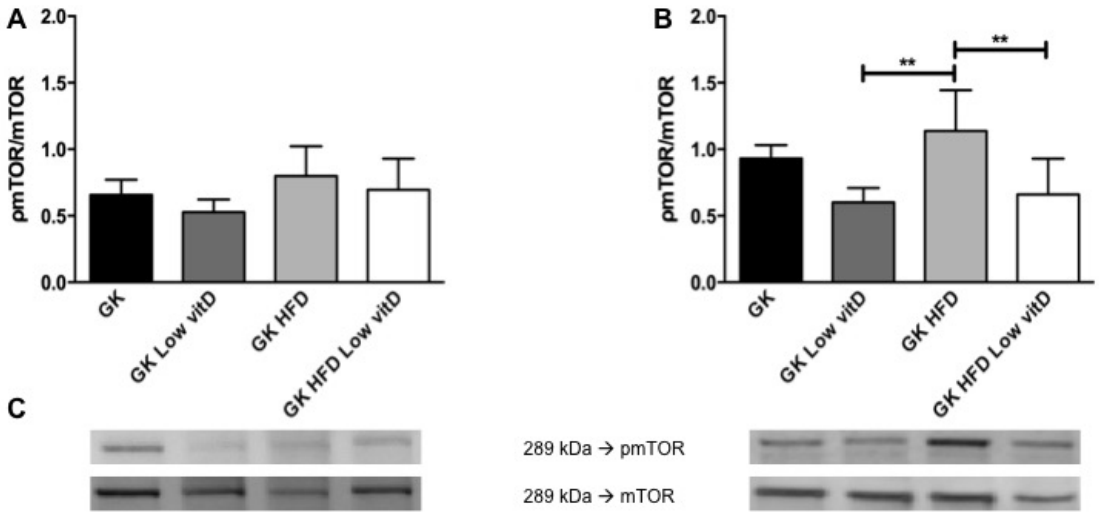


**Figure 4.17 – *Effect of high-fat and/or low vitD diets in brain cortex and hippocampus mTOR phosphorylation.*** Brain cortex **(A)** and hippocampus **(B)** ρmTOR/mTOR levels and western blot representative images **(C).** Data shown are the means ± SEM of 6 animals from each condition studied. Statistical significance: **p<0.01.

## 4.10 – High-fat and low vitD diets affect ρCREB and PKA levels

cAMP, an important biological second messenger, is widely described as being involved in the activation of several proteins such as AMPK, CREB and PKA. In our study, we observed that cAMP levels did not present any statistically significant alteration in brain cortex and hippocampus, although the combination of low vitD and HFD decreased slightly the levels of cAMP in brain cortex **(Figure 4.18 A and B)**.

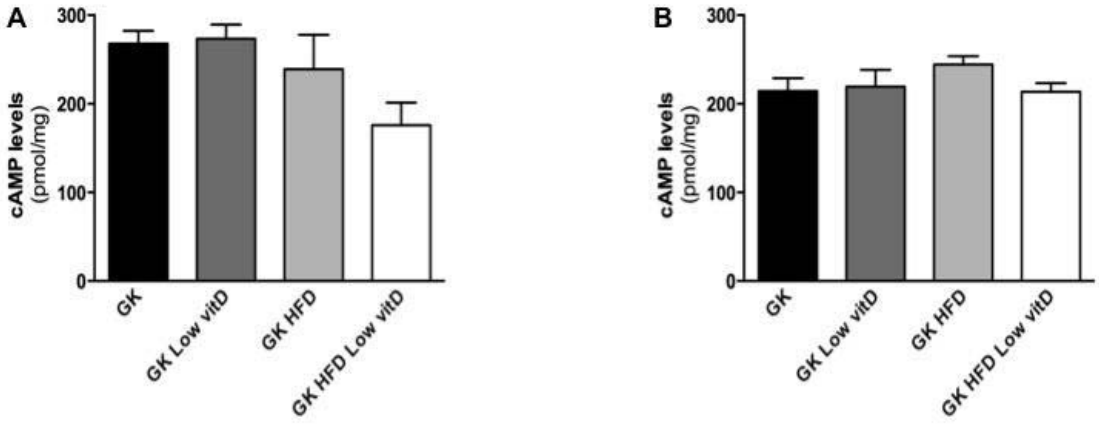


**Figure 4.18 –** ***Effect of high-fat and/or low vitD diets in brain cortex and hippocampus cAMP levels.*** Brain cortex **(A)** and hippocampus **(B)** cAMP levels. Data shown are the means ± SEM of 3-5 animals from each condition studied.

CREB is a transcription factor highly associated with synaptic plasticity and long-term memory, being activated by AKT and cAMP. The activation pattern of CREB was different in brain cortex and hippocampus. Specifically, in brain cortex there was an increase in ρCREB/CREB levels, reaching statistical significance in HFD low vitD group when compared with GK rats **(Figure 4.19 A)**. In hippocampus, the opposite occurs, with a significant decrease observed in the HFD low vitD group when compared with GK rats **(Figure 4.19 B).**

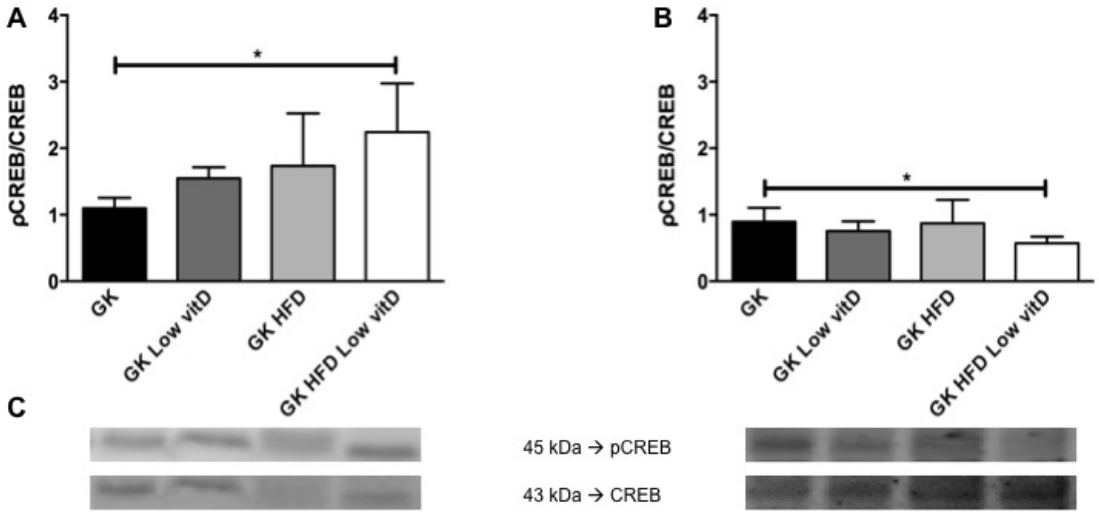


**Figure 4.19 –** ***Effect of high-fat and/or low vitD diets in brain cortex and hippocampus CREB phosphorylation.*** Brain cortex **(A)** and hippocampus **(B)** ρCREB levels and western blot representative images **(C).** Data shown are the means ± SEM of 6-7 animals from each group studied. Statistical significance: *p<0.05.

PKA is a protein kinase also regulated by cAMP levels and it is also involved in synaptic plasticity and memory. Here, despite the absence of alterations observed in cAMP, a decrease in active PKA levels was observed in both brain cortex and hippocampus of HFD and HFD low vitD groups when compared with the GK group **(Figure 4.20 A and B)**. Interestingly, low vitD diet alone also induced a decrease in PKA activity in the hippocampus. Moreover, in the hippocampus, HFD induced a lower decrease in PKA levels than that promoted by low vitD **(Figure 4.20 B)**.

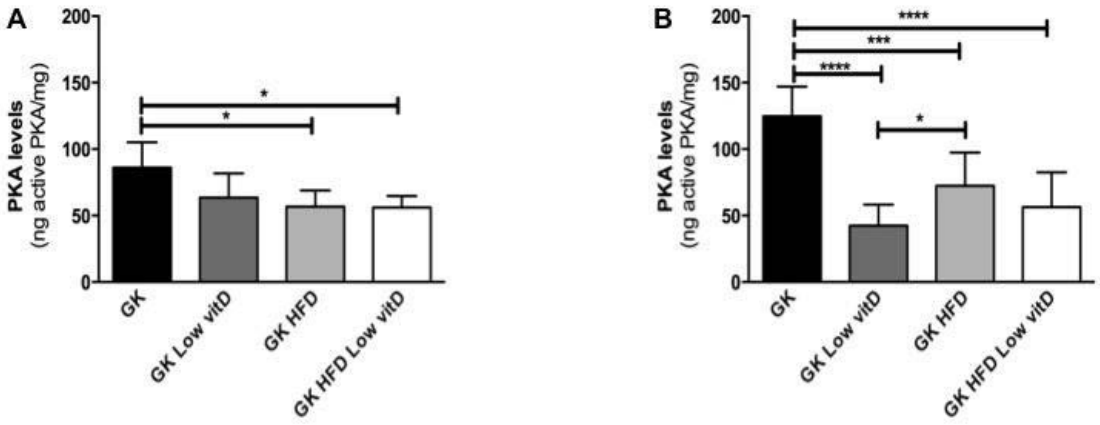


**Figure 4.20 –** ***Effect of high-fat and/or low vitD diets in brain cortex and hippocampus PKA levels.*** Brain cortex **(A)** and hippocampus **(B)** active PKA levels. Data shown are the means ± SEM of 6 animals from each condition studied. Statistical significance: *p<0.05; ***p<0.001; ****p<0.0001.

## 4.11 – High-fat and/or low vitD diets promote GSK3β activation, p35 and p25 levels alterations and Tau protein hyperphosphorylation

The insulin pathway can also regulate GSK3β activity, a kinase that is intimately related with the neuropathological hallmarks of AD such as tau hyperphosphorylation and Aβ formation. In present study no significant alterations were observed in the levels of GSK3β inactive form in brain cortex **(Figure 4.21 A)** while in the hippocampus the combination of low vitD and HFD promoted a significant decrease in the levels of GSK3β inactive form, when compared with HFD group **(Figure 4.21 B).** No significant alterations were observed in GSK3β active form in both brain cortex and hippocampus **(Figure 4.22 A and B).**

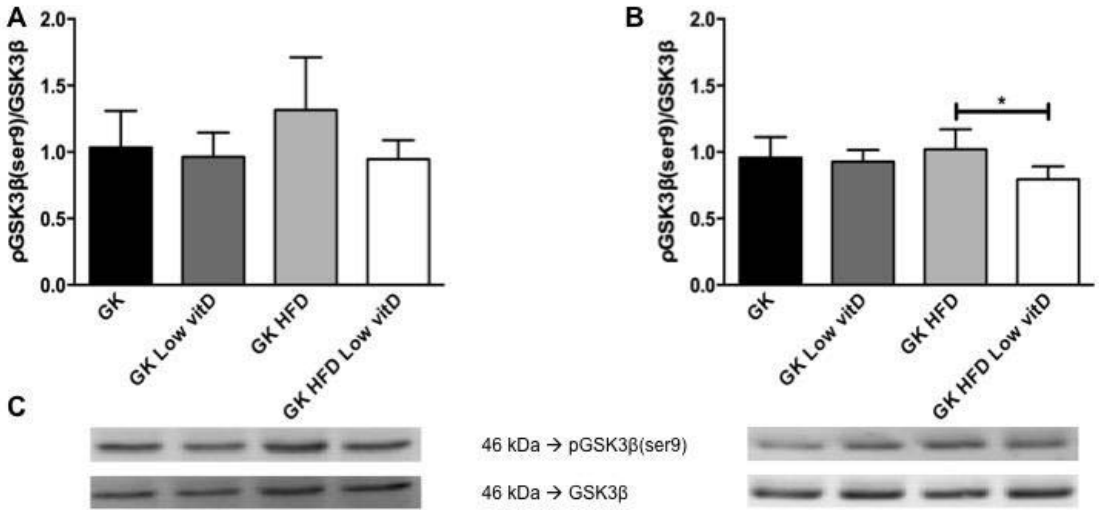


**Figure 4.21 –** ***Effect of high-fat and/or low vitD diets in GSK3β phosphorylated at serine 9 (inactive form) in brain cortex and hippocampus.*** Brain cortex **(A)** and hippocampus **(B)** ρGSK3β(ser9)/GSK3β levels and western blot representative images **(C).** Data shown are the means ± SEM of 7 animals from each condition studied. Statistical significance: *p<0.05.

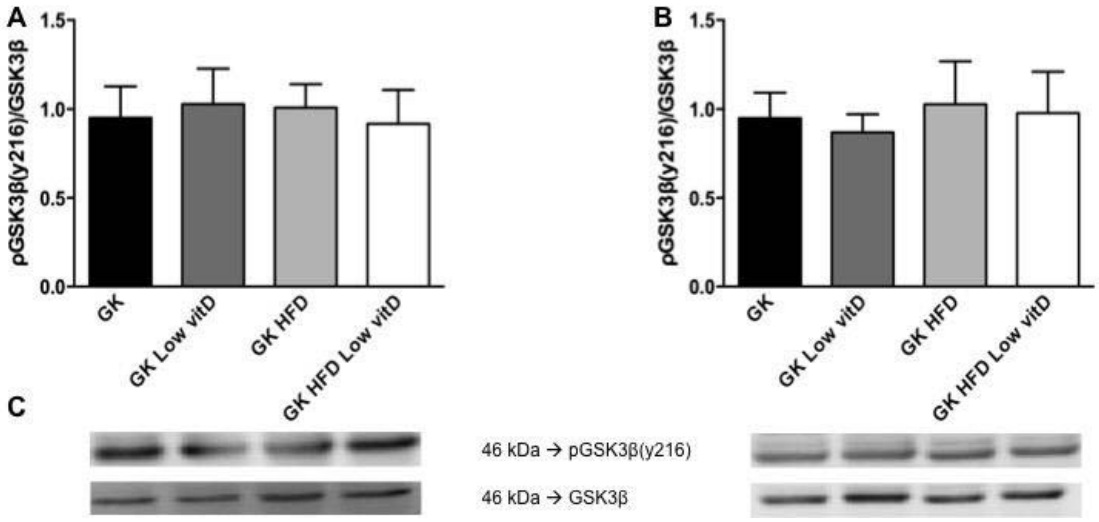


**Figure 4.22 –** ***Effect of high-fat and/or low vitD diets GSK3β phosphorylated at tyrosine 216 (active form) in brain cortex and hippocampus.*** Brain cortex **(A)** and hippocampus **(B)** ρGSK3β(y216)/GSK3β levels and western blot representative images **(C).** Data shown are the means ± SEM of 7 animals from each condition studied.

Besides GSK3β, the complex cyclin-dependent kinase 5 (CDK5)-p25 can also be responsible for tau phosphorylation. Concerning CDK5 levels, no significant alterations were observed in both brain cortex and hippocampus, although HFD seem to induce a slight decrease in CDK5 levels in brain cortex **(Figure 4.23 A and B)**.

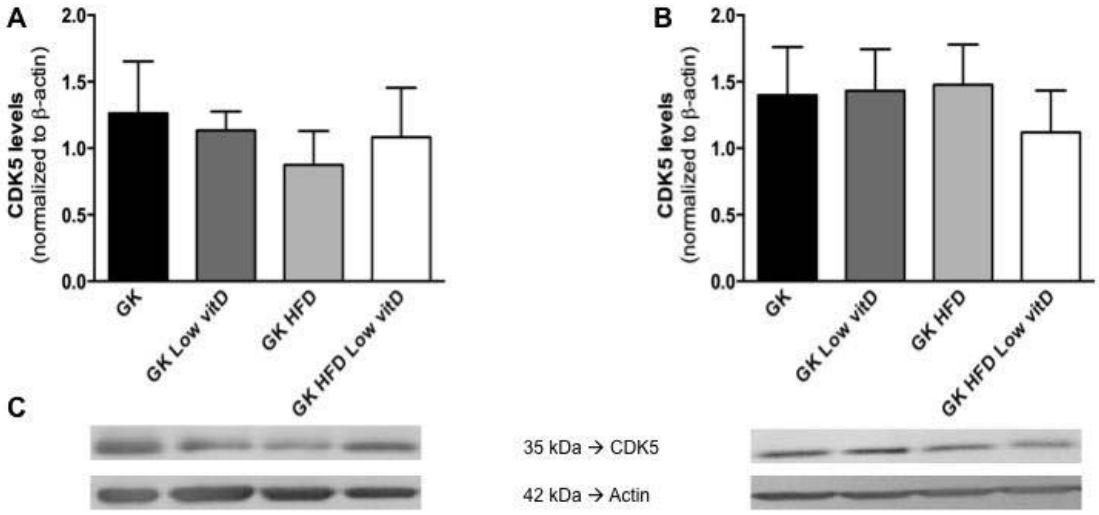


**Figure 4.23 –** ***Effect of high-fat and/or low vitD diets in brain cortex and hippocampus CDK5 levels.*** Brain cortex **(A)** and hippocampus **(B)** CDK5 levels and western blot representative images **(C).** Data shown are the means ± SEM of 7 animals from each condition studied.

However, although not statistically significant, brain cortex from GK low vitD and GK HFD low vitD groups presented an increase in p35 levels **(Figure 4.24 A)** On the other hand, in hippocampus p35 levels are significantly decreased in GK HFD low vitD group when compared with GK and GK HFD groups **(Figure 4.24 B)**.

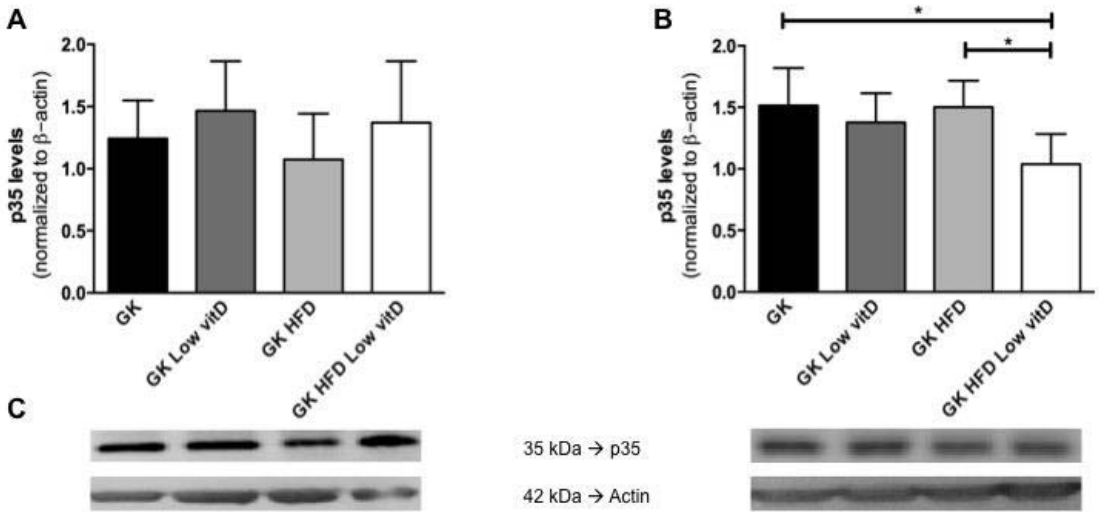


**Figure 4.24 – *Effect of high-fat and/or low vitD diets in brain cortex and hippocampus p35 levels.*** Brain cortex **(A)** and hippocampus **(B)** p35 levels and western blot representative images **(C).** Data shown are the means ± SEM of 6 animals from each condition studied. Statistical significance: *p<0.05.

Concerning p25 levels, a significant decrease in the levels of this protein were observed in brain cortex in HFD rats when compared with GK and GK low vitD groups **(Figure 4.25 A).** However, in hippocampus a significant increase in p25 levels in GK low vitD diet animals occurred when compared with GK rats being this effect attenuated by the combination with HFD **(Figure 4.25 B)**.

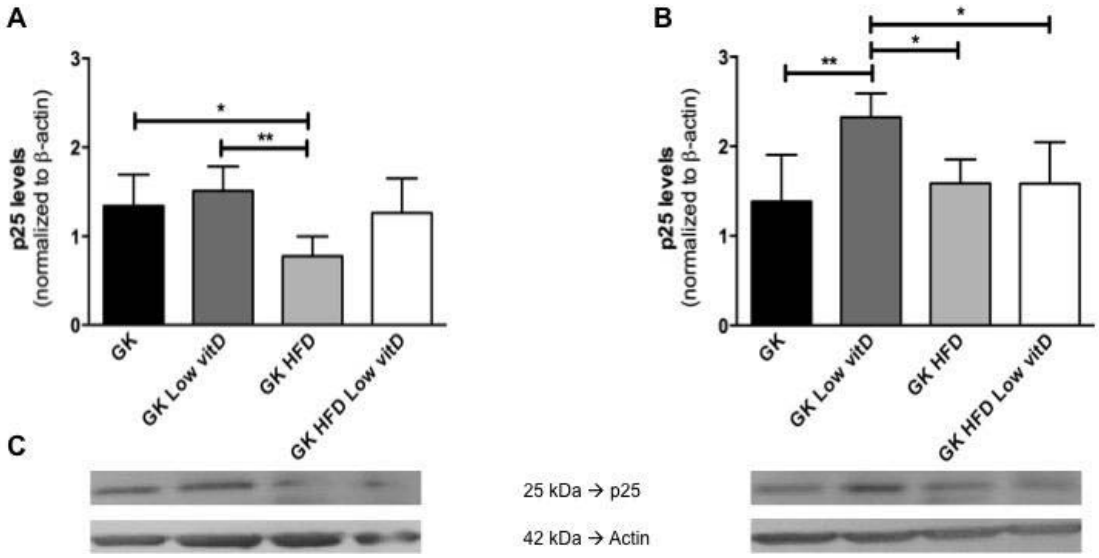


**Figure 4.25 –** ***Effect of high-fat and/or low vitD diets in brain cortex and hippocampus p25 levels.*** Brain cortex **(A)** and hippocampus **(B)** p25 levels and western blot representative images **(C).** Data shown are the means ± SEM of 6 animals from each condition studied. Statistical significance: *p<0.05; **p<0.01.

We next evaluated tau phosphorylation levels and we observed that in brain cortex, HFD induces an increase in the levels of tau phosphorylated at serine 396 residue when compared with GK rats, while in threonine 181 the increase is only statistically significant when compared with GK low vitD rats **(Figure 4.26 A and C)**. A similar pattern was also observed in the hippocampus **(Figure 4.26 B and D)**. No significant alterations were induced by low vitD **(Figure 4.26 A - D).**

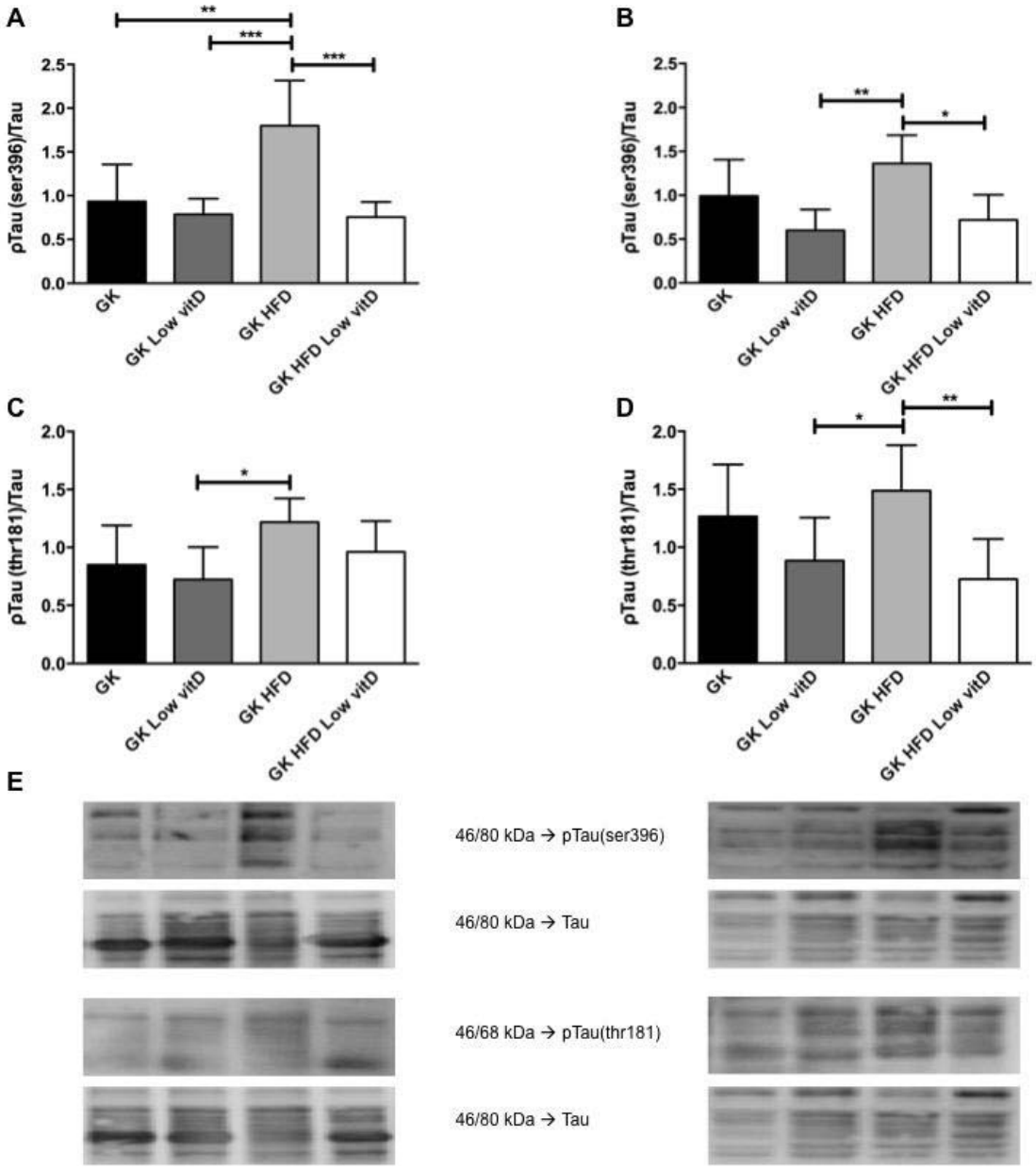


**Figure 4.26 –** ***Effect of high-fat and/or low vitD diets in brain cortex and hippocampus Tau phosphorylation.*** Brain cortex ρTau(ser396)/Tau **(A)**; hippocampus ρTau(ser396)/Tau **(B)**; brain cortex ρTau(thr181)/Tau **(C);** hippocampus ρTau(thr181)/Tau **(D)** and western blot representative images **(E).** Data shown are the means ± SEM of 7 animals from each condition studied. Statistical significance: *p<0.05; **p<0.01; ***p<0.001.

Additionally, the levels of $A\beta_{1-42}$, whose increased accumulation is a feature of degenerative brains, namely AD, were also measured and no alterations in this parameter were observed neither in brain cortex nor in hippocampus of our experimental groups **(Figure 4.27 A and B).**

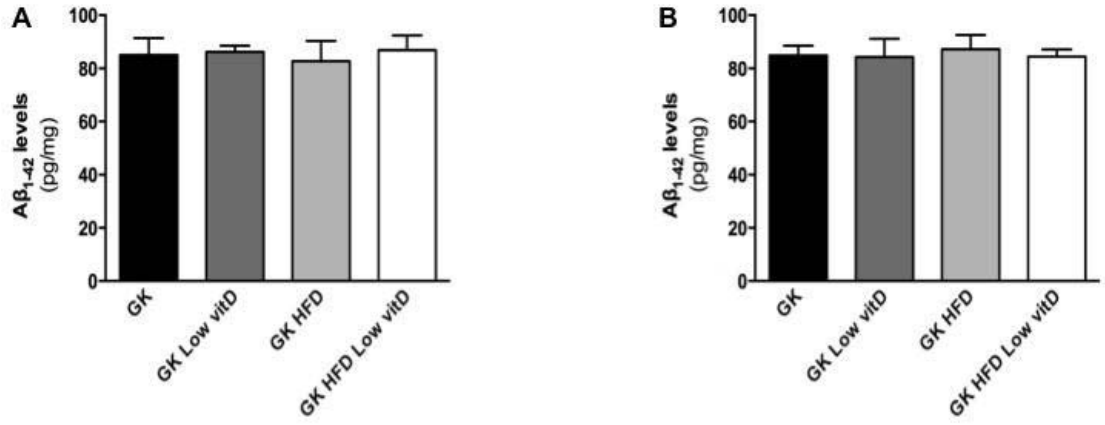


**Figure 4.27 – *Effect of high-fat and/or low vitD diets in brain cortex and hippocampus $A\beta_{1-42}$ levels.*** Brain cortex **(A)** and hippocampus **(B)** $A\beta_{1-42}$ levels. Data shown are the means ± SEM of 7 animals from each condition studied.

## 4.12 – High-fat and low vitD diets decrease cholesterol levels but do not affect synaptic integrity

In order to evaluate synaptic integrity, the levels of cholesterol and of three synaptic markers were determined. Brain is the most cholesterol-rich organ and its regulation seems to be extremely important for correct synapse formation and function (Orth and Bellosta, 2012). Furthermore, cholesterol levels can also be regulated by insulin (Suzuki et al., 2010). When compared with low vitD group, a decrease in brain cortex cholesterol levels was observed in the HFD low vitD group **(Figure 4.28 A)**. However, in

hippocampus no statistically significant differences were observed **(Figure 4.28 B).**

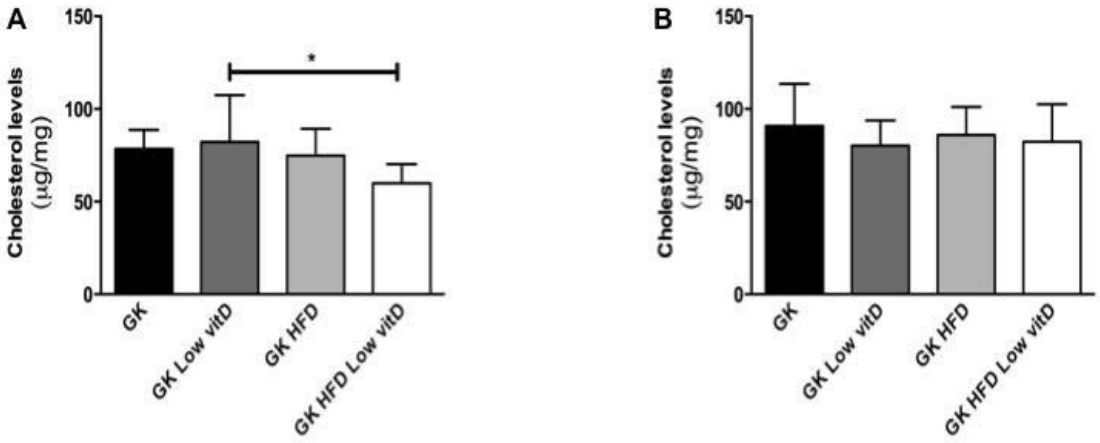


**Figure 4.28 –** ***Effect of high-fat and/or low vitD diets in brain cortex and hippocampus cholesterol levels.*** Brain cortex **(A)** and hippocampus **(B)** cholesterol levels. Data shown are the means ± SEM of 8 animals from each condition studied. Statistical significance: *p<0.05.

Concerning the synaptic integrity markers, we evaluate the levels of synaptophysin, a protein present in presynaptic vesicles, synaptosomal-associated protein 25 (SNAP25), which helps the docking of presynaptic vesicles in order to release neurotransmitters to the synapse, and post-synaptic density protein 95 (PSD95) that is present on postsynaptic density. No significant alterations were observed in both brain cortex and hippocampus exposed to the different dietary regimens **(Figure 4.29 A-F)**.

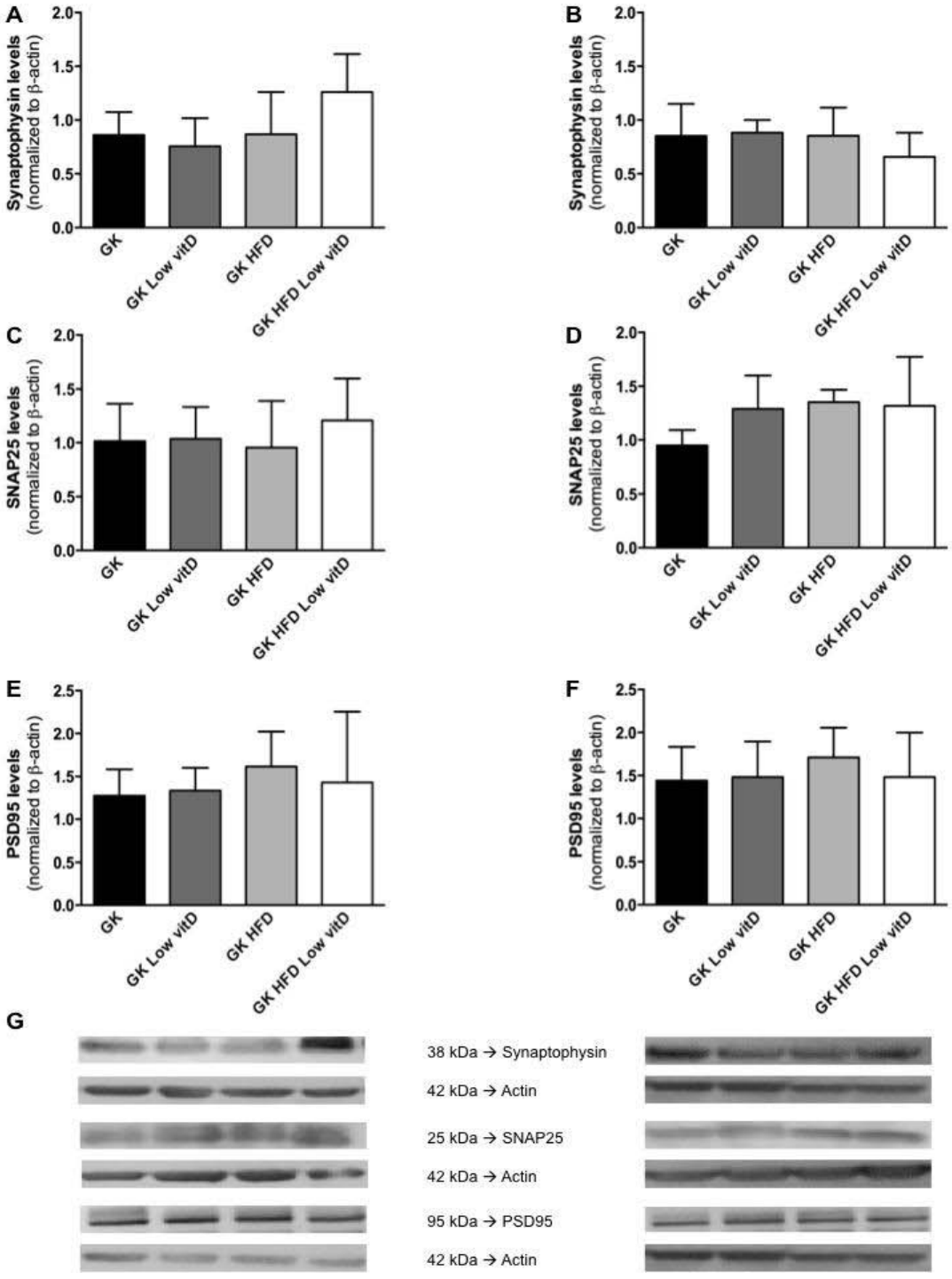


**Figure 4.29 –** ***Effect of high-fat and/or low vitD diets in brain cortex and hippocampus synaptic markers.*** Brain cortex **(A)** and hippocampus **(B)** synaptophysin levels; brain cortex **(C)** and hippocampus **(D)** SNAP25 levels; brain cortex **(E)** and hippocampus **(F)** PSD95 levels and western blot representative images **(G).** Data shown are the means ± SEM of 7 animals from each condition studied.

# *Chapter 5 – Discussion*

## 5.1 – Discussion

Our work reveals that high-fat and/or low vitamin D diets affect insulin-mediated signaling pathways in the diabetic brain, which may increase the risk of neurodegeneration.

Insulin resistance is a common feature of T2D and metabolic syndrome (Umegaki, 2012, Moreira, 2013, Hussain et al., 2014), a situation that can be aggravated by poor diets (e.g. high intake of fats) and lack of physical exercise (Zimmet et al., 2001, American Diabetes, 2014). Insulin resistance underlies several diabetes-associated complications, including alterations in the biochemistry, structure and function of CNS (Sima, 2010). It was already demonstrated that insulin resistance plays a key role in neuronal degeneration and loss (Hussain et al., 2014), which may affect several cognitive functions, particularly learning and memory abilities (Cardoso et al., 2009, Candeias et al., 2012, Duarte et al., 2012). In fact, T2D has been considered a major risk factor for vascular dementia and AD (Sebastiao et al., 2014).

Interestingly, hypovitaminosis D has been associated not only with diabetes (Norman et al., 1980, Hirani et al., 2014, Reddy et al., 2015) but also with dementia (Evatt et al., 2008). In fact, vitD is involved in neuromodulation, neuroprotection and neurotrophic support (Eyles et al., 2013, Annweiler et al., 2014) sharing similar functions with insulin (Alkharfy et al., 2013).

In this study we used GK rats, an animal model of non-obese T2D, exposed to distinct diets (standard, low vitD, HFD and low vitD + HFD) as previously referred. We started by performing a systemic characterization of the animals to evaluate the impact of the diets. GK rats presented an increase

in fasting glycemia and $HbA_{1C}$ and a significant decrease in body weight, as previously reported (Duarte et al., 2000, Santos et al., 2014). Although HFD only promoted a slight increase in $HbA_{1C}$ and body weight, it significantly increased the levels of plasma total and non-HDL cholesterol and triglycerides, which is in accordance with previous studies (Srinivasan et al., 2005, Petrov et al., 2015). In the literature we can find several effects of HFD depending on the duration and composition of the diet and animal model. A previous study showed that distinct HFD (42% energy; fat sources: HF-L - lard; HF-O - olive oil; HF-C - coconut fat; HF-F - fish oil) induced dissimilar alterations in Wistar rats; while HF-L and HF-O fed rats showed the most pronounced obesity and insulin resistance, insulin sensitivity in HF-C and HF-F was close to normal (Buettner et al., 2006). Lee and coworkers (2011) showed that wild type mice fed a short-term HFD developed insulin resistance and presented increased body weight and deposition of lipid content in liver and skeletal muscle. More recently, it was demonstrated that HFD-induced obese rats do present insulin resistance but not increased serum free fatty acids (Jiang et al., 2015). Interestingly, our study demonstrated that low vitD attenuated HFD-induced increased body weight and total cholesterol, non-HDL and triglycerides levels. Although low levels of serum vitamin D are associated with obesity and diabetes in humans (Liel et al., 1988), a study performed in vitD deficient rats showed that these animals present less body weight gain and lower amounts of visceral fat when compared with control rats (Bhat et al., 2014). In our study, the low vitamin D diet did not affect blood glucose. Although some evidence exists demonstrating that low levels of serum vitamin D are associated to high blood glucose levels (Kostoglou-

Athanassiou et al., 2013, Hirani et al., 2014), this relation remains a matter of debate. Also our rats fed a low vitD diet did not present alterations in triglycerides levels, which confirm the observations made by De Novellis and coworkers (1994).

For a long time, brain was considered an insulin-insensitive organ. Nowadays, it is widely accepted the fundamental role of insulin in CNS and disturbed insulin signaling is associated with altered cerebral biochemistry, structure and function (Cardoso et al., 2009). Although most of the insulin present in the brain is of peripheral origin, there is also a local production of this hormone (Cardoso et al., 2009, Duarte et al., 2012).

We evaluated insulin signaling in brain cortex and hippocampus because these structures are highly enriched in insulin receptors and also VDR (Eyles et al., 2005), are intimately involved in learning and memory processes and are target areas in T2D and AD (Cardoso et al., 2009). Our study revealed that in brain cortex low vitD diet and HFD increase the levels of insulin, while in the hippocampus only HFD and the combination HFD and low vitD increases the levels of the hormone. These alterations seem to indicate a central insulin resistance resembling the peripheral hyperinsulinemia that occur in early stages of T2D. It was demonstrated that peripheral HFD induces a secondary hyperinsulinemia (Pratchayasakul et al., 2011) as a response to insulin resistance that later will evolve to a state of hypoinsulinemia due to pancreatic β-cells dysfunction and consequent failure of insulin production (Butler et al., 2003). So, we hypothesize that in our animals, a compensation mechanism is occurring in an attempt to overcome central insulin resistance (Liu et al., 2015a).

IGF-1 is a growth factor that can exert insulin-like functions and can also activate IR (Duarte et al., 2012). Nevertheless, no significant alterations were observed in the levels of this growth factor. It was previously reported that IGF-1 levels are downregulated in the heart of streptozotocin (STZ)-induced T1D rats (Jiang and Steinle, 2010) and friend virus-b type mouse (FVB) mice under HFD (Zhang et al., 2012). Cheng and colleagues (2003) also found decreased IGF-1 messenger ribonucleic acid (mRNA) levels in the temporal cortex of mice under ketogenic HFD. But, as said above, different experimental protocols and models may lead to distinct observations. Concerning vitD, it was shown that adolescents with severe vitD deficiency present low plasma levels of IGF-1 (Soliman et al., 2014) and vitD supplementation was shown to increase IGF-1 levels (Hypponen et al., 2008, Ameri et al., 2013). More studies are necessary to better understand the effects of HFD and low vitD IGF-1 levels, particularly in the brain.

The action of insulin is a process under tight regulation. IDE is a zinc metallopeptidase that degrades intracellular insulin, and thereby terminates insulin activity, as well as participating in intercellular peptide signaling by degrading diverse peptides such as glucagon, amylin, bradykinin, and Aβ (Kim et al., 2011). Deficiencies in this protein's function are associated with AD and T2D (Maianti et al., 2014). Although a previous study demonstrated that HFD fed wild-type mice presented lower levels of IDE in the hippocampus (Petrov et al., 2015), we did not observe any significant alterations induced by the diets.

To properly exert their functions, insulin and IGF-1 bind to IR and IGF-1R initiating a cascade of events. Of notice, under insulin resistant conditions

some tissues or organs may compensate by increasing IR and/or IGF-1R expression (Sesti, 2000), a situation that was observed in the brain cortex of rats exposed to the combination HFD and low vitD. Interestingly, in the same group of animals a significant decrease in VDR levels was observed. Because vitD share similar functions with insulin (Deeb et al., 2007, Vuolo et al., 2012), the increase in IR levels may compensate for the decrease of VDR levels. It was also reported that VDR mutant mice suffer premature aging (Keisala et al., 2009), suggesting that the loss of VDR in the brain may accelerate brain cortex aging predisposing to neurodegenerative events. It was previously reported that under chronic inflammation, a characteristic of T2D, a significantly decreased VDR expression was observed in lung tissue (Agrawal et al., 2012), suggesting that in our study the lower levels of VDR could reflect a chronic inflammation of brain cortex. In accordance with our observations, Pratchayasakul and coworkers (2011) did not find alterations in IR levels in the hippocampus of HFD fed Wistar rats. Concerning IGF-1R, although it was previously showed an increase in brain cortex of HFD fed Sprague-Dawley rats (Cheng et al., 2003), our results revealed no alterations in the levels of this receptor induced by the diets. No significant alterations were observed in low vitD fed rats. As far as we know, there is no literature dealing with vitD and IGF-1R levels in the brain.

When insulin binds to IR, IRS1 and IRS2 are phosphorylated in order to further activate downstream pathways. Our results showed no alterations in ρIRS1 levels in any of the studied conditions however, a significant increase in ρIRS2 levels was observed in brain cortex of HFD rats. Previous studies have demonstrated that IRS2 in endothelial cells plays a crucial role in

pancreatic insulin secretion (Hashimoto et al., 2015). Perhaps in brain cortex increased ρIRS2 may help in insulin synthesis or action. Concerning the hippocampus, our observations are in accordance with those of Petrov et al (2015), showing that HFD fed mice hippocampus do not have significant alterations in ρIRS levels.

Diabetes and obesity are associated with an increased activation of the stress-response kinase JNK (Kaneto et al., 2005). However, we did not observed significant alterations in JNK. It should be noted that we are comparing the effects of low vitD diet and/or HFD in brain insulin signaling of T2D brains. The lack of significant alterations observed in our study may simply demonstrate that our diets were insufficient to aggravate T2D-associated alterations.

Nevertheless, we observed a significant increase in ρERK levels in brain cortex and hippocampus of HFD fed rats, which is in accordance with the observations made by Petrov and coworkers (2015). On the other hand, low vitD alone or in combination with HFD lead to a decrease in activation of ERK. Indeed, it was previously shown that vitD could be involved in the activation of this pathway since it was observed that vitD supplementation in T-helper cells was responsible for an increase in ρERK (Nanduri et al., 2015).

Consistent with the unaltered ρIRS1 levels we did not observe differences in the activation of PI3K among all groups of rats. Besides insulin, also vitD has been shown to activate PI3K (Wu and Sun, 2011). It was previously reported an increase in PI3K levels in the hypothalamus of 4-week-old FVB/N mice fed a HFD for 19 weeks an effect that was not yet observed after 4 weeks of treatment (Metlakunta et al., 2008). Thus, we can speculate

that PI3K activation may be differently modulated depending on the stages of disease progression. Interestingly, although we did not observed significant differences in PI3K (p110) levels, ρAKT was decreased in the brain cortex of GK rats fed a HFD. In the hippocampus, ρAKT levels decreased in low vitD plus HFD diet fed rats.

These results are in agreement with previous studies demonstrating a decreased activation of AKT in the heart of STZ-induced diabetic rats (Jiang and Steinle, 2010) and in the hippocampus of wild-type mice fed a HFD (Petrov et al., 2015). A decrease in ρAKT was also observed in the amygdala of diet-induced obese rats (Castro et al., 2013). Another study demonstrated that in 7-week-old male C57/BL mice exposed to a high energy diet the expressions of PI3K and AKT protein in brain tissue were significantly lower than those of the control group after 30 weeks of treatment (Ma et al., 2014). Interestingly, high-fat diet-induced, insulin-dependent PI3K activation in neurons of ventromedial hypothalamus contributes to obesity development (Klockener et al., 2011).

AKT activation leads to a negative regulation of AMPK, through the regulation of ATP levels. AMPK is an energy sensor and plays a role in insulin sensitivity and glucose homeostasis (Tao et al., 2010). A decrease in AMPK activation was observed in both brain cortex and hippocampus of low vitD diet and/or HFD. These observations are in agreement with a previous study (Lindholm et al., 2013) demonstrating that HFD decreased AMPK activation in adipose tissue, heart and liver of Sprague-Dawley rats. Also Greig and coworkers (2015) reported that the aortic tissue of alipoprotein E (ApoE) -/- mice on high fat diet for 6 weeks present a decrease in AMPK activation.

Similarly, HFD-induced obese mice presented a decreased activation of AMPK in the hippocampus (Wang et al., 2015). It was also reported that 1,25-dyhydroxyvitamin $D_3$ attenuated rotenone-induced neurotoxicity in SH-SY5Y cells through induction of autophagy, a phenomenon that also involves increased expression of AMPK (Jang et al., 2014). VitD in skeletal muscle was shown to improve muscle function, mass and strength (Teegarden and Donkin, 2009). AMPK is also able to increase glucose uptake from exercise (Teegarden and Donkin, 2009), raising the hypothesis that vitD is able to improve insulin sensitivity through AMPK-dependent glucose uptake.

The PI3K/AKT pathway activates several proteins including mTOR (Scott et al., 1998), a protein involved in protein synthesis and cell growth (Vander Haar et al., 2007). The measurement of mTOR phosphorylation levels is one of the best ways to assess its activation and in our study we observed an increase in mTOR activation in the hippocampus of HFD fed GK animals, an effect that was abolished by low vitD. A previous study from our laboratory showed an increase in mTOR phosphorylation in GK rats brain cortex (Santos et al., 2014). There is evidence of increased mTOR activation in the liver of mice under HFD (Korsheninnikova et al., 2006). Also HFD fed rainbow trout showed increased activation of mTOR in the liver and hypothalamus (Libran-Perez et al., 2015). Evidence concerning the effects of low vitD in mTOR activation is scarce, with few studies showing a decrease in mTOR activation in HL60 cancer cells (Wu and Sun, 2011). It was also shown that 1,25$(OH)_2D_3$ protects β cells against high glucose-induced apoptosis through mTOR suppression (Yang et al., 2015) while in murine C2C12 skeletal myotubes it enhances mTOR activation enhances the stimulating the

effect of leucine and insulin on protein synthesis (Salles et al., 2013). Of notice, mTOR phosphorylation can occur in different residues and we only measured the phosphorylation of serine 2448, the most common residue.

The activation of PI3K pathway inhibits GSK3β activation and in insulin resistance conditions an increased activation of this protein is observed (Dokken et al., 2008). In our study no significant alterations were observed, except for the significant decrease in GSK3β inactivation (phosphorylation of serine 9) observed in the hippocampus of low vitD plus HFD fed rats. An increase GSK3β activation was observed in the hippocampus of wild-type rats and C57BL/6 mice fed a HFD (Petrov et al., 2015). In this line, Sharma and Taliyan (Sharma and Taliyan, 2014) observed that indirubin-3'-monoxime, a GSKβ inhibitor, plays a neuroprotective role in HFD-induced cognitive impairment in mice, suggesting that GSK3β inhibition could prove to be beneficial in insulin resistant conditions. As far as we know, no studies exist concerning the role of vitD in GSK3β activation.

GSK3β plays a key role in tau phosphorylation and Aβ production. We did not find significant alterations in the levels of $A\beta_{1-42}$. A similar observation was made in the hippocampus of HFD fed wild-type rats treated (Petrov et al., 2015). However, several other studies demonstrate that diabetes and insulin resistant conditions are associated with increased levels of Aβ (Carvalho et al., 2012, Son et al., 2012). Although we did not find a pronounced alteration in the activity of GSK3β, a significant increase in tau phosphorylation was observed in both brain cortex and hippocampus of HFD fed rats, an effect that was avoided when HFD was combined with low vitD. Accordingly, it was previously shown that STZ-induced diabetic rats (Santos et al., 2014a) and

HFD fed mice (Julien et al., 2010) present increased levels of tau phosphorylation in the brain.

CDK5 is a modulator of tau phosphorylation and APP maturation and when activated CDK5 converts p35 in p25 (Patrick et al., 1999). No significant alterations were observed in the levels of CDK5. However, a decrease in p35 levels was observed in hippocampus of GK rats treated with HFD low vitD diet. Concerning p25 levels a decrease was observed in the brain cortex of HFD rats and an increase in the hippocampus of low vitD diet fed rats, reflecting brain area specific alterations. Teegarden and coworkers (2009) showed an increase in CDK5 levels in ventral striatum of HFD fed mice. Furthermore, in hippocampus of HFD fed wild-type rats p35 levels were increased (Petrov et al., 2015). Also, vitD was shown to increase p35 levels in HL60 cancer cell-line (Chen et al., 2004). p25 can form a complex with CDK5 promoting tau hyperphosphorylation. In fact in AD patients, p25 is increased (Patrick et al., 1999), which may support our observations concerning the increase in p25 in low vitD diet fed mice. In fact, low serum levels of vitD are commonly observed in AD patients (Przybelski and Binkley, 2007, Evatt et al., 2008, Littlejohns et al., 2014). These results seem to be in contradiction with tau phosphorylation levels however, we should note that tau can be phosphorylated in several residues and we only measured the phosphorylation at serine 396 and threonine 181.

cAMP is involved in several cellular processes including the insulin signaling pathway and synaptic plasticity (Rivera et al., 2015). Also, it is associated with Aβ levels regulation (Rivera et al., 2015). In brain cortex a slight decrease in cAMP was observed in low vitD, HFD fed rats. Makar and

coworkers (1995) reported that T2D db/db mice had reduced ability to synthesize cAMP. It is also known that vitD stimulates the activity of adenylyl cyclase, responsible for cAMP production (Deeb et al., 2007), which lead us to hypothesize that the low vitD diet can also contribute for the decrease in cAMP levels.

CREB, a well-known transcription factor responsive to cAMP, has many functions in many different organs, and some of its functions have been studied in relation to the brain. In fact, CREB has a well-documented role in neuronal plasticity and long-term memory formation (Silva et al., 1998). In our study CREB levels increased significantly in brain cortex of rats exposed to low vitD combined to HFD while in the hippocampus the opposite was observed. It was previously shown that CREB mRNA levels decreased in HFD fed Sprague-Dawley rats hippocampus (Molteni et al (2002), although no alterations were observed in brain cortex. Accordingly, Miao and coworkers (2015) showed that the hippocampal levels of CREB are decreased in diabetic rats and 3 month-old C57BL/6J mice exposed to HFD for 12 weeks showed a suppression of the ERK/CREB pathway in the brain (Liu et al., 2015). Also, mouse skeletal muscle cells supplemented with vitamin D showed an enhancement of ρCREB levels through ERK activation (Ronda et al., 2007), these results being in accordance with our observations.

PKA, whose activity in dependent on cAMP levels, is also involved in memory formation and synaptic plasticity (Rivera et al., 2015). Our study revealed that PKA activation is decreased in both brain cortex and hippocampus of rats fed a HFD and/or low vitD diet. It was previously reported that disturbances of CaMKII/PKA/PKC phosphorylation in the hippocampus is

an early change that may be associated with the development and progression of diabetes-related cognitive dysfunction (Liao et al., 2013). However, impaired working memory and hippocampal LTD deficits in juvenile-onset diabetes mellitus rats were shown to be associated with an excessive PKA activity, which may play a role in altered NMDA receptor function and impaired LTD (Sacai et al., 2014). Recently, it was demonstrated that learning and memory deficits of STZ-induced diabetic rats were ameliorated by the administration of vitD (Calgaroto et al., 2014, Moghadamnia et al., 2015) corroborating the idea that vitD play a key role in cognitive function. However, we must be aware that distinct patterns of PKA activation may occur depending on the causes and duration of the diabetic status.

Cholesterol is essential for neuronal physiology: it is a major component of cell membranes and precursor of steroid hormones, it contributes to the regulation of ion permeability, cell shape, cell-cell interaction, and transmembrane signaling (Vance, 2012). Defects in brain cholesterol metabolism may contribute to neurological syndromes such as AD, and even to the cognitive deficits typical of the old age (Refolo et al., 2000). A significant decrease in brain cortex cholesterol was observed in low vitD, HFD fed rats suggesting that these animals are more susceptible to neurodegenerative events. However, no significant alterations were observed in the hippocampus, which is in accordance with a previous study (Granholm et al., 2008).

The brain depends upon glucose as its main source of energy, and tight regulation of glucose metabolism is critical for brain physiology. Glucose is transported across the cell membranes by facilitated diffusion mediated by

Gluts (Glut 1 mainly in brain endothelial and glial cells; Glut 3 and Glut 4 mainly in neuronal cells) (Duelli and Kuschinsky, 2001). Interestingly, we observed alterations in the levels of insulin sensitive and insensitive Gluts. The decrease in Glut 4 (insulin sensitive) was the most pronounced alteration in both brain cortex and hippocampus. Also, the levels of the partially insulin sensitive Glut 3 decreased in the hippocampus of rats fed the HDF diet alone or combined with low vitD. These alterations possible reflect the disturbances in insulin signaling, as previously discussed. In fact, it was previously shown that HFD fed C57BL/6J mice presented decreased expression and plasma membrane localization of the insulin-sensitive neuronal glucose transporters Glut 3/Glut 4 (Liu et al., 2015) as well as reduced adipose tissue Glut 4 mRNA expression (McAllan et al., 2013). In skeletal muscle of HFD fed rats a decrease in Glut 4 levels was also observed (Tremblay et al., 2001). Another study showed that the brain cortex of HFD fed rats do not present alterations in Glut 3 and Glut 1 mRNA levels (Cheng et al., 2003). But some compensations also seem to happen: an increase in Glut 3 levels in the brain cortex of rats fed a HFD combined with low vitD and an increase in Glut 1 levels in low vitD fed rats. However, it was previously reported that Glut 3 mRNA expression in brainstem might be activated by vitamin D (Peeyush Kumar et al., 2011). But we must be aware that mRNA expression does not always correlate with protein levels.

Adequate ATP production and regulation of the metabolic processes are essential for the maintenance of synaptic transmission and neuronal function. Brain's function depends on the maintenance of synaptic integrity and plasticity, processes that are highly affected in AD and T2D-associated

neurodegeneration (Cardoso et al., 2009, Correia et al., 2012, Moreira, 2013, Carvalho et al., 2015). In order to evaluate the impact of diets-induced alterations discussed above, we evaluated the levels of 3 synaptic integrity markers: synaptophysin, SNAP25 and PSD95. We did not observe statistically significant alterations in the levels of those markers. Accordingly, previous studies reported no significant alterations in the levels of those synaptic markers in brain cortex (Julien et al., 2010) and hippocampus (Bhat and Thirumangalakudi, 2013) of HFD fed mice. However, Arnold and collaborators (Arnold et al., 2014) found decreased expression of PSD95 and synaptopodin, an actin-associated protein enriched in spine apparatuses in brain cortex of HFD mice. Also vitamin D supplementation has been shown to improve synaptic function (Latimer et al., 2014).

In summary, our work shows that low vitD and/or HFD disturb(s) insulin-mediated signaling pathways in T2D brains. However, some compensation mechanisms seem to occur that may help overcome the metabolic changes induced by those diets. Although the diets-induced alterations potentially increased the susceptibility to neurodegenerative processes and cognitive deficits (e.g. increase in tau protein phosphorylation) no changes in synaptic integrity were detected. In fact, we can find many contradictory observations in the literature, which may result from different durations and composition of the diets and different animal models. We hypothesize that more pronounced effects would be observed in older rats and/or by increasing the duration of the diets. Another interesting observation is that both brain cortex and hippocampus behave differently demonstrating brain-structure specific susceptibilities. However, more studies are warranted

to elucidate the impact of excessive consumption of fats and deficiency in vitD in brain biochemistry, structure and function.

# *Chapter 6 – References*

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