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Insulin signalling depends on the endocannabinoid system in the brain.

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ABBREVIATION LIST

[^{3}H]DG - 2-^{3}H(N)-deoxy-D-glucose

2-AG - 2-arachidonoyl-glycerol

2-AGE - 2-arachidonyl-glyceryl ether

Aβ - amyloid β

AD - Alzheimer's disease

AEA - *N*-arachidonoyl-ethanolamine

CB_1R - cannabinoid receptor type 1 (or 2)

CNS - central nervous system

COX-2 - cycloxygenase-2

DAG - diacylglycerol

DAGL - diacylglycerol lipase (α or β)

DM - diabetes mellitus

DSE/I - depolarization-induced suppression of excitation/inhibition

eCB - endocannabinoid

ERK - extracellular signal-regulated kinase

FAAH - fatty acid amide hydrolase

GABA - γ-aminobutyric acid

$GABA_AR$ - $GABA_A$ receptor

GLUT4 - glucose transporter type 4 (or others, from 1 to 8)

GPCR - G protein-coupled receptor

IGF-1 - insulin-like growth factor 1 (or 2)

IGF-1R - insulin-like growth factor 1 receptor

ILP - insulin-like peptide

IP_3 - inositol 1,4,5-trisphosphate

InR - insulin receptor

InRS - insulin receptor substrate

KO - knockout

LTD - long-term depression

MAGL - monoacylglycerol lipase

MAPK - mitogen-activated protein kinase

mAChRs - metabotropic muscarinic receptors

mGluRs - metabotropic glutamate receptors

MRI - magnetic resonance imaging

MSE/I - metabotropic-induced suppression of excitation/inhibition

NADA - *N*-arachidonoyl-dopamine

NAPE - *N*-acyl-phosphatidylethanolamine

NAPE-PLD - N-acyl-phosphatidylethanolamine-hydrolyzing phospholipase D

NArPE - N-arachidonoyl-phosphatidylethanolamine

NFTs - neurofibrillary tangles

OEA - *N*-oleoyl-ethanolamine

PET - positron emission tomography

PI_3K - phosphatidylinositol 3-kinase

PIP_2 - phosphatidylinositol 4,5-bisphosphate

PKB - protein kinase B

PLC_β - phospholipase C_β (or D)

PPF - paired-pulse facilitation

PPR - paired-pulse ratio

PTEN - phosphatase and tension homologue

RTK - receptor tyrosine kinase

STZ - streptozotocin

T1DM - type 1 or insulin-dependent diabetes mellitus

T2DM - type 2 or non-insulin dependent diabetes mellitus

THC - Δ^9-tetrahidrocannabinol

tLTD - timing-dependent long-term depression

$TRPV_1$ - transient receptor potential vanilloid type 1

VGCC - voltage-gated calcium channel

VTA - ventral tegmental area

Virodhamine - *O*-arachidonoyl-ethanolamine

WHO - World Health Organization

RESUMO

A insulina e o sistema endocanabinóide (eCB) exercem funções muito importantes no organismo, tanto na periferia como no cérebro. Algumas destas funções são desempenhadas em comum, uma vez que as vias de sinalização intracelular destes dois sistemas se sobrepõem. Além disso, o recetor de canabinóides do tipo 1 (CB_1R) tende a formar heterodímeros com recetores de tirosina cinase (RTKs). Recentemente, foi demonstrado através de experiências de *voltage-clamp* em *whole-cell* que a perfusão de insulina induz a libertação de eCBs nos corpos celulares dopaminérgicos da área tegmental ventral (VTA), revelando a existência de uma interação direta entre a insulina e o sistema endocanabinóide no sistema nervoso central (CNS).

Assim, com o objetivo de caracterizar algumas das funções da insulina no cérebro e perceber como é que a sua ação depende do CB_1R, foram realizadas experiências de electrofisiologia, para medição da transmissão sináptica, e de captação de glucose.

Com este trabalho verificou-se que o efeito da insulina na transmissão sináptica basal é dependente do CB_1R e das condições metabólicas do organismo (jejum ou diabetes induzida por streptozotocina (STZ)), em fatias de hipocampo de rato. Adicionalmente, o cálculo do *paired-pulse ratio* (PPR) demonstra que a ação da insulina também é pré-sináptica. A insulina diminuiu também a captação de glucose, em fatias de hipocampo e de córtex de rato, sendo este efeito dependente do CB_1R. A deleção genética do CB_1R diminuiu igualmente a captação de glucose. Finalmente, experiências preliminares de Western blot indicam que tanto o recetor de insulina (InR) como o CB_1R estão localizados na zona pós-sináptica, no córtex de ratinhos e humanos. Assim, estes resultados sugerem uma interação entre o InR e o CB_1R no cérebro, que poderá ser direta ou indireta. Experiências

adicionais serão necessárias para perceber se estes dois recetores formam um heterodímero ou interagem indiretamente.

Palavras-chave: insulina; endocanabinóides; transmissão sináptica; captação de glucose.

ABSTRACT

Insulin and the endocannabinoid (eCB) system have important and common functions in both peripheral and brain metabolism. This is due to the overlapping of their intracellular signalling pathways and the tendency of the cannabinoid receptor type 1 (CB_1R) to form heterodimers with receptor tyrosine kinases (RTKs). Recently it was demonstrated by whole-cell voltage-clamp experiments that bath application of insulin triggers eCB release in ventral tegmental area (VTA) dopaminergic cell bodies, revealing a direct interaction between insulin and the eCB system in the central nervous system (CNS). Thus, synaptic transmission and glucose uptake measurements were made in order to characterize some of the functions of insulin in the brain and to understand if these functions depend on the CB_1R.

Here is reported that the effect of insulin on basal synaptic transmission is dependent on the CB_1R and also on metabolic conditions like fasting or diabetes induced by streptozotocin (STZ), in rat hippocampal slices. Besides, a presynaptic action of insulin is suggested by paired-pulse ratio (PPR) calculations. Insulin also inhibited glucose uptake in a CB_1R-dependent fashion in both hippocampal and cortical slices of fed rats. The genetic deletion of the CB_1R also impaired glucose uptake. Finally, preliminary Western blot experiments suggested that both insulin receptor (InR) and CB_1R are localized in the postsynaptic active zone of mice and human cortices.

Hence, this work suggests an interaction between the InR and CB_1R, that might be direct or indirect. Further experiments will be necessary to understand if these two receptors form or not an heterodimers.

Keywords: insulin; endocannabinoids; synaptic transmission; glucose uptake.

1. INTRODUCTION

1.1. Insulin

1.1.1. The insulin molecule

It was in 1923 when Frederick Grant Banting and John Rickard Macleod were jointly awarded the Nobel Prize in Physiology or Medicine for the discovery of insulin (Nobelprize.org, 1923). Since then, many discoveries have been made and now we have a much better understanding of the importance of insulin and its numerous signalling pathways. Nevertheless, there is still a lot to uncover and to understand.

Insulin is a small protein with two polypeptide chains, A and B, joined by two disulphide bonds. In the periphery, insulin is synthesized in the pancreas as an inactive precursor called preproinsulin, which has an amino-terminal signal sequence that addresses its passage into secretory vesicles. Proinsulin is produced by the proteolytic cleavage of the signal sequence of preproinsulin and consequent formation of three disulphide bonds. It is stored in secretory granules in pancreatic β-cells and converted to active insulin by specific proteases, when blood glucose is elevated, in order to be released into the circulatory system (Nelson and Cox, 2005).

1.1.2. Insulin signalling

Insulin is an anabolic hormone that regulates both metabolism and gene expression. It promotes the synthesis and storage of carbohydrates, lipids and proteins, while inhibiting their degradation and release into the circulation. Insulin increases glucose uptake in muscle and fat, stimulating the translocation of the glucose transporter type 4 (GLUT4) from intracellular sites to the cell surface. It inhibits hepatic glucose production by blocking gluconeogenesis and glycogenolysis, acting as the main regulator of blood glucose

concentration. Insulin also regulates the expression or activity of enzymes that catalyse glycogen, lipid and protein synthesis, whilst hindering the activity or expression of those that catalyse degradation. Thus, insulin stimulates cell growth and differentiation and promotes the storage of substrates in fat, liver and muscle by stimulating lipogenesis, glycogenesis and protein synthesis, while it inhibits lipolysis, glycogenolysis and protein breakdown (Saltiel and Kahn, 2001).

Insulin signalling is mediated by a complex and highly integrated network that controls several processes. Its action involves a series of signalling cascades which start with insulin binding to its specific cell-surface receptor (Cheatham and Kahn, 1995; Cuatrecasas, 1972 and Freychet *et al.*, 1971), the consequent receptor autophosphorylation and activation of the receptor tyrosine kinases (RTKs), resulting in the phosphorylation of tyrosine residues of insulin receptor substrates (InRSs) (Kapeller *et al.*, 1999; Sato *et al.*, 1999 and Soumaya, 2012).

In its native conformation, the insulin receptor (InR) is composed by two identical α-chains and two β-subunits covalently linked through disulphide bonds to form an $\alpha_2\beta_2$-hereotetramer (Cheatham and Kahn, 1995 and Nelson and Cox, 2005). It has two splice forms which are usually co-expressed in cells that also express the related insulin-like growth factor 1 receptor (IGF-1R), which is activated by the insulin-like growth factor 1 (IGF-1) and can also be activated by insulin (Taniguchi *et al.*, 2006). The InR and the IGF-1R belong to the class II of the subfamily of RTKs, because they both contain cysteine-rich motifs in their extracellular α-subunit and are disulphide-linked heterotetramers. The InR α-subunit contains the extracellular insulin binding domain, whereas the β-subunit holds sites for *N*- and *O*-linked glycosylation, a transmembrane domain and an intracellular domain that contains an insulin-stimulated protein tyrosine kinase (Cheatham and Kahn, 1995). Insulin binding to the α-subunit leads to activation of

the kinase activity in β-subunits, followed by transphosphorylation of those β-subunits and a conformational change which further increases the kinase activity (Patti and Kahn, 1998 and Saltiel and Kahn, 2001). Therefore, in the presence of insulin, the InR phosphorylates InRSs which are linked to the activation of two main signalling pathways: the phosphatidylinositol 3-kinase (PI_3K)-Akt/protein kinase B (PKB) pathway, which starts with the activation of PI_3K and is responsible for most of the metabolic actions of insulin, including glucose transport and GLUT4 translocation (Cheatham *et al.*, 1994); and the Ras-mitogen-activated protein kinase (MAPK-ERK, of extracellular signal-regulated kinases) pathway, which regulates the expression of some genes and cooperates with the PI_3K-Akt pathway to control cell growth and differentiation (Avruch, 1998 and Taniguchi *et al.*, 2006). Following the PI_3K-Akt pathway, activated (phosphorylated) Akt phosphorylates AS160 (its 160 kDa substrate) which, in turn, stimulates the translocation of GLUT4 from intracellular vesicles to the plasma membrane (Sano *et al.*, 2003 and 2007). GLUT4 is the transporter found in adipose tissues and skeletal and cardiac muscle responsible for glucose uptake into the cells.

The InR is dephosphorylated and inactivated by protein tyrosine phosphatases (Bourdeau *et al.*, 2005 and Harley and Levens, 2003) like the phosphatase and tensin homologue (PTEN) deleted on chromosome 10, which serves as an important negative modulator for the PI_3K-Akt pathway (Sasaoka *et al.*, 2006; Vereshchagina *et al.*, 2008 and Vinciguerra and Foti, 2006). Thus, the physiological regulation of insulin action is mainly controlled by the balance between phosphorylation and dephosphorylation reactions (Soumaya, 2012).

1.1.3. Insulin resistance and diabetes

Western lifestyle combines the easily accessible high caloric carbohydrate and fat-rich food, with a sedentary lifestyle leading to obesity and associated diseases. The World Health

Organization (WHO) has already considered overweight and obesity the fifth leading risk factor for global deaths and calculated that 44 % of diabetes, 23 % of ischemic heart disease and between 7 and 41 % of certain cancer burdens are attributable to overweight and obesity (Who.int, 2013). With time, the human metabolism exposed to these conditions develops resistance to insulin, resulting in diabetes and neuropathy in the periphery and neurodegenerative diseases in the brain.

Diabetes mellitus (DM) is the most common endocrine disorder, currently affecting over 380 million people world-wide and prospectively over 365 million in the year 2030 (Wild *et al.*, 2004). DM is represented mostly by two types: type 1 or insulin-dependent DM (T1DM), which is mostly due to autoimmune-mediated destruction of the pancreatic islets of Langerhans, resulting in insulin deficiency; and type 2 diabetes or non-insulin dependent DM (T2DM), that is characterized by insulin resistance and is usually associated with abnormal insulin secretion. Most cases of diabetes are of T2DM (Bhattacharya *et al.*, 2007; Zimmet, 1999 and Zimmet *et al.*, 2001). Gestational diabetes affects 10 % of pregnant women and is probably a natural process, but also requires management.

T2DM is a complex disease characterized by a combination of impaired insulin action, increased hepatic glucose production and insulin secretory defects. For insulin resistant individuals who are not diabetic, control of blood glucose levels can be arranged by compensatory increases in insulin secretion by pancreatic β-cells (Rhodes, 2005). But when insulin resistance goes along with the dysfunction of pancreatic islet β-cells this control fails. Therefore, together with β-cell failure, the major pathophysiological source of T2DM is the resistance of target tissues to insulin (Ginsberg, 2000 and Larsen, 2009). Insulin resistance is the initial measurable symptom in patients who are in risk of developing T2DM (Utzschneider *et al.*, 2011) and also contributes to the morbidity of obesity.

1.1.4. Insulin in the brain

1.1.4.1. Insulin and insulin receptors in the brain

The brain was thought to be insulin insensitive for many years, but it has been suggested that insulin in the brain regulates both peripheral and central glucose metabolism, neurotransmission, learning and memory and confers neuroprotection (Duarte *et al.*, 2012b). The presence of InRs in the central nervous system (CNS) was first demonstrated by *in vitro* binding studies (Havrankova *et al.*, 1978a, 1981, 1983 and Plum *et al.*, 2005). InRs were then identified in both neurons and glial cells of the rat brain (Wozniak *et al.*, 1993), but their sub-cellular localization was found just a few years later. Using immunohistochemistry and western blotting techniques, the InR was localized at synapses from cultured hippocampal neurons (Abbott *et al.*, 1999) as well as insulin (Baskin *et al.*, 1983a, 1983b, 1987; Havrankova *et al.*, 1978a, 1981 and Plum *et al.*, 2005). Besides, InR expression was also described in the olfactory bulb, hypothalamus, cerebral cortex, hippocampus and the pituitary intermediate lobe (Havrankova *et al.*, 1981; Plum *et al.*, 2005; Unger and Betz, 1998 and van der Heide *et al.*, 2006).

The origin of brain insulin is still not fully understood, but it is known to be present in the cerebrospinal fluid (Baura *et al.*, 1993 and Fernandez and Torrez-Alemán, 2012). Glucose was also shown to significantly enhance the immunoreactive insulin released in rat brain synaptosomes (Santos *et al.*, 1999). Hence, it has been suggested that insulin in the brain can have two different sources: it can be transferred through the blood-brain barrier (Banks, 2004; Schwartz *et al.*, 1992 and Woods *et al.* 2003), with lower concentration when compared to insulin levels in the blood stream; and it can also be locally synthesised (Woods *et al.*, 2003 and van der Heide *et al.*, 2006), which seems to occur in pyramidal neurons from hippocampus, prefrontal cortex, enthorhinal cortex and olfactory bulb, but not in glial cells

(Hoyer, 2003). Thus, insulin can be found in brain cortex, olfactory bulb, hippocampus, hypothalamus and amygdala (Havrankova *et al.*, 1978b and Duarte *et al.*, 2012b).

1.1.4.2. Insulin signalling in the brain

Some data also suggests that insulin is neuroprotective, having an important role in neuronal maintenance and survival. For instance, it was shown that insulin is capable of inhibiting apoptosis of cerebellar granule cells, acting through the InR rather than the IGF-1R (Schubert *et al.*, 2004 and van der Heide *et al.*, 2006).

Just like in the periphery, insulin in the brain binds to its receptor and activates two signal transduction cascades: the PI_3K-Akt pathway and the MAPK-ERK pathway (van der Heide *et al.*, 2006). In the brain, the PI_3K-Akt pathway is of a major importance because, among other reasons, it mediates the effects of PI_3K in neuronal survival, since the overactivation of PI_3K and Akt kinase prevents apoptosis (Philpott *et al.*, 1997; Rodgers and Theibert, 2002 and van der Heide *et al.*, 2006). On the contrary, the hypoactivity of the PI_3K-Akt pathway leads to tau hyperphosphorylation, resulting in the retraction of neuronal processes and the accumulation of neurofibrillary tangles (NFTs) - both are major hallmarks of Alzheimer's disease (AD) (Liu *et al.*, 2008).

1.1.4.2.1. Insulin signalling and glucose metabolism in the brain

The brain is dependent on a strictly regulated supply of glucose (Erecinska and Silver, 1989). Glucose uptake in the brain occurs mainly through GLUT1 and GLUT3 isoforms (Emmanuel *et al.*, 2013 and Mueckler, 1994), although there is evidence of expression of GLUT2, GLUT4, GLUT5 and GLUT8 in neurons (Choeiri *et al.*, 2002 and Uemura and Greenlee, 2006). GLUT1 mediates resting glucose uptake independently of insulin action (Vannuci, 1994) while GLUT3 enables glucose uptake from the interstitium into neurons

(Emmanuel *et al.*, 2013; Mueckler, 1994 and Simpson *et al.*, 2007). In primary cultures of rat neurons and astroglia, glucose deficiency and hypoxia increase glucose uptake by facilitating the translocation of GLUT proteins to the plasma membrane (Bruckner *et al.*, 1999; Loike *et al.*, 1992 and Sivitz *et al.*, 1992). Also in culture, insulin and membrane depolarization combined stimulate the fusion of GLUT3 with the neuronal plasma membrane, resulting in increased glucose uptake (Uemura and Greenlee, 2001). Then, studies in human subjects with positron emission tomography (PET) showed that insulin modulates neural activity and, probably as a consequence, glucose uptake in those brain areas which are normally also involved in control of food intake, especially in regions related to appetite and reward as the hypothalamus and orbitofrontal cortex. Therefore, the lack of these responses can serve as measures of cerebral insulin resistance (Anthony *et al.*, 2006).

1.1.4.2.2. Insulin signalling and neurotransmission

The main inhibitory neurotransmitter in the CNS is γ-aminobutyric acid (GABA), which is released from the axon terminals to bind to synaptic and extrasynaptic $GABA_A$ channels and to decrease neuronal excitability. By this mechanism, GABA mediates phasic and tonic neural inhibition, respectively. It is worth noting that tonic conductance regulates basal neuronal excitability, it is controlled by hormones and it seems to modulate cognitive functions in the hippocampus (Caraiscos *et al.*, 2004; Crestani *et al.*, 2002; Martin *et al.*, 2010; Pavlov *et al.*, 2009 and Prut *et al.*, 2010). It was described that insulin inhibits spontaneous firing of rat hippocampal pyramidal neurons (Palovcik *et al.*, 1984) and that insulin increases the number of $GABA_A$ channels (Wan *et al.*, 1997). Then, it was found that insulin has an important effect in the hippocampal CA1 pyramidal neuronal excitability,

increasing the GABA mediated tonic inhibitory conductance and, consequently, decreasing the action potential firing frequency in the CA1 pyramidal neurons (Jin *et al.*, 2011).

1.1.4.2.3. Insulin signalling and synaptic plasticity - learning and memory

As it was previously said, InRs have been found in higher density in the brain structure directly related to learning and memory, the hippocampus. In the last few years, pieces of evidence linking neuronal insulin signalling and cognitive function have been found in the hippocampus (Duarte *et al.*, 2012b; Ghasemi *et al.*, 2013 and McNay and Recknagel, 2011). For instance, it was demonstrated in animal studies that the hippocampal expression of proteins involved in the insulin signalling cascade increases in response to cognitive activity (Dou *et al.*, 2005; McNay *et al.*, 2010 and Zhao *et al.*, 1999). Cognitive activity and long-term memory storage are centred in synaptic neural transmission and molecular changes at the postsynaptic density. And it was found both InRs and intracellular InRS proteins in that synaptic active zone (Abbott *et al.*, 1999). Localized insulin synthesis in neurons and excitatory neurotransmitters induced changes in neuronal insulin signalling capacity (Hori *et al.*, 2005), supporting the idea that insulin signalling affects neuronal activity and, specifically, hippocampal cognitive activity (Emmanuel *et al.*, 2013). Short-term memory formation after a spatial learning task is also associated with an up-regulation of the InR mRNA in the rat hippocampal CA1 area. Increases in InR protein levels have also been found in hippocampal synaptic membrane fractions after short-term memory formation (van der Heide *et al.*, 2006 and Zhao *et al.*, 1999). Furthermore, specific defects in neuronal insulin signalling were already associated with a deficit in NMDA receptor-dependent synaptic plasticity in the hippocampus and loss of metaplasticity (modulation of synaptic plasticity by the previous activity of a synapse), which means that

defects in the hippocampal insulin signalling leads to cognitive deficits (Costello *et al.*, 2012).

Long-term depression (LTD) is considered a synaptic mechanism underlying learning though new experiences in the mammalian brain (Bear and Abraham, 1996 and Manahan-Vaughan and Braunewell, 1999). Besides the LTD induced by low-frequency stimulation (LFS) at the hippocampal Schaffer collateral CA1 synapses, it was reported that insulin induces a protein phosphatase (PP)-independent form of LTD, or insulin-LTD. The insulin-LTD is induced and expressed postsynaptically and it is dependent on the activation of the PI_3K-Akt pathway and a local protein synthesis at the synaptic site of dendrites. Thus, the insulin-LTD might be considered another important role of insulin in learning and memory (Huang *et al.*, 2004).

Apart from PET studies, magnetic resonance imaging (MRI) has already been used as an indirect but non-invasive technique to measure changes in neuronal activity levels, *in vivo*, in the brain of human subjects. These studies revealed that insulin, not only changes the hypothalamic response allowing us to perceive some differences between lean and obese subjects neural metabolism (Matsuda *et al.*, 1999 and Smeets *et al.*, 2005), but also that it influences the intrinsic brain activity besides homeostatic systems of the brain modulating the orbitofrontal cortex, anterior cingulate cortex, prefrontal cortex and hypothalamus (Kullmann *et al.*, 2012).

1.1.4.2.4. Insulin as a neuroprotective agent

Insulin has also been shown to have an important neuroprotective role against damaging conditions like oxidative stress (D'Mello *et al.*, 1993; Duarte *et al.*, 2005, 2008; Gwag *et al.*, 1997; Hamabe *et al.*, 2003; Hong *et al.*, 2001; Ryu *et al.*, 1999 and Wu *et al.*, 2004) and reduction of neuronal apoptotic death (Duarte *et al.*, 2005 and Fülöp *et al.*, 2003).

The signalling pathway underlying this role is still under discussion. Nowadays it is thought to be caused by the reestablishment of InR/IGF-1R signalling gene transcription (Duarte *et al.*, 2008), improving neuronal glucose metabolism (Duarte *et al.*, 2006; Fülöp *et al.*, 2003; Gerozissis, 2003 Hoyer, 2003; and Watson and Craft, 2003) and antioxidant defenses (Duarte *et al.*, 2005), or by an antiapoptotic cascade involving IGF-1R and PI_3K-Akt signalling activation with consequent prevention of caspases inactivation (Chin *et al.*, 2005 and Leinninger *et al.*, 2004), or even by an antiapoptotic insulin effect, through neuronal stress-activated protein kinase inhibition (Kim and Han, 2005). Regardless, it is important to notice that some of those injuries may constitute the causes for brain dysfunction, associated with several pathologies (as DM), aging and age related diseases (like AD) and that insulin seems to play a key role in those mechanisms (Duarte *et al.*, 2012b).

1.1.4.3. Insulin-like peptide signalling in the brain

Insulin-like peptides (ILPs) include insulin, IGF-1 and IGF-2 among others, and - like insulin - both IGF-1 and IGF-2 exert many effects in the brain (Fernandez and Torrez-Alemán, 2012). IGF-1 is produced by all cell types in the brain but its expression is the highest perinatally. Despite the decreased levels in adults, IGF-1 can be found in the cortex, hippocampus, cerebellum, brainstem, hypothalamus and spinal cord (Bach *et al.*, 1991 and Bondy and Lee, 1993). IGF-2 is highly produced during development in the choroid plexus, leptomeninges and in the hippothalamus (Ayer-le Lievre *et al.*, 1991) but it is also present in the adult brain (Fernandez and Torrez-Alemán, 2012 and Stylianopouiou *et al.*, 1988).

Most studies tackling at the roles of ILPs in the brain are carried out under pathological conditions. The probable main source of IGFs in the adult brain is the microglia while their targets, IGF-1Rs, are overexpressed in neurons and astrocytes under brain injury (Walter *et*

al., 1997). IGF-1 and IGF-2 levels, as well as IGF binding protein expression change in a coordinated manner. Therefore, they are thought to work in a cooperative way of action in glia to preserve neural tissue and energy homeostasis (Lee *et al.*, 1996 and Walter *et al.*, 1997). ILPs specific functions depend on the physiological context where they are found, conditioning their sources, pathways and effects (Fernandez and Torrez-Alemán, 2012).

1.1.4.4. Insulin signalling and Alzheimer's disease

With the ageing of the population of developed countries and the increase in life expectancy, the age-related metabolic and neurodegenerative diseases are becoming a global health problem (Correia *et al.*, 2012). Most sporadic forms of AD are detected in the middle-aged and the elderly and it is the most common form of dementia, affecting more than 35 million people worldwide (Who.int, 2012). AD is a progressive neurodegenerative disease clinically characterized by gradual cognitive impairment and adaptive function, culminating in premature death (Moreira *et al.*, 2009 and Querfurth and LaFerla, 2010). Neuropathologically, AD is recognized by the presence of intracellular NFTs, mostly composed of hyperphosphorylated tau protein and senile plaques, which are massive aggregates of the amyloid β (Aβ) peptide in the extracellular space (Castellani *et al.*, 2010; Goedert and Spillantini, 2006 and Moreira *et al.*, 2009). AD is also identified by severe neuronal atrophy, primarily in the entorhinal region, then at the temporal lobe, later progressing to the limbic system and finally to major areas of the neocortex (Braak and Braak, 1995). AD can have genetic causes, constituting the rare familial form, or more commonly it has sporadic origin (Correia *et al.*, 2012).

The major risk factors for sporadic AD seems to be aging, T2DM and apolipoprotein E type 4 allele (Corder *et al.*, 1993; Hoyer, 2004; Irie *et al.*, 2008; Kivipelto *et al.*, 2002; Luchsinger *et al.*, 2007 and Ott *et al.*, 1999). Indeed, human subjects with T2DM have a

large increase in the risk of AD, independently of the risk for vascular dementia, when compared with non-diabetic subjects (Kroner, 2009). A study has already reported that more than 80 % of AD patients also suffer from T2DM or irregular blood glucose levels (Janson *et al.*, 2004). Both AD and T2DM are diseases characterized by the presence of insoluble protein aggregates with fibrillar conformation in the brain and the pancreas, respectively. Human islet amyloid polypeptide aggregation is related to pancreatic β-cells loss, while Aβ aggregation is related with neuronal and synaptic function, as previously said (Ciccotosto *et al.*, 2011; Correia *et al.*, 2012 and Lim *et al.*, 2008). Therefore, these associations suggest that AD and T2DM may share common pathomechanisms leading to brain cell and β-cells loss. Hence, AD has been termed a brain-type diabetes or a type 3 diabetes (Duarte *et al.*, 2012b and Kroner, 2009).

Because of insulin's neuromodulatory functions, neurotrophic effects (Farrar *et al.*, 2005 and Schubert *et al.*, 2003) and neuroprotection against apoptosis, oxidative stress, Aβ toxicity and ischemia (Duarte *et al.*, 2005; Rensink *et al.*, 2004; Ryu *et al.*, 1999 and Voll and Auer, 1991), insulin deregulation was already suggested to have a role in a variety of neurodegenerative and psychiatric disorders, including not only AD, but also Parkinson's disease, Huntington' disease, depression and schizophrenia, all of them related to neuroinflammation and neurodegeneration (Ghasemi *et al.*, 2013).

1.2. The endocannabinoid system

1.2.1. The discovery of the endocannabinoid system

The study of the endocannabinoid (eCB) system started in the late 80's with the discovery of specific cannabinoid receptors for the pharmacological actions of

Δ^9-tetrahydrocannabinol (THC), the main psychoactive constituent of cannabis (Nagy *et al.*, 2008 and Pertwee, 1997). Definitive evidence for the existence of specific cannabinoid receptors resulted from the demonstration of high-affinity and saturable, stereospecific binding sites for the synthetic radiolabelled cannabinoid agonist [^{3}H]CP55940 in mouse brain plasma membranes, which correlated with the *in vitro* inhibition of andenylate cyclase and the *in vivo* analgesic effect of the compound (Devane *et al.*, 1988 and Pacher *et al.*, 2006). Autoradiography with this radioligand allowed the precise localization of cannabinoid receptors in rat brain sections (Herkenham *et al.*, 1991). A G protein-coupled receptor (GPCR) was also identified and molecular characterized as the brain receptor for cannabinoids (Matsuda *et al.*, 1990) and it is now referred to as cannabinoid receptor type 1 (CB_1R) (Pacher *et al.*, 2006).

1.2.2. Cannabinoid receptors

The CB_1R is considered the most abundant GPCR in the mammalian brain but it is also present in a variety of peripheral tissues and cells. Besides, there is a related second cannabinoid GPCR, the cannabinoid receptor type 2 (CB_2R), which is mostly associated with cells of the immune and hematopoietic systems (Munro *et al.*, 1993), but it can also be found in the brain (Gong *et al.*, 2006 and van Sickle *et al.*, 2005), in non-parenchymal cells of the cirrhotic liver (Julien *et al.*, 2005), in the endocrine pancreas (Juan-Picó *et al.*, 2006) and in the bone (Idris *et al.*, 2005; Ofek *et al.*, 2006 and Pacher *et al.*, 2006).

Besides the CB_1R and the CB_2R, ionotropic transient receptor potential vanilloid type 1 ($TRPV_1$) channels also participate in eCB signalling (De Petrocellis and Di Marzo, 2010 and Pertwee *et al.*, 2010). $TRPV_1$ is largely expressed in afferent peripheral sensory neurons, an area associated with pain sensation (Caterina and Julius, 2001) and is capable of binding lipophilic molecules, like *N*-arachidonoyl-ethanolamine (AEA, also known as anandamide)

(Di Marzo *et al.*, 2002). If in one hand AEA is a partial agonist of the CB_1R, on the other hand it is a full agonist at $TRPV_1$ channels (Smart *et al.*, 2000 and Zygmunt *et al.*, 1999). $TRPV_1$ channels have also been detected in the CNS, apparently regulating the synaptic activity (Roberts *et al.*, 2004; Köles *et al.*, 2013).

1.2.3. Endocannabinoids and endocannabinoid biosynthesis

The discovery of CB_1R and CB_2R clearly indicated that there should be endogenous ligands for these receptors. Hence, AEA and 2-arachidonoyl-glycerol (2-AG) were the first eCBs identified (Devane *et al.*, 1992; Mechoulam *et al.*, 1995 and Sugiura *et al.*, 1995). Until now, several more cannabinoid receptor agonists have been proposed, including 2-arachidonyl-glyceryl ether (2-AGE or noladin), *O*-arachidonoyl-ethanolamine (virodhamine) and *N*-arachidonoyl-dopamine (NADA) (Bisogno *et al.*, 2000; Huang *et al.*, 2002 and Porter *et al.*, 2002).

AEA is synthesized and released into the extracellular space and synaptic cleft on demand of physiological or pathological stimuli (Di Marzo *et al.*, 1994). Research on the class of *N*-acylethanolamines, to which AEA belongs, revealed that it is biosynthesised via a phospholipid dependent pathway, through the enzymatic hydrolysis of the corresponding *N*-acyl-phosphatidylethanolamine (NAPE) catalysed by a phospholipase D (PLD), selective and specific for NAPEs. NAPEs, in turn, are produced through the acyl group transfer from the *sn*-1 position of phospholipids to the *N*-position of phosphatidylethanolamine, catalysed by a Ca^{2+}-dependent *trans*-acylase (De Petrocellis *et al.*, 2004; Schmid *et al.*, 1996 and 2002).

2-AG levels detected in cells and tissues are usually much higher than those of AEA and enough to activate both CB_1R and CB_2R (Stella *et al.*, 1997 and Sugiura *et al.*, 1995). Furthermore, 2-AG is present in many other signalling pathways and is an important

precursor and/or degradation product of phospho-, di-, and triglycirides and also of arachidonic acid. So, there are several biosynthetic pathways known for 2-AG formation (Ueda *et al.*, 2013). Mainly, like AEA, 2-AG is synthesised via the hydrolysis of diacylglycerols (DAGs) by DAG lipase-α (DAGLα) and -β (DAGLβ) activity, at the *sn*-1 position. DAG, together with inositol 1,4,5-trisphosphate (IP_3), is the cleavage product of phosphatidylinositol 4,5-bisphosphate (PIP_2) by the enzyme PLC_β, which, in turn, is activated by glutamate release (Maejima *et al.*, 2001 and Varma *et al.*, 2001). In the brain, 2-AG synthesis may also be induced by postsynaptic neuronal depolarization, through increases in the intracellular Ca^{2+} promoted by voltage-gated Ca^{2+} channels (VGCCs) (Chevaleyre *et al.*, 2006). DAGLs are the major biosynthetic enzymes for 2-AG in the brain, spinal cord, liver and other tissues and studies show that, although they have different contributions within each tissue, there seems to be some cooperation between DAGLα and DAGLβ. In the CNS, the DAGLα has shown to be more important, regulating the retrograde synaptic plasticity and adult neurogenesis (Best and Regehr, 2010; Gao *et al.*, 2010; Min *et al.*, 2010 and Reisenberg *et al.*, 2012).

Still very little is known about 2-AGE, virodhamine and NADA biosynthesis and, as 2-AG and AEA are the major eCBs in the brain, they will not be discussed in detail in this work.

After their biosynthesis, AEA and 2-AG are promptly released into the extracellular medium to selectively act upon the cannabinoid receptors and, in the case of AEA, also upon the $TRPV_1$. For termination of eCBs effect on extracellular targets, cells reuptake them, as explained below (De Petrocellis *et al.*, 2004).

1.2.4. Inactivation of endocannabinoids

Inactivation of eCBs starts with their removal from their extracellular molecular targets and reuptake. Even though some authors defend that fatty acid amide hydrolase (FAAH) is enough to drive the facilitated diffusion of eCBs (Glaser *et al.*, 2003), it is largely thought that AEA and 2-AG are taken up by cells through selective, saturable, temperature-dependent and sodium (Na^+)-independent facilitated transport mechanisms, via the FAAH-like transporter and other transport proteins (Fowler, 2013 and Fu *et al.*, 2011).

Once inside the cell, the hydrolysis of AEA to arachidonic acid and ethanolamine is catalysed by the FAAH-1. FAAH is an enzyme with an alkaline optimal pH, which can be found in microsomal membranes, which structure revealed by X-ray cristallography also suggests that it might be able to integrate the plasma membrane (Bracey *et al.*, 2002). FAAH-1 hydrolyzes other long-chain fatty acid amides too, like *N*-oleoyl-ethanolamine (OEA), which is an anorexigenic and neuroprotective mediator (Hansen, 2010 and Piscitelli and Di Marzo, 2012). Although FAAH-1 is capable of hydrolysing 2-AG to some extent (Blankman *et al.*, 2007), 2-AG is mainly cleaved by monoacylglycerol lipase (MAGL) and also other hydrolases such as α,β-hydrolases 6 and 12 in the synapse (Dinh, *et al.*, 2002; Savinainen *et al.*, 2012 and Schlosburg *et al.*, 2010). 2-AG, unlike AEA, can also be directly esterified into phospho-glycerides through phosphorylation and/or acylation of its free hydroxyl groups, before FAAH or MAGL hydrolysis (Di Marzo *et al.*, 1998a, b and 1999). As it was previously said, 2-AG is also a precursor for arachidonic acid production in sensory neurons (Gammon *et al.*, 1989).

Both AEA and 2-AG may even be converted into the corresponding prostaglandin ethanolamides and prostaglandin glyceryl esters, by the cycloxygenase-2 (COX-2) and subsequent action of the various prostaglandin synthases, but those metabolites are not

capable of acting under cannabinoid or prostanoid receptors (Ross *et al.*, 2002 and Woodward *et al.*, 2001). Hence, as COX-2 catalyzes the formation of endoperoxides, eCBs can serve as percursors for potential new lipid mediators (Kozak *et al.*, 2002 and Piscitelli and Di Marzo, 2012). Besides COX-2, AEA and 2-AG were shown to be good substrates for several other enzymes that oxidize polyunsaturated fatty acids, including the cytochrome p450 monoxygenases and lipoxygenases (Kozak and Marnett, 2002; Piscitelli and Di Marzo, 2012 and Snider *et al.*, 2010). Furthermore, 12,15-lipoxygenase (and subsequent lipoxin A4-hydrolase product) is an allosteric enhancer of the CB_1R (Pamplona *et al.*, 2012).

Hence, AEA and 2-AG oxidation, by COX-2 or other enzymes, can result in their inactivation as eCBs, but might also produce other active molecules capable of changing the kind of signalling observed (De Petrocellis *et al.*, 2004 and Piscitelli and Di Marzo, 2012).

1.2.5. The endocannabinoid system in the brain

1.2.5.1. AEA and 2-AG in the brain

AEA biosynthesis in neurons seems to be similar to the pathway followed in other cells, being induced by postsynaptic depolarization and intracellular Ca^{2+} influx (Di Marzo, 2011). It is synthesized by NAPE-hydrolyzing PLD (NAPE-PLD) which converts the NAPE precursor of AEA *N*-arachidonoyl-phosphatidylethanolamine (NArPE) into the eCB agonist (Cristino *et al.*, 2008). NAPE-PLD expression was detected postsynaptically and also on axonal membranes, especially at CA3 mossy fiber terminals of the hippocampus (Cristino *et al.*, 2008; Egertová *et al.*, 2008 and Nyilas *et al.*, 2008). NArPE was also detected in murine brain, testes and leukocytes (Cadas *et al.*, 1997; Di Marzo *et al.*, 1996 and Sugiura *et al.*, 1996b). Both NArPE and AEA were found with a similar distribution in several different brain areas (Bisogno *et al.*, 1999a and De Petrocellis *et al.*, 2004).

Like AEA, 2-AG *de novo* synthesis in neurons is induced by membrane depolarization (Bisogno *et al.*, 1997 and Stella *et al.*, 1997). In retrograde eCB signalling, postsynaptic neuronal depolarization increases intracellular Ca^{2+} through VGCCs and enhances 2-AG production. Postsynaptic metabotropic glutamate receptors (mGluRs) activation by glutamate can also lead to 2-AG formation, through PLC_{β} activation (Hashimotodani *et al.*, 2007). Both metabolic pathways are thought to converge in order to mobilize 2-AG (Castillo *et al.*, 2012). An important finding for the understanding of synaptic plasticity was that PLC_{β} showed to act as a detector for simultaneous postsynaptic Ca^{2+} and GPCR signalling (Brenowitz and Regehr, 2005; Castillo *et al.*, 2012; Hashimotodani *et al.*, 2005 and Maejima *et al.*, 2005). Besides, mGluRs activation was revealed as enough to mobilize eCBs to trigger both short- and long-term synaptic plasticity (Castillo *et al.*, 2012 and Chevaleyre *et al.*, 2006).

1.2.5.2. The endocannabinoid signalling in the brain

AEA, 2-AGE and NADA are functionally more selective for CB_1Rs, while virodhamine is more selective for CB_2Rs and 2-AG is equally selective for both receptors (De Petrocellis *et al.*, 2004). As already mentioned, both CB_1R and CB_2R are coupled to $G_{i/o}$ proteins in most cases (Köfalvi, 2008). The prevalence of eCBs through the brain suggests they are primary modulators of the synaptic function and the main mechanism by which they do it is through retrograde signalling.

First it was found that eCBs mediate two forms of short-term synaptic plasticity: depolarization-induced suppression of excitation (DSE), suppressing the glutamate release (Kreitzer and Regehr, 2001); and depolarization-induced suppression of inhibition (DSI), which results in the decrease of GABA release (in the presynaptic cell) by retrograde signalling of a strongly depolarized postsynaptic cell (Diana and Marty, 2004;

Ohno-Shosaku *et al.*, 2001 and Wilson and Nicoll, 2002). DSE was first detected in the hippocampus (Diana and Marty, 2004; Wilson and Nicoll, 2002 and Zachariou *et al.*, 2013) and DSI was first studied in the hippocampus (Llano *et al.*, 1991) and the cerebellum (Pitler and Alger, 1992). For DSE and DSI, the release of eCBs is driven by a large increase in postsynaptic calcium (Best and Regehr, 2010).

Endocannabinoids can induce another form of short-term synaptic plasticity: metabotropic-induced suppression of excitation (MSE) or inhibition (MSI). MSE and MSI are induced by the activation of some $G_{q/11}$-linked receptors which, in the hippocampus, are the group I of mGluRs, specifically $mGluR_1$ and $mGluR_5$ and the metabotropic muscarinic receptors (mAChRs), specifically M_1 and M_3 receptors (Straiker and Mackie, 2005 and Zachariou *et al.*, 2013).

Now it is also known that eCBs also mediate presynaptic forms of LTD at both excitatory and inhibitory synapses in the hippocampus (Chevaleyre and Castillo, 2003). Thus, when the glutamatergic axons are repetitively stimulated it might leads to homo-synaptic LTD on glutamatergic (excitatory) synapses or to hetero-synaptic LTD on GABAergic (inhibitory) synapses (Zachariou *et al.*, 2013).

1.2.5.3. Nonretrograde endocannabinoid signalling

Endocannabinoids also can modulate synaptic function in a non-retrograde fashion: it has been found that repetitive activation of the dendritic tree of a neocortical GABAergic interneurons and pyramidal cells may trigger self-inhibition following locally produced 2-AG activating somatodendritic CB_1Rs in an autocrin manner (Bacci *et al.*, 2004; Marinelli *et al.*, 2008). This is likely achieved through the activation of GIRK channels and the consequent long-lasting hypopolarization.

1.2.5.4. Endocannabinoid signalling and astrocytes

Another form of non-retrograde synaptic plasticity leads to the field of synaptic metaplasticity, when post-synaptic eCB release activates astroglial CB_1Rs. Astrocytic CB_1Rs are coupled to PLC-mediated increase in intracellular Ca^{2+} and subsequent vesicular glutamate release onto parallel synapses where this glutamate will modulate synaptic function acting on mGluRs (Navarrete and Araque, 2008 and 2010).

Although *in vivo* studies are still controversial, recent studies serve evidence for eCB synthesis in oligondendrocytes, astrocytes and microglial cells (Gomez *et al.*, 2010; Hashimotodani *et al.*, 2011 and Hegyi *et al.*, 2012). More relevant was the finding that astrocytes can influence eCBs signalling and mediate synaptic function. It has been recently observed through immunoelectron microscopy eCBs released postsynaptically acting under both presynaptic and astrocytic CB_1Rs, at Schaffer collateral excitatory synapses of CA1 pyramidal neurons of the hippocampus (Han *et al.*, 2012). Also in some synapses of CA1 pyramidal neurons, glutamate activated *N*-methyl-D-aspartate receptors (NMDARs) and triggered short-term facilitation of transmitters release, probably by stimulating mGluRs (Navarrete and Araque, 2008 and 2010). So, eCBs might induce a DSE and, at the same time, induce synaptic transmission through astrocytes, both dependent on CB_1Rs (Castillo *et al.*, 2012).

Neuron-astrocyte communication mediated by eCBs has also been related to long-term plasticity. Presynaptic NMDARs and CB_1Rs activation are known to be necessary for spike timing-dependent LTD (tLTD) between neocortical pyramidal neurons (Bender *et al.*, 2006; Nevian and Sakmann, 2006 and Sjöström *et al.*, 2003). But a recent study showed that astrocytic CB_1Rs were necessary and enough to induce tLTD (Min and Nevian, 2012). CB_1Rs anatomy and function in neocortex was already investigated and eCBs produced in

pyramidal neurons seem to activate astrocytic CB_1Rs increase intracellular Ca^{2+}, promote glutamate release and stimulate presynaptic NMDARs (Castillo *et al.*, 2012; Domenici *et al.*, 2006; Hill *et al.*, 2007 and Lafourcade *et al.*, 2007).

In addition, it was recently found that the CB_1R activation inhibits not only neuronal but also astrocytic intermediary metabolism in the rat hippocampus (Duarte *et al.*, 2012). Hence, further studies will be definitely necessary to understand this interaction between neurons and astrocytes.

1.2.5.5. The plasticity of the endocannabinoid signalling

The existence of several eCB ligands, the variety of synthetic pathways for the same ligand, the various forms of eCB release and the multiplicity of the signalling pathways activated through different eCB receptors make the cerebral eCB signalling redundant (Di Marzo and De Petrocellis, 2012 and Harkany *et al.*, 2008), with significant roles in cellular adaptation and regulation of learning and memory, by mediating short- and long-term synaptic changes (Gerdeman and Lovinger, 2003 and Zachariou *et al.*, 2013). By modulating brain metabolism plasticity, eCBs regulate several neural functions such as cognition, motor control, feeding behaviour and pain (Castillo *et al.*, 2012). *In vivo* and *in vitro* experiments, these changes can be induced by the application of the CB_1R agonists or blocked with CB_1R antagonists (Ohno-Shosaku *et al.*, 2001 and Wilson and Nicoll, 2001). Besides, the eCB signalling is intricately involved in several brain disorders such as AD, Parkinson's and Huntington's diseases, schizophrenia and depression among others (Blázquez *et al.*, 2011; Köfalvi and Fritzsche, 2008; Micale *et al.*, 2013; Mulder *et al.*, 2011 and Pertwee, 2012), yet this involvement invites further studies in both animal models and man.

1.2.6. The endocannabinoid system in food intake and reward

The eCB system is especially known as a food intake modulator (Banni and Di Marzo, 2010 and DiPatrizio and Piomelli, 2012). In fact, THC and the eCBs AEA and 2-AG were early shown to stimulate appetite in the CNS in both animal models and human subjects (Di Marzo and Matias, 2005; Matias *et al.*, 2008; Sacks *et al.*, 1990 and Williams and Kirkham, 1999), while antagonists reduce food consumption. Research using CB_1R knockout (KO) models showed that the absence of the CB_1R in brain prompts reduced food intake after food deprivation, leanness, resistance to diet-induced obesity and increased leptin sensitivity, a hormone that regulates appetite and reward senses (Cota *et al.*, 2003; Di Marzo *et al.*, 2001; Matias *et al.*, 2008; and Ravinet-Trillou *et al.*, 2004).

Recently, it was demonstrated that diet-induced obesity in mice increases DAGLα levels, AEA and 2-AG synthesis and CB_1R expression in the hippocampus, as well as DSI and eCB-LTD, but DAGLβ, MAGL and FAAH levels remained unchanged (Massa *et al.*, 2010). The other way around, food deprivation downregulates CB_1R signalling in hypothalamic feeding circuits, changing eCB-LTD into LTD dependent on nitric oxide (Crosby *et al.*, 2011). Besides, experiments with polyunsaturated fatty acid diet-deficient mice exhibited impaired eCB-LTD in prefrontal cortex and nucleus accumbens, both areas related to reward (Lafourcade *et al.*, 2011). This can be either explained by a reduced coupling of the CB_1R with its $G_{i/o}$ protein or a decrease in the levels of eCB precursors due to their depletion in the diet. These mice also showed changes in mood and emotional behaviour, connecting the eCB signalling with affective behaviours, respectively (Castillo *et al.*, 2012).

1.2.7. The endocannabinoid system and glucose homeostasis

It has been long known that cannabis increases blood glucose levels in rats and dogs as a result of the decrease in glucose tolerance (Mahfouz *et al.*, 1975; Matias *et al.*, 2008 and de Pasquale *et al.*, 1978). In rats injected intraperitoneally with a glucose load, a preceding injection of CB_1R antagonists, including AEA, prolonged high plasma glucose levels (Bermúdez-Silva *et al.*, 2006). In another experiment, high fat diet-induced obesity increased blood glucose levels, but the chronic treatment of these obese mice with the CB_1R inverse agonist SR141716A (also known as Rimonabant or Acomplia) decreased glucose, insulin and leptin levels (Poirer *et al.*, 2005), as well as improved the high/low density lipoproteins (HDL/LDL) cholesterol ratio. After 14 days of treatment, SR141716A still reduced feeding, blood glucose and insulin levels and increased insulin sensitivity (Doyon *et al.*, 2006 and Matias *et al.*, 2008).

Therefore, the eCB system has been long known as a carbohydrate metabolism modulator (Matias *et al.*, 2008). In fact, the CB_1R inverse agonist SR141716A was introduced in the market a few years ago to fight cardiometabolic risk factors including low insulin sensitivity, high blood glucose, fatty acid and cholesterol levels and abdominal obesity. However, it was withdrawn due to rare but serious psychiatric side effects like anxiety, depression and even suicide (Christensen *et al.*, 2007; Lazary *et al.*, 2011 and McPartland, 2009). Now it is thought that those side effects are caused by the interference with the constitutive CB_1R activity and that CB_1R neutral antagonists might constitute safer anti-obesity drugs, because they leave the constitutive CB_1R coupling to intracellular cascades unaffected, while dampening extra eCB tone at the CB_1R signalling (Meye *et al.*, 2012).

The role of the eCBs in cerebral glucose homeostasis has been further studied in the last few years. Our group has previously demonstrated that 1) the CB_1R KO mice has impaired hippocampal glucose homeostasis (Lemos *et al.*, 2012), 2) the activation of the CB_1R slows down the oxidative glucose metabolism in the citric acid cycle in rat hippocampal astrocytes and neurons (Duarte *et al.*, 2012), 3) and finally in the streptozotocin (STZ) model of T1DM, the hippocampal expression of the CB_1R was altered in the rat (Duarte *et al.*, 2007). These altogether suggest the involvement of the eCB system in diabetic encephalopathy and glucose homeostasis.

1.2.8. The endocannabinoid system and Alzheimer's disease

As shown in the subchapter 1.2, the eCB system is a very complex network that interferes with both peripheral and central signalling pathways. It is considered a neuromodulatory system, regulating several neurotransmitter systems, including dopaminergic, cholinergic, serotoninergic, adrenergic, opiate, glutamatergic and GABAergic systems (Degroot *et al.*, 2006; Farkas *et al.*, 2012; Ferreira *et al.*, 2012; Freund *et al.*, 2003; Köfalvi *et al.*, 2005 and Richter *et al.*, 2012).

The involvement of the eCB system in chronic neurodegenerative diseases has also been studied in the last decade, as in AD (Micale *et al.*, 2007; Mulder *et al.*, 2011 and Ramírez *et al.*, 2005), Parkinson's and Huntington's disease (Blázquez *et al.*, 2011; Di Marzo *et al.*, 2000 and Pertwee, 2012). CB_1R and CB_2R are thought to act reducing excitotoxicity, hypothermia, inflammation (inhibiting microglia) and facilitation of neurogenesis (Pacher and Kunos, 2013). For instance, Sativex® is a medicine composed by the mixture of THC and cannabidiol, approved for the treatment of spasticity in multiple sclerosis (Collin *et al.*, 2007 and Novotna *et al.*, 2011). It acts on both CB_1R and CB_2R and recently demonstrated

significant neuroprotective effects in a mice model of tauopathy, making it a promising therapeutic agent in multi-systemic neurological disorders (Casarejos *et al.*, 2013).

1.3. Insulin and the CB_1 receptor

In the previous sections, it was summarized that both insulin and the eCB system can have common functions, which is due to their highly overlapping intracellular signalling pathway and the fact that the CB_1R tends to form heterodimers with RTKs (Dalton and Howlett, 2012).

Peripheral CB_1R blockade facilitates while CB_1R activators counteract InR signalling in pancreatic β-cells, suggesting a functional interaction between the CB_1R and InR signalling (Kim *et al.*, 2011). This lead to the discovery of that the CB_1R and the InR establish physical and functional interactions, through a mechanism by which CB_1R antagonists improve insulin action in insulin sensitive tissues, peripherally and independently of other metabolic effects of CB_1Rs (Figure 1.1) (Kim *et al.*, 2012). On the other hand, acute CB_1Rs modulation affects skeletal muscle insulin sensitivity in mice, but not by CNS CB_1Rs modulation (Song *et al.*, 2011).

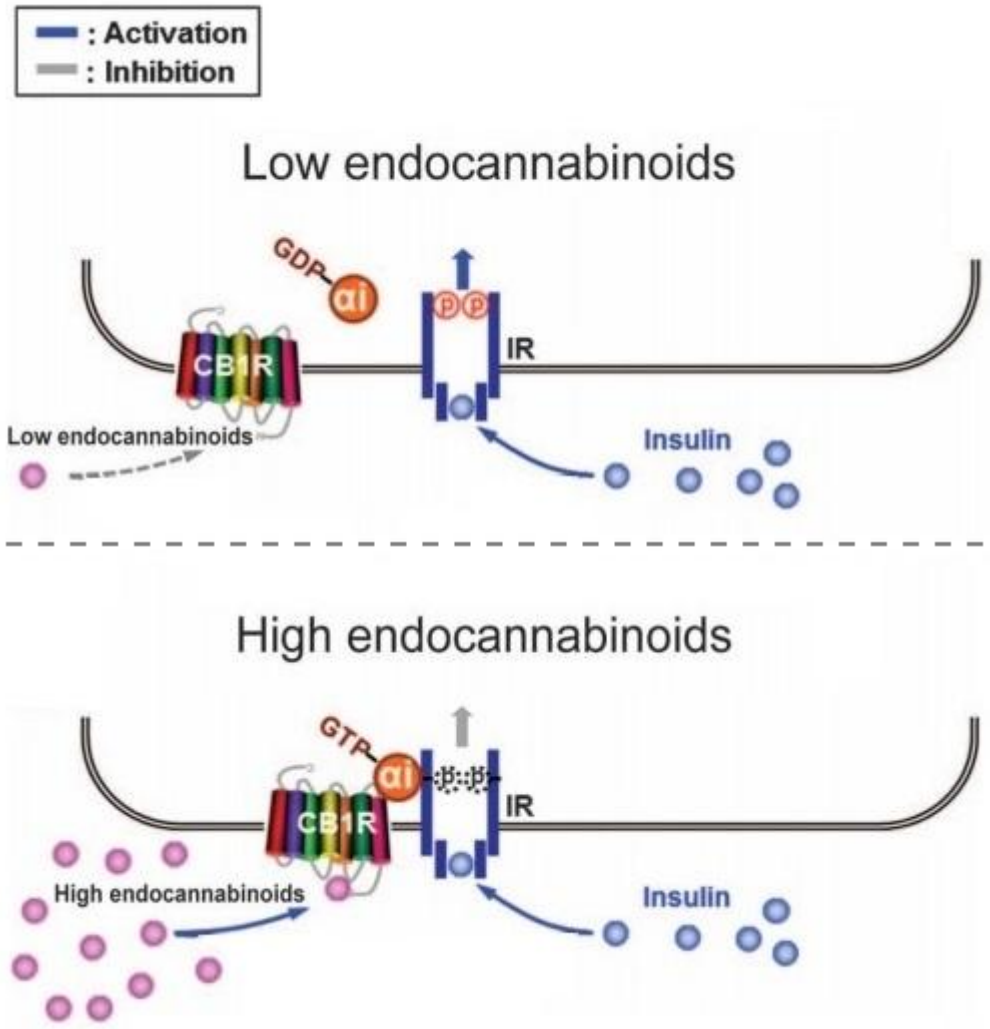


Figure 1.1. Schematic ilustration of the regulation of IR signalling by eCBs, in pancreatic β-cells. Binding of eCBs to CB_1Rs activates the Gαi class of heterotrimeric proteins and increases the association between Gαi and the IR that counteracts the effects of insulin on autophosphorylation and kinase activity of the IR (adapted from Kim *et al.*, 2012).

Very recently, it was demonstrated in whole-cell voltage-clamp recording that bath application of insulin triggers eCB release, acting at the presynaptic glutamatergic terminals stimulating dopaminergic activity, in ventral tegmental area (VTA) dopaminergic cell bodies. This might represent a new mechanism of postprandial feedback, related to satiety (Labouèbe *et al.*, 2013). But more importantly than pointing to a new type of LTD mediated by eCBs, these results reveal a direct interaction between insulin and the eCB system in the CNS.

1.4. Aims

The main aim of this study is to characterise some of the functions of insulin in the brain, with emphasis in synaptic transmission and glucose uptake.

The dependence of the action of insulin on the CB_1R will also be explored, with an attempt to characterize the molecular substrates responsible for this possible interaction, such as a direct heterodimer vs. indirect action through eCBs release.

2. MATERIALS AND METHODS

2.1. Samples

2.1.1. Animals

All experiments were performed in accordance with the principles and procedures outlined as "3Rs" in the European Union (EU) guidelines (directive 86/609/EEC), the Federation of Laboratory Animal Science Associations (FELASA) and the National Centre for 3Rs (Kilkenny *et al.*, 2010), and were approved by the Animal Care Committee of the Center for Neuroscience and Cell Biology of Coimbra. The ARRIVE guideline for the design and the execution of *in vitro* pharmacological experiments and for data management and interpretation was also applied (McGrath *et al.*, 2010).

Male Wistar rats (180-240 g, 6-13-week old) and C57BL/6j mice (28-32 g, 8-10-week old) were purchased from Charles-River (Barcelona, Spain). CB_1R KO mice on the CD-1 strain (Ledent *et al.*, 1999) and their wild-type littermates were genotyped and provided by collaborators (Catherine Ledent, IRIBHN, Brussels). Animals were housed in a conventional facility, with a 12 h light/dark cycle and *ad libitum* access to food and water. All efforts were made to reduce the number of animals used and to minimize their stress and discomfort.

In a group of these rats, we induced insulin-dependent diabetes with a single injection of STZ (Calbiochem, Merck Biosciences, Germany). STZ was dissolved in citrate buffer (226 mM, pH 4.5) and injected intraperitonealy one high dose of 60 mg/kg body weight in rats fasted for 5 h (7 weeks old).

Blood glucose was determined using an Accu-Chek® system (Roche) and body weights were measured 3 and 15 days after injection. Rats were considered diabetic when their non-fasted blood glucose levels were ≥ 300 mg/dL. Up to two of these diabetic animals were kept in cages for 15 days with elevated grid bottom due to frequent urination. They

were frequently evaluated by two independent researchers for levels of suffering, according to FELASA recommendations, and received *ad libitum* food and water and daily change of bedding.

Another group of rats was food-restricted for 16 hours until sacrifice (fasted rats). These animals were housed individually in grid bottom cages to avoid the consumption of the bedding or the fur of the cage mates, but otherwise housed as above.

Animals were deeply anesthetized with halothane (no reaction to handling or tail pinch, while still breathing) before decapitation with a guillotine (rats) or scissors (mice), between 10:00 and 14:00 o'clock to reduce putative circadian hormonal effects. Hippocampi and cortices were removed and sliced or homogenized, depending on the aim of the experiment.

2.1.2. Human samples

Human samples were obtained at autopsy from the National Institute of Legal Medicine of Coimbra, Portugal, thanks to the collaboration between Beatriz da Silva and the Purines' group in Center for Neuroscience and Cell Biology of Coimbra (CNC), headed by Rodrigo A. Cunha. Samples were generously provided by Paula M. Canas.

2.2. Experimental procedures

2.2.1. Extracellular electrophysiology

2.2.1.1. Hippocampal slices preparation for electrophysiology recordings

Hippocampal slices were prepared from 8-13 week-old Wistar rats of the three groups (control, STZ and fasted) using standard techniques (Costenla *et al.*, 2011 and Sebastião *et*

al., 2000). Brains were rapidly removed and placed in ice-cold modified artificial cerebrospinal fluid (ACSF) containing in mM: 124 NaCl, 3 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 26 $NaHCO_3$, 1.25 NaH_2PO_4 and 10 D-glucose, pH 7.4, saturated and continuously gassed with with a mixture of 95 % O_2 and 5 % CO_2. The isolated hippocampi were transversely cut in 400 μm-thick slices using a McIlwain tissue chopper and allowed to recover in the ACSF solution, previously and continuously oxygenated at room temperature, for at least 60 min. Hippocampal slices were then transferred to a recording chamber (Harvard Apparatus, CT, USA) and continuously superfused at a rate of 2 to 3 mL/min with oxygenated ACSF solution, at 30.5 ºC, during electrophysiology recordings.

2.2.1.2. Electrophysiology recordings protocol

A bipolar stainless steel stimulating electrode was placed on the Schäffer collateral fibers of the hippocampus to stimulate presynaptic fibers arising from the CA3 pyramidal cells. Slices were stimulated with a S44 stimulator (Grass Instruments, West Warwick, RI) once every 15 ms, with 100 μs of pulse duration. The recording electrodes were glass microelectrodes (1-2 MΩ) pulled on a multistage puller (Sutter P-97, Novato, CA) and filled with a 4 M NaCl solution. Field excitatory postsynaptic potentials (fEPSPs) were recorded in the CA1 pyramidal cells (Figure 2.1) and then amplified with an ISO-80 amplifier (World Precision Instruments, Hertfordshire, UK). Responses were digitalized using an ADC-42 board (Pico Technologies, Pelham, NY, USA). Information was gathered about the slope.

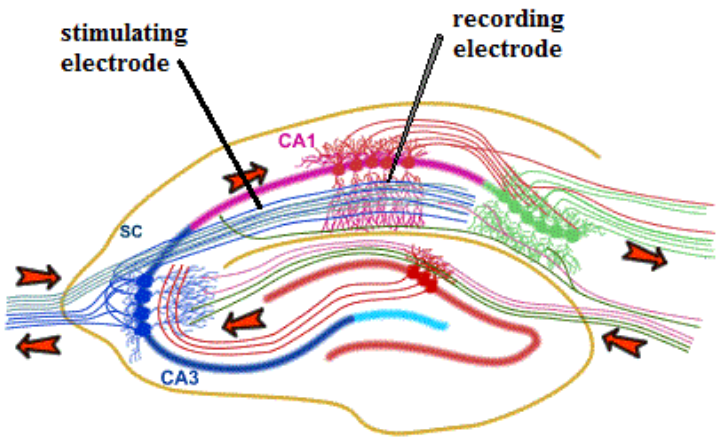


Figure 2.1. Schematic illustration of electrode placements in the hippocampal slice (adapted from Bristol.ac.uk).

Stable baseline measurements were obtained within 30 min after placing hippocampal slices in the recording chamber. Postsynaptic responses were recorded for at least 10 min prior to the application of paired pulse stimulation (PPS), when applied, or drugs perfusion. During this time, input/output (I/O) relations were generated by varying the stimulation intensity in steps of 2 μA from minimum to maximal fEPSPs amplitude (Figure 2.2). The baseline presynaptic stimulation was then delivered, with one pulse (Figure 2.3 A) or two pulses with 50 ms pulse interval and 100 μs duration (in order to induce paired pulse facilitation, PPF) (Figure 2.3 B), using a stimulation intensity that evoked approximately 60 % of maximal postsynaptic response (stimulus amplitude range between 5 and 7.2 μA). The evoked responses were considered stable, forming a baseline, when changed less than 10 % for 10 min of recording and the signal obtained had a shape similar to the one exemplified in Figure 2.4. Then, insulin (300 nM) and/or the CB_1R neutral antagonist O-2050 (500 nM) (Meye *et al.*, 2012) were bath applied alone for 15-20 min, then in combination.

All results were monitored and treated using the WinLTP 2.01 software (Anderson, 2012).

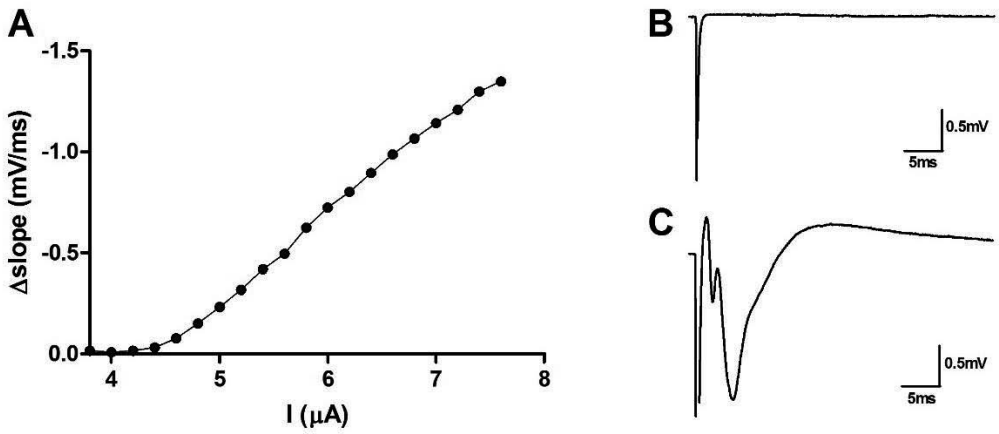


Figure 2.2. (A) Input/output (I/O) curve example, obtained by varying the stimulation intensity in steps of 2 μA from **(B)** minimum to **(C)** maximal fEPSPs amplitude.

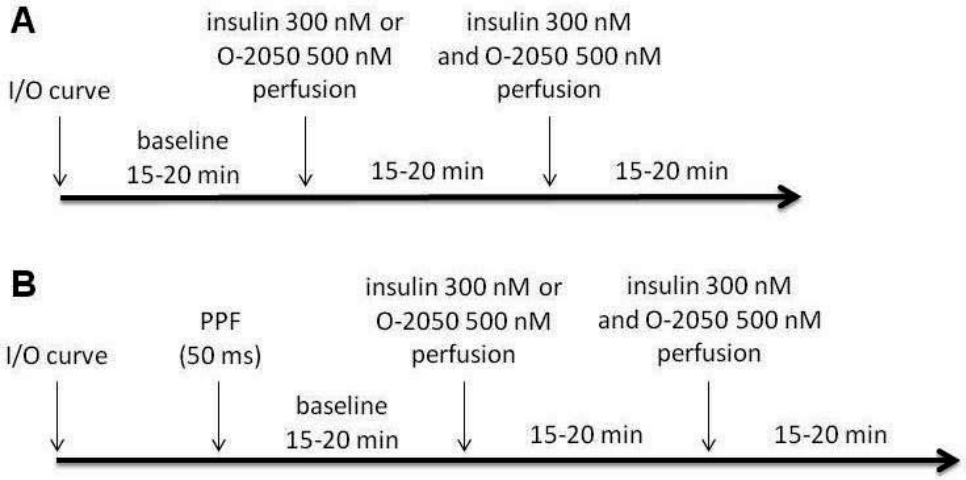


Figure 2.3. Experimental design for **(A)** extracellular recording of fEPSPs and **(B)** for paired pulse facilitation (PPF).

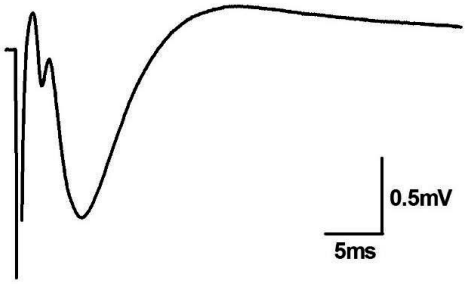


Figure 2.4. Shape of a stable hippocampal fEPSPs.

2.2.2. Glucose uptake

2.2.2.1. Hippocampal and cortex slices preparation for glucose uptake experiments

Hippocampal and cortex slices were prepared from 7-13 week-old Wistar rats and CD-1 WT and CB_1R KO mice using a previously optimized procedure (Lemos *et al.*, 2012). Brains were rapidly removed and collected into ice-cold Krebs'-HEPES solution (composition in mM): 113 NaCl, 3 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 25 $NaHCO_3$, 1.2 KH_2PO_4, 10 HEPES and 5.5 D-glucose, pH 7.4, saturated and continuously gassed with a mixture of 95 % O_2 and 5 % CO_2). Transversal 450 μm-thick hippocampal and cortical slices were cut using a McIlwain tissue chopper (Ted Pella, CA, USA). It was previously characterized with 50 μm steps from 300 μm to 1 mm thickness that 450 μm is optimal for oxygenation, drug penetration in the centre of the slice vs. physical resistance (Lemos *et al.*, 2012). Slices were gently separated in the same assay solution and allowed to recover for 1 h to reach steady-state glucose uptake/metabolism in the slice (Lemos *et al.*, 2012), in a multichamber slice incubator, in 50 ml of assay solution, under continuous carboxigenation at 37 ºC.

2.2.2.2. Glucose uptake protocol

After 60 min of recovery incubation, drugs (the CB_1R agonist WIN55212-2 (500 nM), O-2050 (500 nM), insulin (30 and 300 nM), the $GABA_A$ receptor ($GABA_AR$) antagonist bicuculline (20 μM) or the sodium channel blocker tetrodotoxin (1 μM)) were added into the incubating bath. Five min later, the radioactive analogue 2-^{3}H(N)-deoxy-D-glucose ([^{3}H]DG, 2.5 nM, 60 Ci/mmol, ARC) was bath applied for 30 min. In the end of the incubation time, slices were washed twice in the iced-cold assay solution and denatured in 1 mL of 0.5 M NaOH.

After dissolving the slices in NaOH, 800 µl of the samples were assayed for ^{3}H (X disintegration/minute, dpm) count with the help of a Tricarb β-counter (PerkinElmer, USA), and the rest for protein concentration (P·mg) with the bicinchoninic acid assay (Merck Biosciences, Germany). The incubation bath containing the radioactive glucose analogue was also sampled and assayed for ^{3}H: 181.8 µl volume of the assay solution contains 1 µmol amount of cold glucose molecules in a 5.5 mM glucose solution, which is represented as A dpm ^{3}H. This allows calculating how many nanomoles of glucose (cold + radioactive) represent the actual count in the slices, which finally can be expressed as nmol·(mg of protein)$^{-1}$. Note that the same figures are obtained when the X dpm values with the specific isotope activities (dpm/mmol) are transformed into nmol ^{3}H (Lemos *et al.*, 2012).

2.2.3. Western blotting

2.2.3.1. Preparation of samples for Western blotting

Total synaptosomal fraction, the pre- and post-synaptic active zone and the extra-synaptic fractions of mice and human cortices were prepared as previously described (Köfalvi *et al.*, 2005; Phillips *et al.*, 2001 and Rebola *et al.*, 2005). Briefly, cortices from 10 mice C57BL/6j and a sample of a human cortex were homogenized with a Teflon potter, at 4 ºC in 2 mL of 0.32 M sucrose solution (containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$). Twelve milliliters of 2 M sucrose solution and 5 mL of 0.1 mM $CaCl_2$ were added to the resulting homogenates and were resuspended. Solutions were distributed into centrifuge tubes and filled carefully onto the top with 1 M sucrose solution containing 0.1 mM $CaCl_2$. Those tubes were centrifuged in a SW41Ti rotor (Beckman Coulter) at 100,000 g for 3 h at 4 ºC. The middle layer, between the 1.25 M and 1 M sucrose interphase, corresponded to

synaptosomes which were collected. Synaptosomes were ten times diluted in 0.32 M sucrose solution and centrifuged at 15,000 g for 30 min at 4 ºC with an Avanti J-26 XPI rotor (Beckman Coulter). The pellets were resuspended in sucrose solution and part of it was centrifuged again at 12,000 g for 5 min, resuspended in SDS 5 % and collected as the total synaptosomal fraction.

The protease inhibitor phenylmethylsulfonyl fluoride (PMSF, 0.1 mM final concentration) was added freshly to the remaining portion of the synaptosomal suspension, which was then slowly ten-times diluted with a 0.1 mM $CaCl_2$ solution. The same volume of solubilisation buffer (2 % Triton X-100, 40 mM Tris, at pH 6.0) was added to the solutions and they were re-incubated for 30 min on ice with mild agitation. The insoluble material (synaptic junctions, which represent both pre and postsynaptic fractions) was pelleted at 40,000 g for 30 min at 4 ºC. The supernatant represents the extrasynaptic fraction. The synaptic junction pellet was washed with solubilisation buffer (1 % Triton X-100 in 20 mM Tris, at pH 6.0) and resuspended in a second buffer (1 % Triton X-100, 20 mM Tris, at pH 8.0). This increase in pH allowed the dissociation of the extracellular matrix that maintains the presynaptic active zone tightly bound to the postsynaptic density. The active zone was solubilised while the postsynaptic density was preserved because the amount of detergent was not enough for its solubilisation (Köfalvi *et al.*, 2005; Phillips *et al.*, 2001 and Rebola *et al.*, 2005). After incubation for 30 min on ice with mild agitation, the mixture was centrifuged at 40,000 g for 30 min. The pellet corresponded to the postsynaptic fraction and was resuspended in SDS 5 %. The supernatant represented the presynaptic fraction. All the fractions were further concentrated by overnight treatment with 30-40 mL of pre-cooled acetone (-20 ºC). Solutions with the acetone were centrifuged at 18,000 g for 30 min at -15 ºC, with a SS34 rotor in a Sorvall centrifuge (Thermo Fisher Scientific). Pellets were left to dry in the incubator and finally resuspended in SDS 5 %. Protein concentration was

determined by the BCA assay and samples were added to an equal volume of 2-times concentrated SDS-PAGE sample buffer before freezing at -20 ºC.

2.2.3.2. Western blotting protocol

Western blot analysis was carried out in total synaptosomes fraction and synaptosomal active zone (pre, post and extrasynaptic fractions) of mice and human cortices, as previously described by us (Köfalvi *et al.*, 2005; Köles *et al.*, 2013; Rebola *et al.*, 2002 and Rebola *et al.*, 2005). Briefly, each sample was denatured at 80 ºC, for 5 min. Then, the samples containing 1-2 µg of protein were loaded in the wells of the concentrating gel and the proteins were separated by SDS-PAGE (10 % with a 4 % concentrating gel) electrophoresis under reducing conditions, together with pre-stained molecular weight markers (Amersham, GE Healthcare). Next, proteins were electrotransferred to polyvinylidene difluoride membranes (PVDF of 0.45 µm; GE Healthcare; previously activated with pure methanol for 10 s, followed by rinsing in mili-Q water for 5 min and then in 10 % *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS)/methanol for more than 10 min). After blocking for 1 h at room temperature with 3 % bovine serum albumine (BSA) in Tris-buffered saline containing 0.1 % Tween 20 (TBS-T), membranes were washed and incubated overnight at 4 ºC with the primary antibodies against InRβ (1:500 diluted in the blockage solution) and CB_1R (1:500 dilution). Controls were made with SNAP-25 (1:40,000 dilution), PSD-95 (1:20,000 dilution) and synaptophysin (1:20,000 dilution) primary antibodies. Afterwards, membranes were washed three times, 15 min each, in TBS-T and incubated with alkaline phosphatase-conjugated secondary antibody (1:20,000 dilution; Table 1) in TBS-T containing 3 % BSA, for 2 h at room temperature. After three consecutive washes in TBS-T with 3 % BSA, membranes were exposed to enhanced chemi-fluorescence reagent (ECF) for 1 min and 20 s and subsequently scanned with a VersaDoc 3000 and

Quantity One software (Bio-Rad Laboratories, Amadora, Portugal). The generated photographs were analysed using the ImageLab (Bio-Rad) software.

2.3. Data treatment

Results are presented as mean ± SEM (standard error of the mean), with the number of independent experiments (n) represented in each figure and statistical significance was considered at $p \leq 0.05$. Statistical data and graphs were produced using Microsoft Office Excel and GraphPad Prism 5 software.

2.4. Chemicals

NaCl, KCl, NaOH, HEPES and D-glucose were purchased from Calbiochem, Merck Biosciences (Germany). $CaCl_2$, $MgSO_4$, $NaHCO_3$, KH_2PO_4, $NaH_2PO_4.H_2O$ and DMSO were purchased from Sigma-Aldrich (Sintra, Portugal). Tested drugs (WIN55212-2, O-2050, insulin, bicuculline and tetrodotoxin) were obtained from Tocris Bioscience (UK). Non-water soluble substances were dissolved or reconstituted in DMSO and stored at -20 ºC until use.

Primary and secondary antibodies used in Western blot analysis are resumed in Table 1.

Table 1. Primary and secondary antibodies used in Western blot analysis.

Antibody	Supplier	Host	Type	Dilution
Insulin Rβ (C-19)	Santa Cruz Biotechnology (California, USA)	Rabbit	Polyclonal	1:500
CB1-Rb-Af380	Frontier Institute (Hokkaido, Japan)	Rabbit	Polyclonal	1:500
SNAP-25	Sigma-Aldrich (Sintra, Portugal)	Mouse	Monoclonal	1:40,000
PSD-95	Sigma-Aldrich (Sintra, Portugal)	Mouse	Monoclonal	1:20,000
Synaptophysin	Millipore	Rabbit	Polyclonal	1:20,000
Rabbit-alkaline phosphatase conjugate	GE Healthcare Biosciences (UK)	Goat	IgG + IgM (H+L)	1:20,000
Mouse-alkaline phosphatase conjugate	GE Healthcare Biosciences (UK)	Goat	IgG (H+L)	1:20,000

3. RESULTS AND DISCUSSION

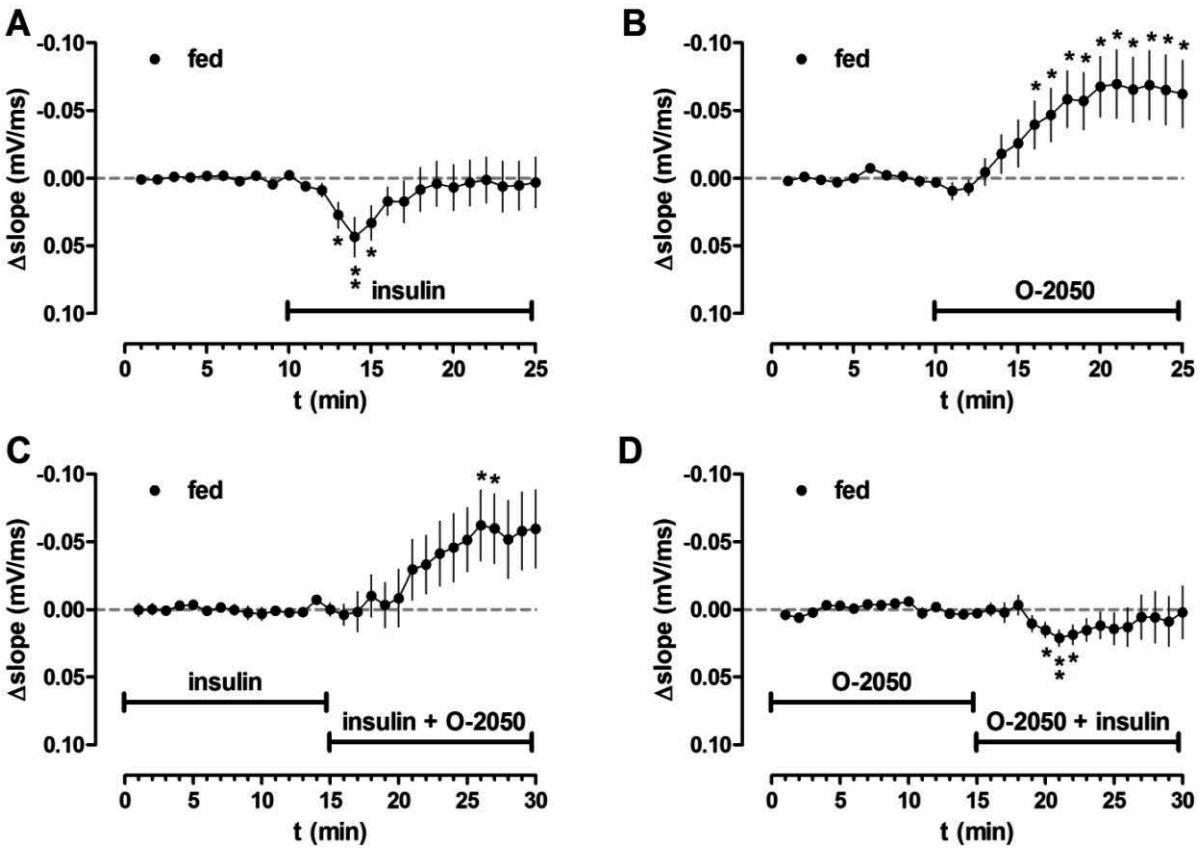
3.1. The effect of insulin on basal synaptic plasticity is dependent on the CB_1R and metabolic conditions in the rat hippocampal slices.

Impaired response to acutely administered insulin in neuronal activity can be measured by MRI and is a marker of (developing) cerebral insulin resistance (Guthof *et al.*, 2010; Kullmann *et al.*, 2012 and Tschritter *et al.*, 2006). Considering that insulin and CB_1R agonists both can control and affect neuronal activity and furthermore, synaptic plasticity, we carried out extracellular electrophysiology recordings, with modifications to our previous study (Martire *et al.*, 2011), with the aim of measuring the effect of insulin on synaptic transmission and its dependence on the CB_1R in rat hippocampal slices.

To define whether the modulation of synaptic transmission by insulin is dependent on the activation of the CB_1R, we tested insulin under the presence or the absence of the neutral CB_1R antagonist, O-2050 (Wiley *et al.*, 2011) (Figures 3.1-3.6). Extracellular recording was performed in hippocampal slices of young adult male Wistar rat (Figure 3.1). Fasted rats, which were food-restricted for 16 hours until sacrifice (Higaki *et al.*, 1999) and rats injected intraperitonealy with STZ two weeks before sacrifice were also included in these experiments with the aim of understanding the effect of insulin and O-2050 (i.e. endogenous CB_1R tone) on action on synaptic transmission under different metabolic conditions.

Insulin (300 nM) statistically significantly and transiently depressed synaptic transmission in the first 5 min of its perfusion, as observed with the help extracellular recording in acute transversal hippocampal slices from *ad libitum* fed rats (n = 19; Figures 3.1 A and 3.4). In contrast, slow perfusion of the CB_1R antagonist O-2050 (500 nM) alleviated synaptic transmission from an apparent inhibitory tone, which effect became statistically significant after 5 min (n = 12; Figures 3.1 B and 3.4). After letting insulin's

effect stabilize for 20 min, O-2050 was added for 15 min in the presence of insulin, which facilitated synaptic transmission similarly to that seen in the absence of insulin (n = 12; Figures 3.1 C and 3.4). This indicates that insulin does not prevent eCB tone in the hippocampal slices of the fed rats. In the other way around, after the 20 min stabilization of the synaptic transmission in the presence of O-2050, insulin's effect was largely prevented: although at individual time points it was significantly different from the previous 10 min of the drug-naïve (preinsulin) baseline (Figure 3.1 D), the overall effect of insulin in the first 5 min or the following 10 min was no longer statistically significantly different from zero (n = 12; Figure 3.4). This suggests that the effect of insulin is dependent on the CB_1R in fed animals.



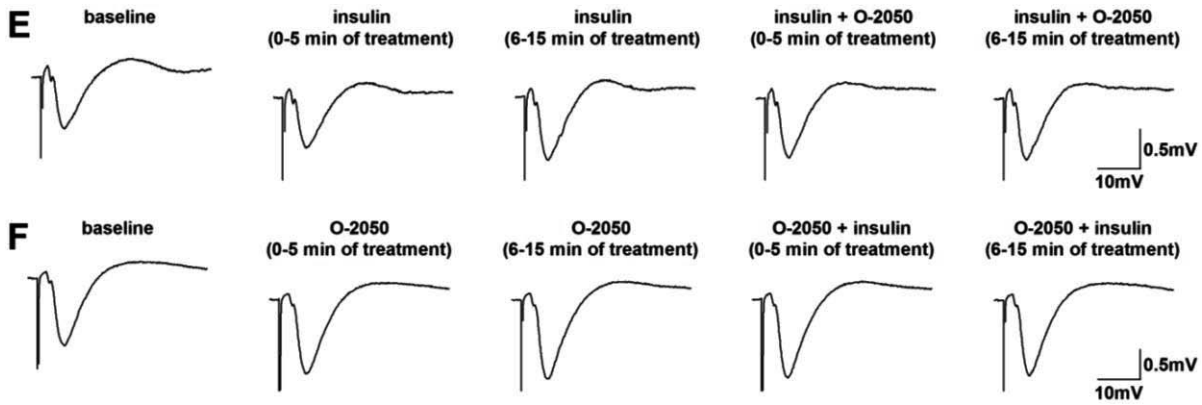


Figure 3.1. Insulin depresses basal synaptic transmission in a CB_1R-dependent fashion in hippocampal slices of the fed rat. (A-D) Time course of the slope variation (mV/ms) of fEPSPs in hippocampal slices of *ad libitum* fed rats, upon the perfusion of **(A)** insulin (300 nM), **(B)** O-2050 (500 nM), **(C)** O-2050 after 20 min stabilization of baseline in the presence of insulin and, finally, **(D)** insulin after 20 min stabilization of the baseline in the presence of O-2050. **(E-F)** Representative traces of fEPSPs from the above experiments. Data are mean ± SEM of $n \geq 12$ independent observations, *$p < 0.05$, **$p < 0.01$.

The subsequent experiments clearly supported that the hippocampal action of insulin and eCBs is not mechanistic as it is subject to modulation by different metabolic states. For instance in fasted rats, insulin's action was drastically different as it induced a rapid and sustained facilitation of synaptic transmission ($n = 8$; Figures 3.2 A and 3.4), which effect was statistically significantly different from that in the *ad libitum* fed animals ($p < 0.0001$, as compared with Two-Way ANOVA to the data from the fed rats). Similarly to the inversion of insulin effect in the fasted animal, the facilitator effect of O-2050 was statistically significantly smaller in the fasted rats ($n = 8$, $p < 0.05$ vs. fed rats, compared with Two-Way ANOVA; Figures 3.2 B and 3.4). Interestingly, in the presence of insulin, O-2050 failed to affect basal transmission and, thus, these data were significantly different from those obtained in the *ad libitum* fed animals ($n = 8$, $p < 0.0001$ vs. fed rats, compared with Two-Way ANOVA; Figures 3.2 C and 3.4). Perhaps the most surprising and striking result was that preincubation with O-2050 fully reverted the action of insulin to what was seen in the fed rats in the absence of O-2050 (compare Figures 3.1 A vs. 3.2 D), and hence the

inhibitory effect of insulin was statistically significantly greater in the fasted rats than in the fed ones in the presence after O-2050 (n = 8, $p < 0.05$ vs. fed rats, compared with Two-Way ANOVA; Figures 3.2 D and 3.4).

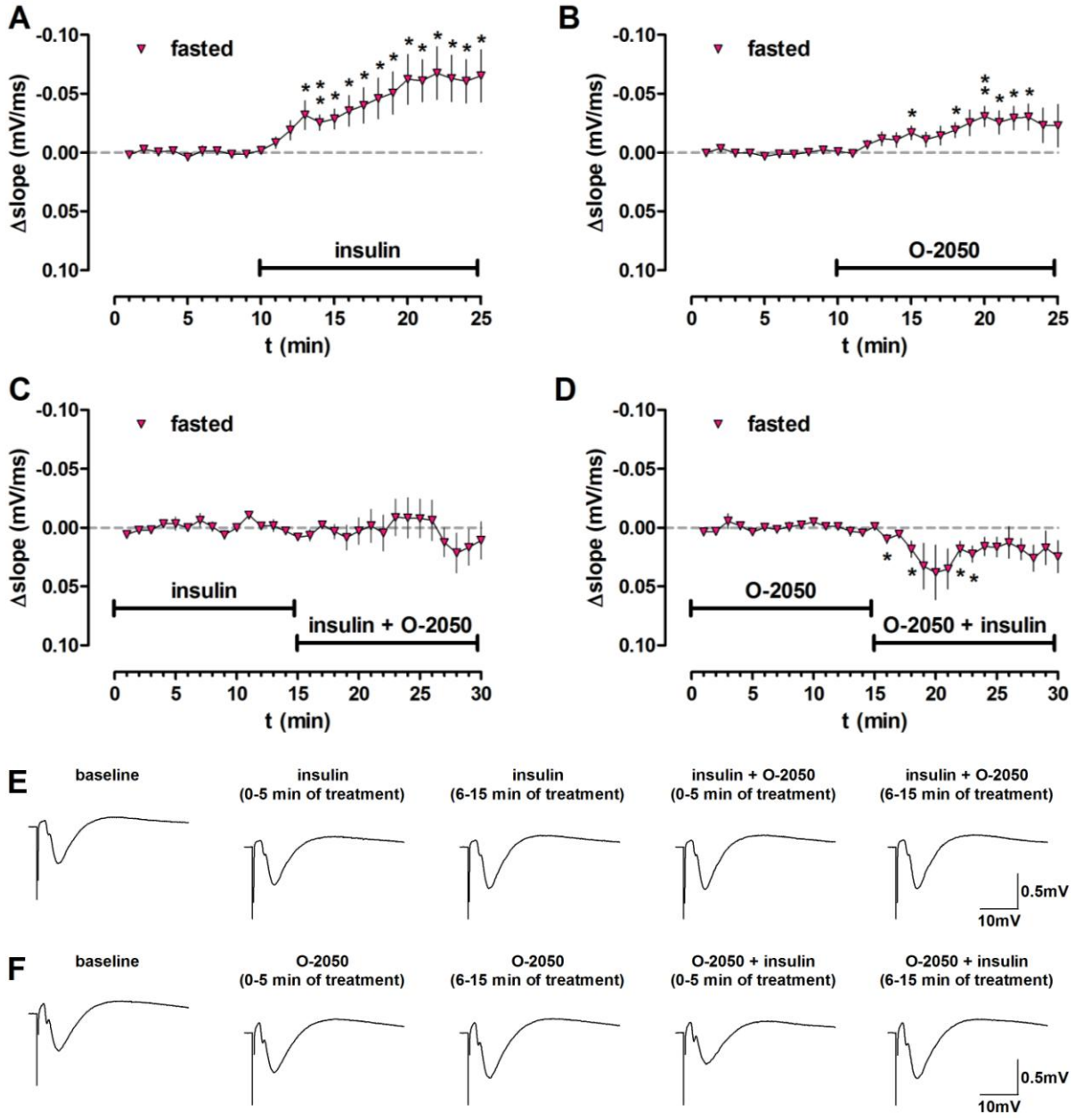


Figure 3.2. Insulin facilitates basal synaptic transmission in a CB_1R-dependent fashion in hippocampal slices of the fasted rat. (A-D) Time course of the slope variation (mV/ms) of fEPSPs in hippocampal slices of 16 h fasted rats, upon the perfusion of **(A)** insulin (300 nM), **(B)** O-2050 (500 nM), **(C)** O-2050 after 20 min stabilization of baseline in the presence of insulin and, finally, **(D)** insulin after 20 min stabilization of the baseline in the presence of O-2050. **(E-F)** Representative

traces of fEPSPs from the above experiments. Data are mean ± SEM of $n \geq 12$ independent observations, *$p < 0.05$, **$p < 0.01$.

Two weeks after STZ injection, insulin and O-2050 failed to alter synaptic transmission alone or in combination (n = 11; Figure 3.3) in the hippocampal slices, beyond tendency. This points out a rapid subacute remodelling of the eCB and insulin signalling systems, which is also reflected in other presynaptic differences measured by us as paired-pulse ratio (PPR; see below).

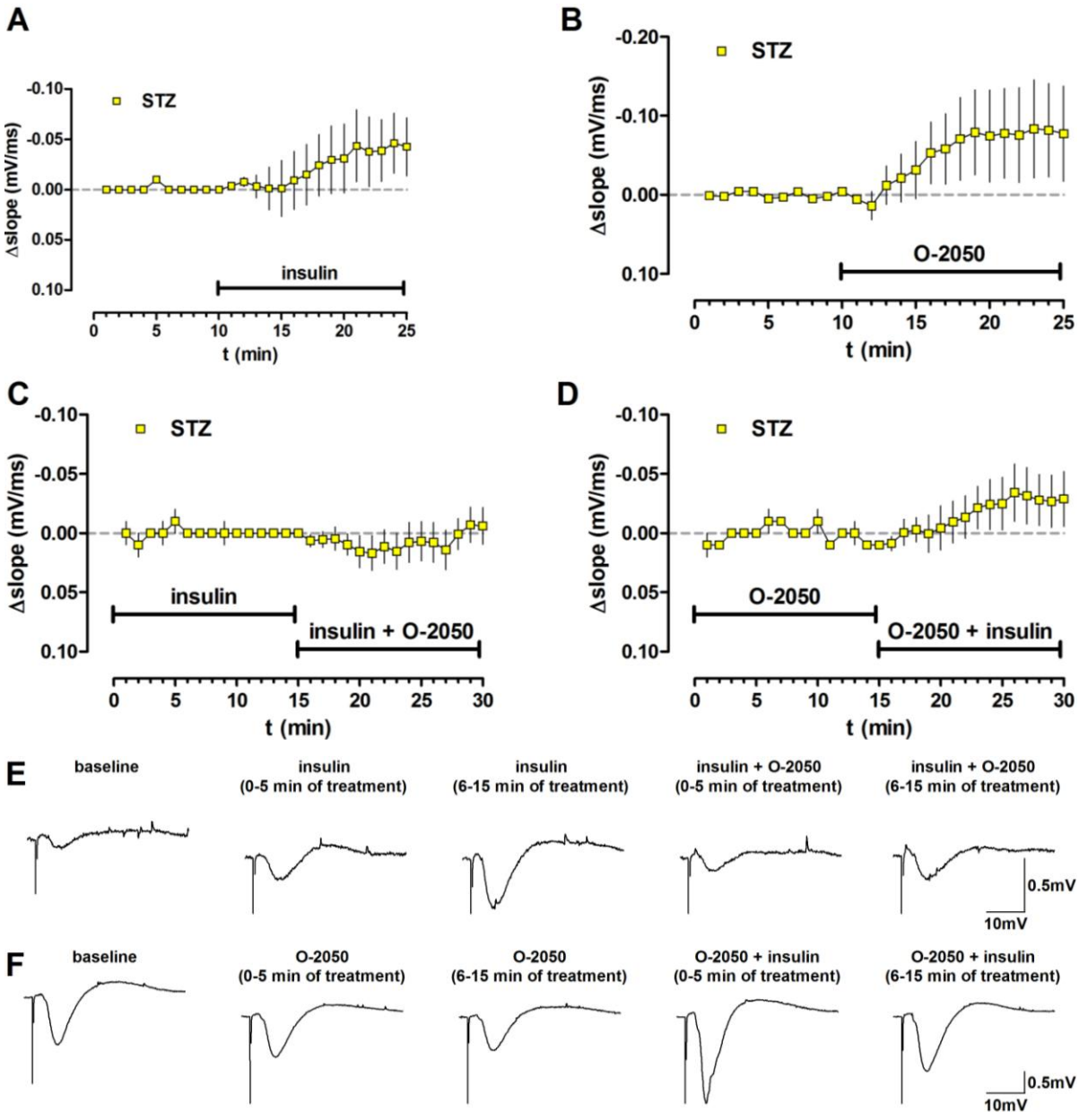


Figure 3.3. Insulin and CB_1R blockade fail to affect basal synaptic transmission in hippocampal slices of the diabetic rat. (A-D) Time course of the slope variation (mV/ms) of fEPSPs in hippocampal slices of diabetic rats 2 weeks post-injection, upon the perfusion of **(A)** insulin (300 nM), **(B)** O-2050 (500 nM), **(C)** O-2050 after 20 min stabilization of baseline in the presence of insulin and finally, **(D)** insulin after 20 min stabilization of the baseline in the presence of O-2050. **(E-F)** Representative traces of fEPSPs from the above experiments. Data are mean ± SEM of $n \geq 12$ independent observations, *$p < 0.05$.

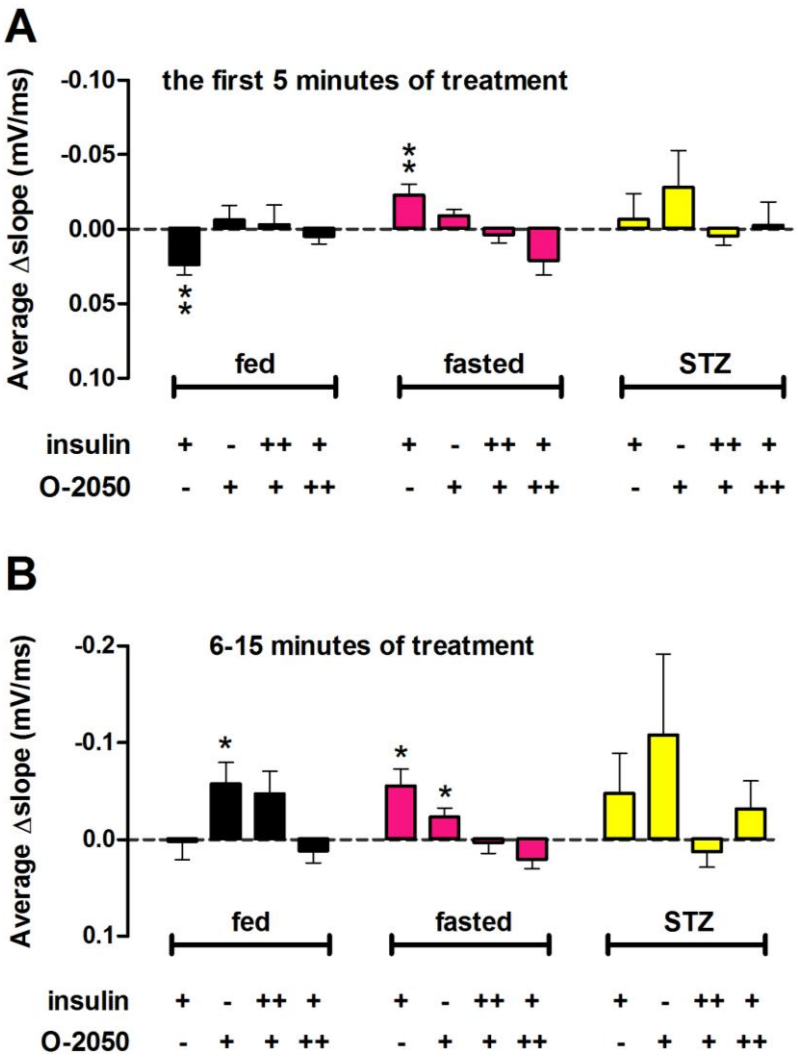


Figure 3.4. Bar graphs summarizing the average changes in synaptic transmission from the extracellular recording experiments. Average slope variation (mV/ms) of fEPSPs in hippocampal slices of fed, fasted and diabetic rats **(A)** from the first 5 minutes of treatment and **(B)** from the 6th to the 15th minutes of treatment with O-2050 (500 nM) or/and insulin (300 nM). **(+)** denotes if the substance was present before the substance marked with **(++)**, while **(-)** marks the absence of either O-2050 or insulin. Data are mean ± SEM of $n \geq 8$ independent experiments, *$p < 0.05$, **$p < 0.01$.

3.2. The subsynaptic side for insuline's action.

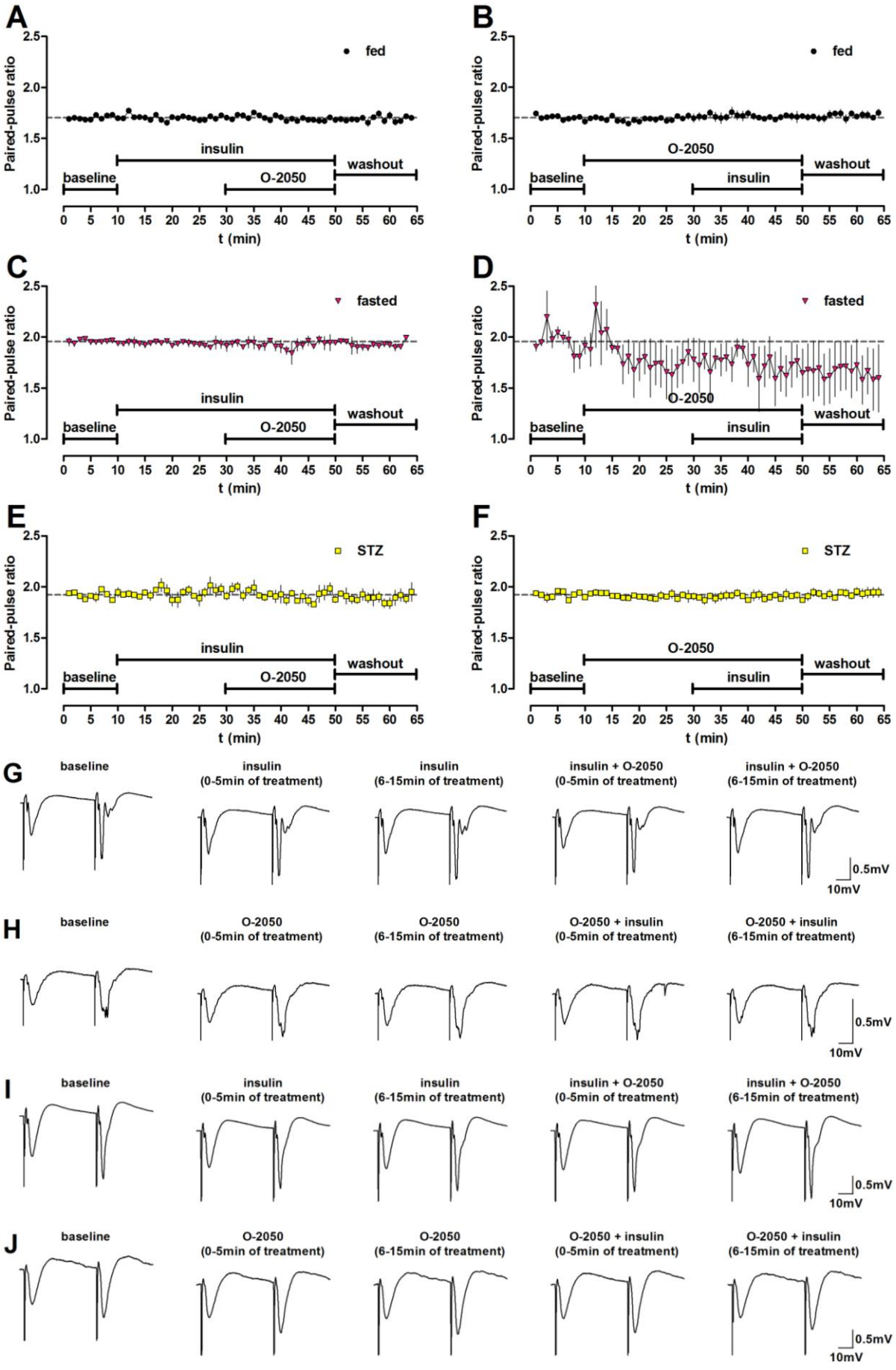
Paired-pulse ratio calculations were carried out alongside the above experiments, i.e. while the above experiments under point 3.1 represent exclusively changes in the response to the first stimulus; here we monitored the change of the response to each second stimulus which followed the first stimulus with a 50 ms interval. This interval is short enough to prevent glial and postsynaptic confounding mechanisms that could alter presynaptic release probability (Zucker and Regehr, 2002). In fed rats, the PPR during the drug-naïve baseline amounted to 1.70 ± 0.05, indicating a so-called paired-pulse facilitation (PPF) according to the literature (Zucker and Regehr, 2002), ($n = 12$; Figures 3.5 A-B and 3.6 D). The PPR value did not increase beyond tendency to for the fasted rats ($n = 6$, $p > 0.05$, estimated with One-Sample *t*-test against the hypothetical value of 1.70), while the diabetic rats had significantly increased PPR ($n = 7$, $p < 0.05$; Figures 3.5 C-F and 3.6 D). This latter finding indicates that in diabetes, hippocampal glutamatergic terminals are under tonic inhibition greater than in the *ad libitum* fed rats, which is in concert with the cognitive deficits and encephalopathy associated with type-1 diabetes (Sima, 2010).

Insulin (300 nM) or in the presence of the CB_1R antagonist O-2050 (500 nM) alone failed to affect the PPR in the fed rat, suggesting that insulin's effect on the synaptic transmission is probably not presynaptic (Figures 3.5 A and 3.6 A). However and as expected since the CB_1R is a major presynaptic regulator of glutamatergic terminals (Domenici *et al.*, 2006), O-2050 slightly but statistically significantly decreased the PPR, indicating the presence of a very small endogenous cannabinoid tone contributing to PPF, i.e. to that the PPR was greater than 1. Notably, an increase in the PPR is virtually always a measure of presynaptic inhibition which was uncovered by O-2050, and this well matches

the effect of O-2050 on the synaptic transmission. Now, what is intriguing is that insulin significantly increased the PPR in the presence of O-2050 which may suggest that an arising presynaptic action of insulin - so far hidden by endogenous CB_1R activity - was now uncovered, contributing to the disappearance of insulin's action on synaptic transmission (Figures 3.5 B and 3.6 A).

In contrast to the small or no changes observed in fed rats, the PPR was much larger and significantly inhibited by either insulin or O-2050 or in combination of the two in fasted animals (n = 6; Figures 3.5 C-D and 3.6 B). This data suggest an increased insulin sensitization of neural activity by fasting in the brain which is fully the opposite in the body, where fasting decreases insulin sensitivity by ~55 % due to the lack of the release of the hepatic insulin sensitizing substance (Lautt, 2004).

Last but not least, no PPR modulation by insulin or O-2050 or by their combination was observed in the diabetic rats (n = 7) bolstering our previous observation about the lack of eCB tone and neuromodulation by insulin in the hippocampal slices under our experimental settings upon diabetes.


A
fed
Paired-pulse ratio
insulin
baseline
O-2050
washout
t (min)
B
fed
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C
fasted
insulin
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G
baseline
insulin (0-5min of treatment)
insulin (6-15min of treatment)
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insulin + O-2050 (6-15min of treatment)
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insulin (6-15min of treatment)
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O-2050 (0-5min of treatment)
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O-2050 + insulin (6-15min of treatment)
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10mV

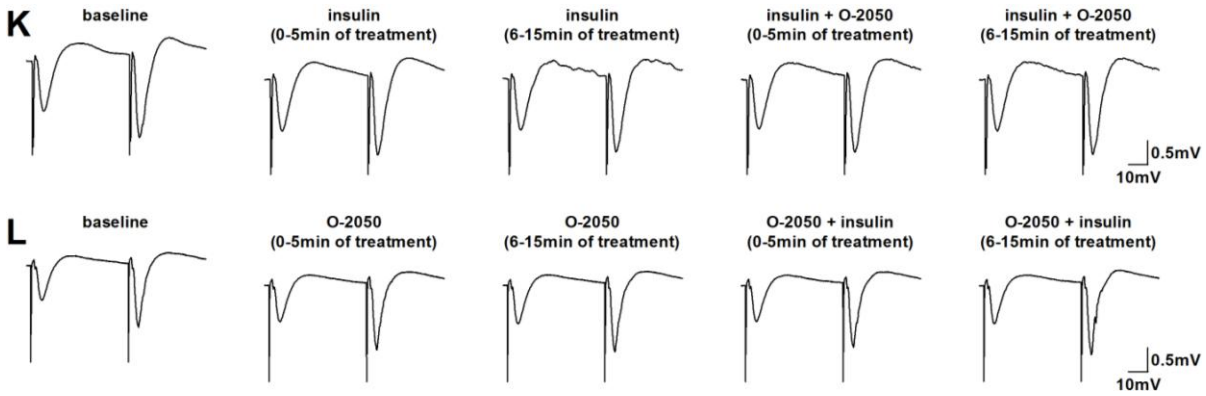

Figure 3.5. The presynaptic effects of insulin and O-2050 alone or in combination as evaluated by changes in PPR. Time course of the effect of insulin (300 nM) and O-2050 (500 nM) alone or in combination as indicated by the horizontal bars, in hippocampal slices of **(A-B)** fed, **(C-D)** fasted and **(E-F)** diabetic rats. **(G-H)** Representative traces of fEPSPs obtained for fed, **(I-J)** fasted and **(K-L)** two weeks STZ rats. Data are of n ≥ 6 independent experiments.

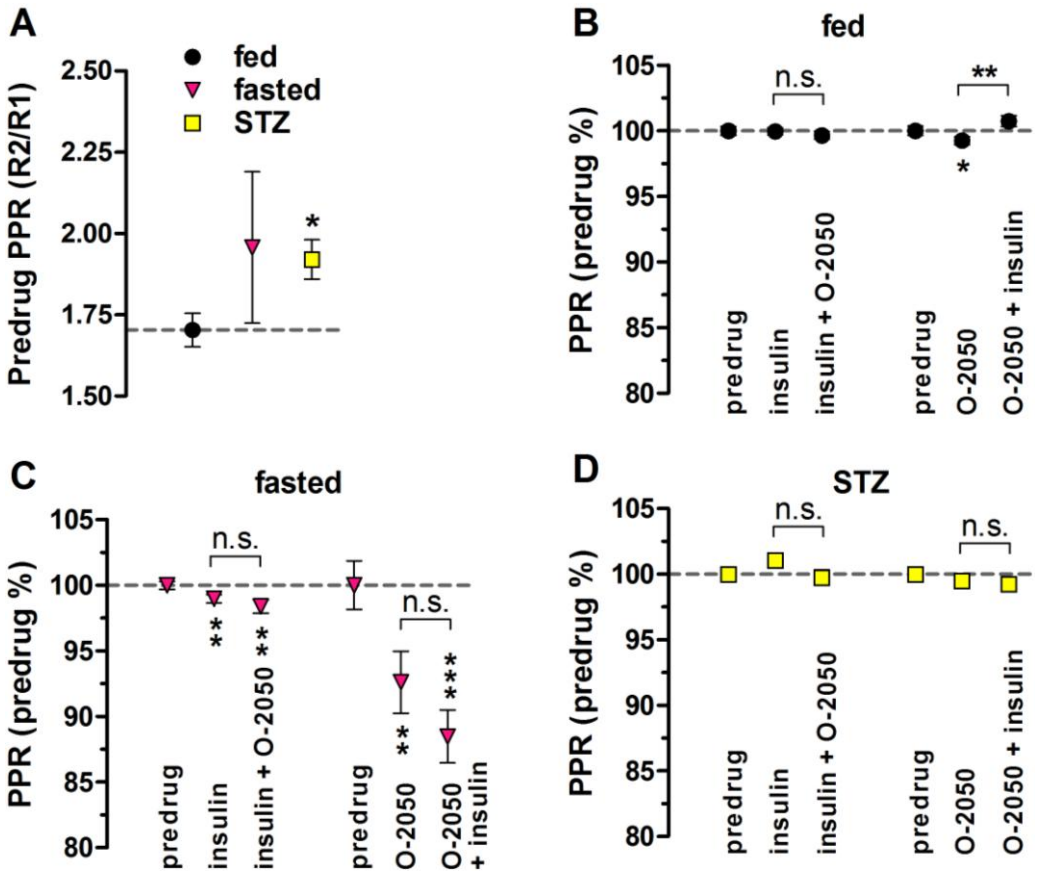

Figure 3.6. Mean PPRs from the above experiments. (A) Drug-naïve PPR values from fed (n = 12), fasted (n = 6) and diabetic (n = 7) rats. **(B-D)** The effect of insulin (300 nM) and O-2050 (500 nM) alone and in combination on the PPR in hippocampal slices prepared from **(A)** fed, **(B)** fasted and **(C)** diabetic rats. All treatment-induced PPR values are expressed to the 10 min average of drug-naïve PPR, taken as 100 %. Combined effect PPR values were compared to the single treatment

PPR values with the help of paired Student's *t*-test. Data are mean ± SEM of n ≥ 6 independent experiments, *p < 0.05, **p < 0.01, ***p < 0.001, n.s.: not significant.

3.3. Insulin inhibits glucose uptake in a CB_1R-dependent fashion.

Glucose is the primary source of energy in the brain and local rates of glucose utilization vary with neuronal activity (Pellerin, 2008). Consequently, as insulin affects neuronal activity we expect that it is also measurable in the rate of glucose uptake. In fact, systemically or nasally applied insulin affects ^{18}F-deoxyglucose PET signals in the human brain, which response is impaired under cerebral insulin resistance (Anthony *et al.*, 2006; Baker *et al.*, 2011 and Bingham *et al.*, 2002). Therefore, impaired response to insulin in glucose utilization is an additional precious indicator of cerebral insulin resistance.

Hence, we were interested if insulin follows the same pattern of effect on glucose uptake and if it is modulated by the same fashion by the CB_1R blockade. Taken that, our protocol allows incubating several slices in the same time in different wells, unlike electrophysiology which allows testing one slice at once. Glucose uptake in the cortex and hippocampus was measured with the help of [^{3}H]DG, which is virtually non-metabolizable and thus indicates total glucose uptake (Lemos *et al.*, 2012). If results coincide with that of the electrophysiology data, this experimental setting will allow us carrying out a large-scale pharmacological analysis otherwise impossible due to the limitations of electrophysiology.

Glucose uptake, as calculated from the [^{3}H]DG uptake, amounted to 81.3 ± 4.1 (n = 26) Insulin at the concentration of 300 nM but not at 30 nM (Figures 3.7 and 3.8) decreased glucose uptake in both fed (n = 15, p < 0.01 and p < 0.05 by One-Sample *t*-test) and fasted (n = 6, p < 0.001) animals, but not in diabetic rats (n = 6, p > 0.05), in cortical and

hippocampal slices (respectively). Besides, fasted animals found to be significantly more sensitive to insulin than fed rats (Figure 3.7).

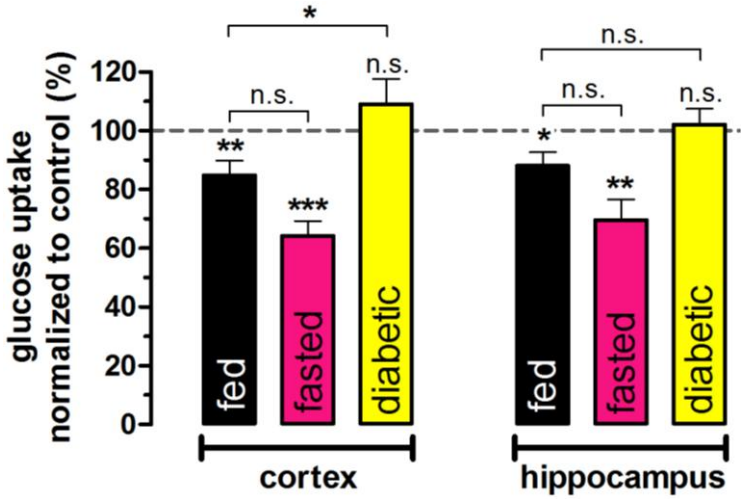

Figure 3.7. Insulin decreases the uptake of glucose in acute cortical and hippocampal slices of fed (CTRL) and fasted, but not diabetic rats. Bar graph representing the uptake of glucose as determined from [^{3}H]DG uptake in fed, fasted and diabetic rat cortical and hippocamapal slices. Slices of 450 µM thickness were first incubated at 37 ºC for metabolic recovery in the presence of 5.5 mM glucose at 37 ºC under carboxygenation, then 2.5 nM [^{3}H]DG was bath applied for 30 min. The effect of insulin (300 nM) is normalized to the respective control (100 %) and was statistically evaluated by One-Way ANOVA. Data are mean ± SEM of $n \geq 6$ independent experiments, *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$, n.s.: not significant.

Then, in order to characterize the possible involvement of the CB_1R in the effect of insulin (30 and 300 nM) on glucose uptake, hippocampal and cortical slices of fed rats were incubated with insulin (30 nM and 300 nM), alone or combined with the neutral CB_1R antagonist O-2050 (500 nM) or with the CB_1R agonist, WIN55212-2 (500 nM) (Figure 3.8). As above detailed, insulin at the concentration of 300 nM diminished resting glucose uptake in both cortical and hippocampal slices while at 30 nM failed to do so ($n = 12$, $p > 0.05$; Figure 3.8). While O-2050 had no effect alone ($n = 8$, $p > 0.5$) it fully prevented the action of insulin (300 nM) in cortical and hippocampal slices. WIN55212-2 also failed to affect the uptake of glucose *per se*, in accordance with previous findings (Lemos *et al.*, 2012),

however, it exerted a permissive effect for 30 nM insulin to diminish glucose uptake in the hippocampal slice (Figure 3.8). Thus, it appears that CB_1R activation positively, while CB_1R blockade negatively influence the action of insulin in both brain areas, and that electrophysiological findings, in large part, correlate with glucose uptake changes. Consequently, we next investigated how the effect of insulin is affected by modulating neuronal and synaptic activity in the slices.

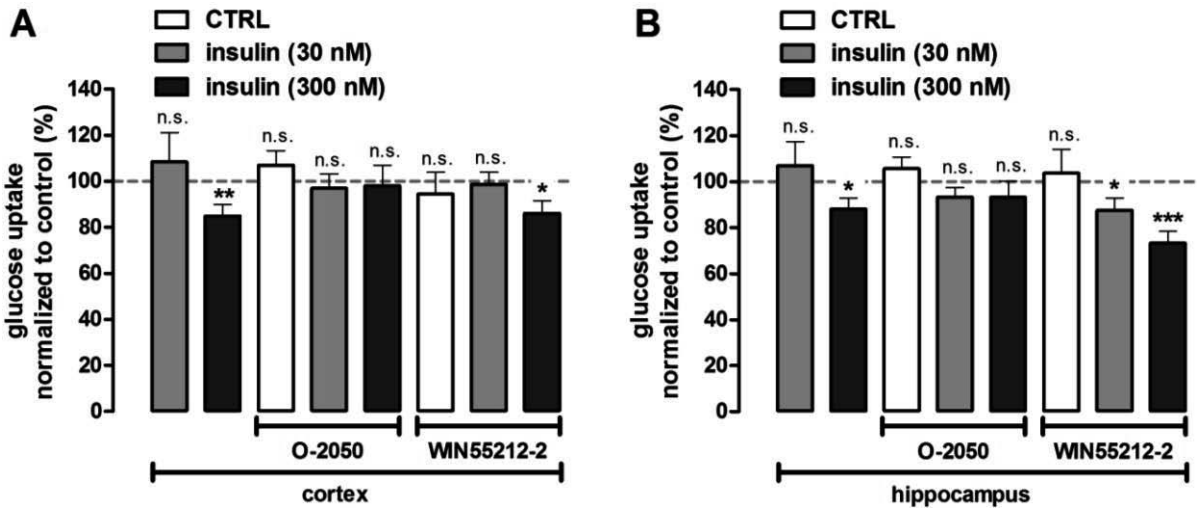

Figure 3.8. The CB_1R neutral antagonist O-2050 (500 nM) prevents insulin's action on glucose uptake, while the CB_1R agonist WIN55212-2 (500 nM) enables it in rat acute cortical and hippocampal slices. Effect of the CB_1R antagonist O-2050 (500 nM) or the CB_1R agonist WIN55212-2 (500 nM) on glucose uptake, alone or in combination, in rat **(A)** cortical and **(B)** hippocampal slices. Data are mean ± SEM of $n \geq 8$ independent experiments, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, n.s.: not significant, by One-Sample *t*-test against the hypothetical value of 100 %.

To this end, we applied two strategies: clamping the slice at high and low activity. Depolarization of slices with 20 mM K^+ increased the uptake of glucose to 192.0 ± 22.85 % of CTRL in the cortex and to 157.7 ± 16.83 % of CTRL in the hippocampus of rat (for both cases: $n = 4$, $p < 0.05$), while 30 mM K^+ increased the uptake of glucose to 284.6 ± 22.73 % in the cortex ($n = 4$, $p < 0.01$) and to 247.7 ± 16.40 % in the hippocampus ($n = 4$, $p < 0.05$; Figure 3.9). These data corroborate that our experimental settings are capable to reveal

glucose uptake changes upon altered activity in the slice. However, under K^+ depolarization, insulin was no longer able to inhibit glucose uptake ($p > 0.05$ vs. respective controls by paired-*t* test; Figure 3.9), indicating that its effect is occluded under clamping the circuitry at high activity.

Previous studies showed that insulin potentiates postsynaptic $GABA_AR$-dependent neurotransmission (Mielke and Wang, 2005 and Wan *et al.*, 1997) and that $GABA_A$ potentiation can decrease the energy demand in the slice (Eintrei *et al.*, 1999). Thus, the effect of insulin (300 nM) under $GABA_AR$ blockade by bicuculline (20 µM) and under the voltage-gated sodium channel blockade by tetrodotoxin (TTX, 1 µM) was also studied (Figure 3.9). Neither bicuculline nor TTX affected the uptake of glucose in the cortex and the hippocampus, either alone or in combination with insulin (300 nM) (for both cases: $n = 8$, $p > 0.05$ by One-Sample *t*-test; Figure 3.9). These data first tell us that the slices were in truly resting state as a further tentative of the inhibition of neural communication resulted in no more decrease in glucose uptake. Together with the data with high-K^+ clamp, these results also point out that insulin's effect is fully dependent on neuronal activity, especially, on GABAergic communication, in accordance with the previous reports (Mielke and Wang, 2005 and Wan *et al.*, 1997).

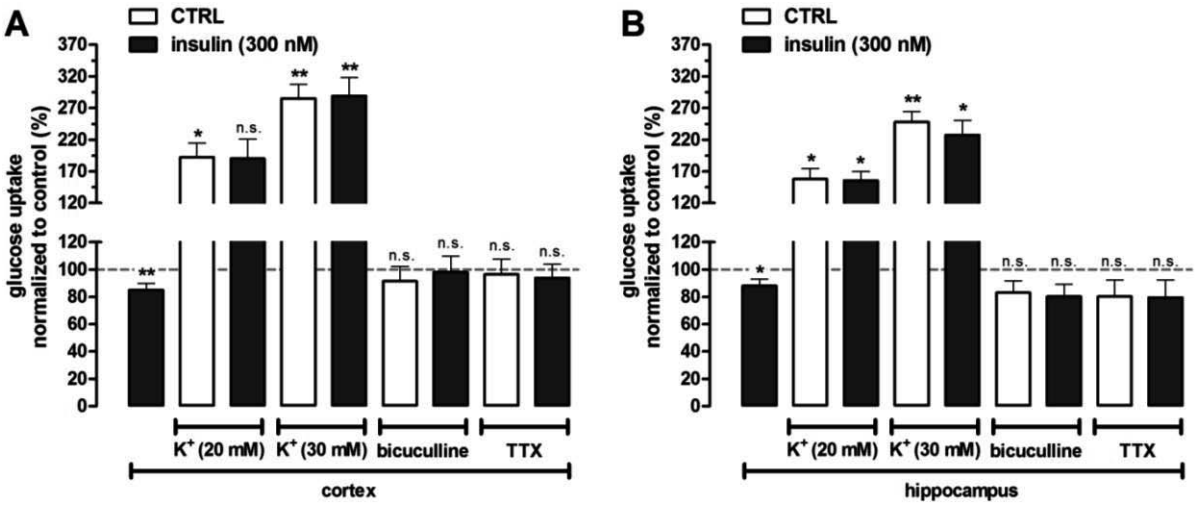

Figure 3.9. Clamping neuronal activity prevents insulin from affecting glucose uptake. Bar graph illustrates the effect of high K^+ (20 or 30 mM), the $GABA_AR$ blocker, bicuculline (20 µM) and the blockade of axonal depolarization by TTX (1 µM) alone or in combination with insulin (300 nM) on glucose uptake in rat **(A)** cortical and **(B)** hippocampal slices. Data are mean ± SEM of n = 4-8 independent experiments, *p < 0.05, **p < 0.01, n.s.: not significant, evaluated by One-Sample *t*-test against the hypothetical value of 100%, while between-bar comparisons were carried out with Student's paired *t*-test.

In vitro experiments on cortical and hippocampal slices of adult male CB_1R KO mice on the CB-1 strain and their wild-type littermates were performed in order to prove the involvement of the CB_1R in the action of insulin (30 and 300 nM) (Figure 3.10). These experiments were carried out on 11 pairs of wild-type and CB_1R KO mice, i.e. each wild-type animal served as a respective control for the CB_1R KO mouse in the same experiment. In the same protocol as above with the rat, glucose uptake amounted to 73.4 ± 4.2 (n = 11) in the wild-type control slices. Incubation with insulin resulted in a significant decrease in glucose uptake in cortical and hippocampal slices of wild-type mice: at 30 nM by 18.49 ± 5.64 % (n = 6, p < 0.05, per One-Sample *t*-test) and at 300 nM by 11.82 ± 4.74 % (n = 11, p < 0.05) in the cortex; and at 30 nM by 22.50 ± 5.78 % (n = 6, p < 0.05) and at 300 nM by 17.78 ± 5.33 % (n = 11, p < 0.05) in the hippocampus (Figure 3.10). As we reported previously (Lemos *et al.*, 2012), the genetic deletion of the CB_1R

impaired glucose uptake ($n = 11$, $p < 0.001$, in the cortex and $n = 12$, $p < 0.01$, in the hippocampus). The amplitude of this impairment was virtually the same as the effect of insulin in the wild-type mice. Furthermore, insulin affected glucose uptake neither in cortical nor in hippocampal slices of the CB_1R KO mice ($p > 0.05$, with One-Way ANOVA followed by Tukey's multiple comparison test; Figure 3.10).

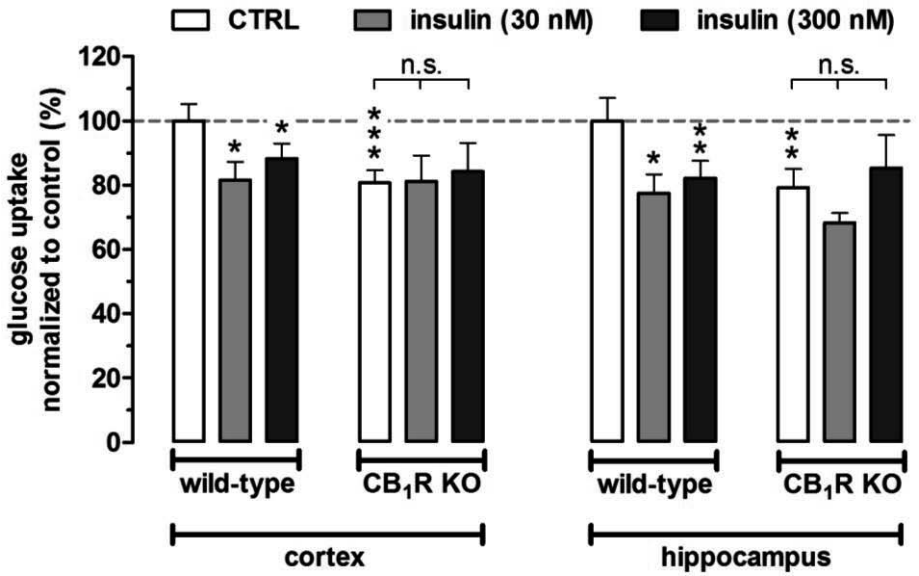

Figure 3.10. Insulin diminishes the uptake of glucose in acute cortical and hippocampal slices of the wild-type but not the CB_1R KO mice. Bar graph exhibiting glucose uptake values normalized to the wild-type control in paired experimental setting. The action of insulin on cerebral glucose uptake in CB_1R KO mice was compared to the respective control group (empty bars) with the help of One-Way ANOVA followed by Tukey's multiple comparison test, while statistical differences from the wild-type control value were evaluated with One-Sample *t*-test. Data are mean ± SEM of $n \geq 6$ independent experiments, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, n.s.: not significant.

Insulin inhibited glucose uptake in rat and mice resting acute slices, similarly to what was previously seen in the amygdala-hippocampus of humans injected with insulin (Anthony *et al.*, 2006). Intranasal insulin spray also decreased intrinsic brain activity in the hypothalamus and orbitofrontal cortex of healthy humans (Kullman *et al.*, 2012). Together with the data presented in this chapter, these may represent the postprandial feed-back

mechanism that terminates food seeking behavior (Brüning *et al.*, 2000). This was previously proposed when cerebral activation by intranasal insulin decreased activity in the brain regions required for food-seeking behaviour (Guthoff *et al.*, 2010). A possible mechanism for the inhibitory effect of insulin under glucose uptake is the previously mentioned recruitment of postsynaptic $GABA_A$Rs, which results in increased GABAergic inhibition of the circuitry (Mielke and Wang, 2005 and Wan *et al.*, 1997) and, consequently, in decreased glucose utilization in limbic areas (Eintrei *et al.*, 1999). $GABA_A$R-mediated transmission in the resting slice is a consequence of spontaneous presynaptic release of GABA. The action potential blocker TTX only prevents the spontaneous release of GABA (Salin and Prince, 1996), which seems to be essential for the action of insulin (Figure 3.9). Besides, bicuculline is an antagonist of $GABA_A$Rs and also inhibits the insulin's effect on glucose uptake (Figure 3.9). Although CB_1Rs were previously found in the presynaptic GABAergic terminals of the rat and human hippocampus, these receptors inhibited only the stimulation evoked by but not the basal release of GABA (Katona *et al.*, 1999 and 2000). Hence, this explains why the CB_1R agonist WIN55212-2 had no effect on the uptake of glucose (Figure 3.8).

Fasting increased insulin-mediated inhibition of glucose uptake in rat hippocampal and cortical slices (Figure 3.7). A possible justification is that has these animals have been with low levels of insulin for such a long time that they become more sensitive to insulin than normal fed rats. On the other hand, glucose uptake was not changed by insulin's action in the diabetic rats. This is probably explained by the measurement of a decrease in cerebral InR density in the diabetic animals by us. Since this decrease was accompanied with a decrease in CB_1R density, it is possible that InR and CB_1R bearing synapses require insulin as a trophic factor (Al-Mubarak *et al.*, 2009) and, in their absence, synaptic dysfunction may take place leading to loss of cerebral insulin and cannabinoid sensitivity.

Finally, the deletion of the CB_1R resulted in similar phenotype as the acute action of insulin and the lack of CB_1Rs occluded the action of insulin. These findings point out that insulin requires the presence of functional CB_1Rs to act upon synaptic transmission and glucose uptake. It is somewhat surprising that the CB_1R KO exhibits an impaired instead of facilitated glucose uptake phenotype, because our research group has recently found that CB_1Rs situated in neuronal and glial mitochondria actually negatively affect glucose metabolism, without impinging on the bioavailability of several other high-energy nutrients such as lactate. These differences may be explained with putative developmental alterations associated with CB_1R deletion.

3.4. Both InR and CB_1R are localized in the postsynaptic active zone of mice and human cortices.

Since there is evidence of the effect of insulin on synaptic transmission and glucose uptake to be dependent on the CB_1R signalling in cortical and hippocampal slices and that this mostly postsynaptic effect, preliminary experiments of Western blot analysis were performed as previously validated (Rebola *et al.*, 2005), with the aim of studying the localization of both InR and CB_1R in the pre and postsynaptic active zones, in tissue preparations, as previously done (Köfalvi *et al.*, 2005).

Before assessing the localization of InR and CB_1R in C57BL/6j mice cortex, the purity of subsynaptic fractions was validated by Western blot, with antibodies that label the presynaptic active zone (anti-SNAP-25), the postsynaptic zone (anti-PSD-95) and the extrasynaptic regions (anti-synaptophysin) (Figure 3.11 A). As expected, an enrichment of subsynaptic proteins was observed in the corresponding subsynaptic fractions. In detail:

SNAP-25 is the synaptosomal-associated protein 25 (with 25 kDa) and is located in the presynaptic fraction, PSD-95 is the post-synaptic density 95 (95 kDa) so it has a higher density in the postsynaptic fraction and synaptophysin is a synaptic vesicle marker (with 38 kDa) which is therefore enriched in the extrasynaptic fraction. Total means the total initial crude from the synaptosomal preparation which was further processed in subsynaptic fractionation as explained in the chapter of Materials and Methods as well as in Köfalvi *et al.* (2005) (Figure 3.11 A).

Afterwards, Western blot analysis carried out in these total synaptosomes and sub-synaptosomal fractions of mice cortices served evidence that the CB_1R (with apparent molecular mass of 55 kDa) was specially enriched in the postsynaptic fraction, but it was also present in lower density at the presynaptic density fraction and in the other cell compartments fraction (extra) (Figure 3.11 B). InR (91 kDa) was also present in presynaptic and postsynaptic fractions, but it was enriched in the extrasynaptic fraction. Hence, CB_1R and InR seem to be localized both pre and especially postsynaptically in the mouse cortex, but it is important to increase the number of experiments to draw definite conclusions about this (Figure 3.11 B).

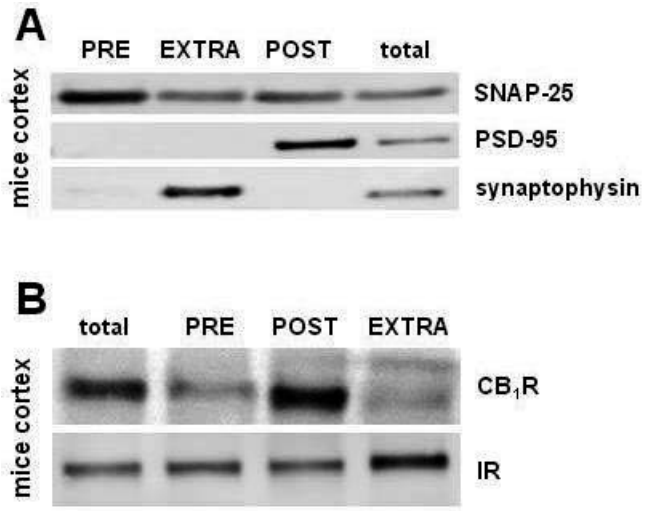

Figure 3.11. CB_1R and InR are localized both pre and postsynaptically in mice cortex. (A) Validation of the subsynaptic fractions by analysis of the enrichment of subsynaptic proteins in the corresponding subsynaptic fractions: SNAP-25 had a higher expression in the presynaptic fraction, PSD-95 was only present in the postsynaptic fraction and synaptophysin was enriched in the extrasynaptic fraction of mice cortices. **(B)** Western blot analysis of the localization of CB_1R and InR in synaptosomal pre, post and extrasynaptic fractions of mice cortices revealed that the CB_1R is mainly localized in the postsynaptic fraction and the InR is present both pre and postsynaptically but it is enriched in the extrasynaptic fraction. Total corresponds to the total synaptosomes which was further subsynaptically fractionated and, thus, it comprehends all fractions working as an internal positive control.

Western blotting of human cortex samples showed that InR and CB_1R are both present in the postsynaptic fraction and InRs are also present in the presynaptic active zone fraction (Figure 3.12). SNAP-25, PSD-95 and synaptophysin were used again to validate the purity of subsynaptic fractions and total synaptosomes were used as an internal positive control of the experiment. Thus, CB_1R as well as InR seem to be localized in the presynaptic active zone of the human cortex (Figure 3.12).

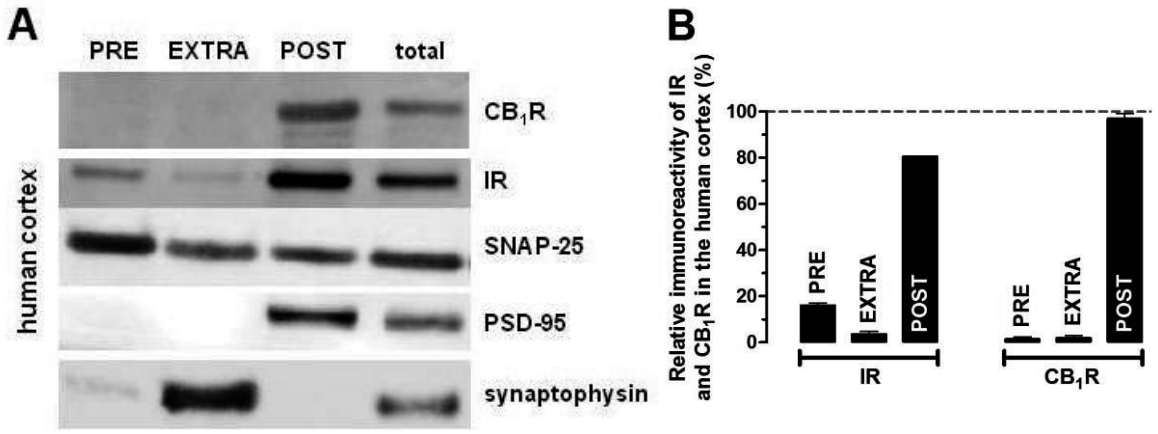

Figure 3.12. Both CB_1R and InR are postsynaptically localized in the human cortex. (A) Representative western blot of the localization of CB_1R and InR in subsynaptic fractions of the human cortex, showing that both CB_1R and InR are mainly localized in the synaptosomal postsynaptic fraction and the InR is also present in the presynaptic fraction. The validation of the purity of subsynaptic fractions was made with SNAP-25, PSD-95 and synaptophysin. The total synaptosomes were used as an internal positive control of the experiment. **(B)** Graphic of the percentage of immunoreactivity for InR and CB_1R bands, which was calculated considering the sum of the pre, extra and postsynaptic fractions value as 100%. Data are mean ± SEM of n = 2 independent experiments.

In spite of the presynaptic CB_1Rs in various brain regions being better established (Freund *et al.*, 2003), CB_1Rs were already detected postsynaptically in rat neocortical GABAergic and pyramidal cells, where their activation promotes slow-self inhibition (Marinelli *et al.*, 2008). Postsynaptic CB_1Rs were also found in rodent striatum (Köfalvi *et al.*, 2005), in rat spinal cord (Salio *et al.*, 2002) and in other brain areas (Freund *et al.*, 2003). Thus, it is possible that both CB_1R and InR are located at the postsynaptic active zone.

Both CB_1R activation (Kim *et al.*, 2011) and inhibition (Bouaboula *et al.*, 1997) have already been reported to inhibit insulin signalling in different conditions, but it is still unknown if the CB_1R and the InR interact through a heteromeric manner or through intracellular messengers in the brain. Recently, the modulation of insulin secretion by eCBs was proposed in pancreatic cells, which may be dependent on local metabolism and cellular conditions (Anderson *et al.*, 2013). Besides, it was recently shown that CB_1Rs form

heteromeric complexes with RTKs, including the InR (Dalton and Howlett, 2012 and Kim *et al.*, 2012). Therefore, after studying that the insulin action in rodent hippocampus synaptic transmission and in hippocampus and cortex glucose uptake are CB_1R dependent and after predicting they are both located postsynaptically, it is possible to hypothesize that probably the interaction between the CB_1R and the InR can be though a heteromeric manner or, most probably, through some secondary messenger molecules. Alternatively, as the recent study of Labouèbe and colleagues (2013) has demonstrated, the activation of InR may promote eCB release which, in turn, would act on CB_1Rs to exert diverse functions. At the present stage we can not speculate with certainty whether the interaction between the two receptors is direct or indirect.

4. CONCLUSIONS

The present study demonstrates that insulin effect under both synaptic transmission and glucose uptake in hippocampal and, for glucose uptake, cortical slices of Wistar rats is disturbed by alterations in the CB_1R activity. Significant alterations were also observed in synaptic transmission of hippocampal slices of fasted and two weeks STZ rats. Furthermore, insulin regulation of glucose uptake on cortical and hippocampal slices of wild-type mice seems to be similar to the pre-existing alterations in the CB_1R KO mice. The CB_1R deletion in mice has also shown to be insulin insensitive. Finally, preliminary studies of IR and CB_1R localization in the subsynaptic fractions of mice and human cortices showed that they are both present in the postsynaptic active zone.

Therefore, this work shows that insulin signalling depends on the eCB system in the brain and provides evidence for a possible functional interaction between an insulin-sensitive receptor (likely the IR) and the CB_1R in cortex and hippocampus (both brain areas related to learning and memory). Both IR and CB_1R signalling pathways have metabolites in common, although it seems to be a direct or a protein mediated physical interaction between the two receptors.

Further molecular studies are necessary to explore if this functional interaction happens between the CB_1R and the IR (rather than the IFG-1R) and if it involves the formation of heterodimmers, which could be verified with a co-immunoprecipitation assay. Then, if the two receptors do not form heterodimmers, it probably means that they interact through another pathway, shorter than through their common metabolites. Hence, co-localization of both receptors could be studied using confocal microscopy, for instance. However, if they interact directly, this reveals a new target of study for neurodegenerative (like AD) and psychiatric disorders. Besides, the methods used in this work could be repeated in human subjects using MRI and PET imaging techniques.

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